Biochimica et Biophysica Acta 1851 (2015) 220–230

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbalip

Role of long-chain acyl-CoA synthetase 4 in formation of polyunsaturated lipid species in hepatic stellate cells

Maidina Tuohetahuntila [a], Bart Spee [b], Hedwig S. Kruitwagen [b], Richard Wubbolts [a], Jos F. Brouwers [a], Chris H. van de Lest [a], Martijn R. Molenaar [a], Martin Houweling [a], J. Bernd Helms [a], Arie B. Vaandrager [a,*]

[a] Department of Biochemistry and Cell Biology, Faculty of Veterinary Medicine & Institute of Biomembranes, Utrecht University, Yalelaan 2, 3584 CM Utrecht, The Netherlands
[b] Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 104, 3584 CM Utrecht, The Netherlands

ARTICLE INFO

Article history:
Received 15 September 2014
Received in revised form 17 November 2014
Accepted 1 December 2014
Available online 9 December 2014

Keywords:
Prostaglandin
Eicosanoid
Phosphatidylcholine
Lipidomics
Heavy isotope labeling
Arachidonic acid

ABSTRACT

Hepatic stellate cell (HSC) activation is a critical step in the development of chronic liver disease. We previously observed that the levels of triacylglycerol (TAG) species containing long polyunsaturated fatty acids (PUFAs) are increased in in vitro activated HSCs. Here we investigated the cause and consequences of the rise in PUFA-TAGs by profiling enzymes involved in PUFA incorporation. We report that acyl CoA synthetase (ACSL) type 4, which has a preference for PUFAs, is the only upregulated ACSL family member in activated HSCs. Inhibition of the activity of ACSL4 by siRNA-mediated knockdown or addition of rosiglitazone specifically inhibited the incorporation of deuterated arachidonic acid (AA-d8) into TAG in HSCs. In agreement with this, ACSL4 was found to be partially localized around lipid droplets (LDs) in HSCs. Inhibition of ACSL4 also prevented the large increase in PUFA-TAGs in HSCs upon activation and to a lesser extent the increase of arachidonate-containing phosphatidylcholine species. Inhibition of ACSL4 by rosiglitazone was associated with an inhibition of HSC activation and prostaglandin secretion. Our combined data show that upregulation of ACSL4 is responsible for the increase in PUFA-TAG species during activation of HSCs, which may serve to protect cells against a shortage of PUFAs required for eicosanoid secretion.

1. Introduction

Hepatic stellate cells (HSCs)[1] are non-parenchymal cells which are located in the spaces of Disse, between the sinusoidal endothelial cells and the hepatocytes [1,2]. In a healthy liver, HSCs are involved in vitamin A (retinol) homeostasis as they are filled with large LDs containing retinylesters, together with triacylglycerols (TAGs) and cholesteryl esters. HSCs also play a role in the turnover of hepatic extracellular matrix (ECM) components such as collagen, glycoprotein and proteoglycan [3]. HSC proliferation and collagen synthesis are regulated by the 3D structure of the ECM [4]. Upon liver injury, quiescent HSCs can transdifferentiate into an activated myofibroblastic phenotype. Activated Kupffer cells may initiate this transition by secreting cytokines, such as transforming growth factor beta (TGF-β), which stimulate the synthesis of matrix proteins and the release of retinoids by HSCs [5]. Production of TGF-β by activated HSCs further stimulates the excess synthesis of ECM and results in liver fibrosis [6].

We previously reported that LD degradation in activated hepatic stellate cell is a highly dynamic and regulated process [7]. Upon activation of the hepatic stellate cells, the LDs reduce in size, but increase in number during the first 7 days in culture. The LDs migrate to cellular extensions in this first phase, before they disappear in a later phase. Raman and lipidomic studies showed that in the initial phase of HSC activation, the retinyl esters are disappearing more rapidly than the triacylglycerols. Interestingly, a large and specific increase in polyunsaturated fatty acid (PUFA)-containing triacylglycerol species was observed during the first 7 days in culture, together with the decrease in retinyl esters [7]. The increase in PUFA-TAGs was caused by a large increase in the uptake of PUFAs from the medium as assessed from deuterium-labeled arachidonic acid uptake studies [7]. So far, the molecular mechanisms and identity of the enzymes involved in the lipid remodeling and breakdown of the LDs during HSC activation are unknown. In this study, we aim to identify lipid enzymes responsible for the accumulation of PUFA-TAG species in activated HSCs and their possible role in the activation or function of HSCs. Our initial targets were isoforms of the family of acyl-CoA synthetases (ACSLs). Members of this family are key enzymes in the synthesis of complex lipids like

Abbreviations: HSC, hepatic stellate cell; LD, lipid droplet; TAG, triacylglycerol; ACSL, long-chain acyl-CoA synthetase; PC, phosphatidylcholine; AA, Arachidonic acid; AA-d8, deuterated arachidonic acid; PL, phospholipid

* Corresponding author at: Dept. Biochemistry and Cell Biology, Utrecht University, Faculty of Veterinary Medicine, Yalelaan 2, 3584 CM Utrecht, The Netherlands. Tel.: +31 30 2535378; fax: +31 30 2535492.
E-mail addresses: T.Maidina@uu.nl (M. Tuohetahuntila), B.Spee@uu.nl (B. Spee), H.S.Kruitwagen@uu.nl (H.S. Kruitwagen), r.wubbolts@uu.nl (R. Wubbolts), j.brouwers@uu.nl (J.F. Brouwers), C.H.A.vandeLest@uu.nl (C.H. van de Lest), m.r.molenaar@uu.nl (M.R. Molenaar), M.Houweling@uu.nl (M. Houweling), J.B.Helms@uu.nl (J.B. Helms), A.B.Vaandrager@uu.nl (A.B. Vaandrager).

http://dx.doi.org/10.1016/j.bbalip.2014.12.003
1388-1981/

phospholipids (PLs), cholesterol esters and TAGs by converting free long-chain fatty acids into acyl-CoAs [8]. Furthermore, uptake of long chain fatty acids is tightly linked to CoA-esterification by acyl-CoA synthetases. Five isoforms of ACSL have been identified in mammals. The ACSL family members differ in their specificity for fatty acid species and in their tissue distributions. Among the ACSLs, ACSL4 is a peripheral membrane protein that was found to be selective for arachidonic acid (AA) and other longer chain polyunsaturated fatty acids and is highly expressed in adrenal gland, brain, ovary and testis [9].

The identification of the enzyme responsible for the increase in PUFA-TAGs will allow us to investigate the role of these lipids in the functioning of HSCs. Elevation of TAG-PUFA species may be physiologically relevant as storage pools for AA, waiting to be incorporated in PLs. AA is also a precursor for eicosanoids, which are signaling lipids that play a role in a broad range of processes, such as modulation of inflammation and the immune system [10,11]. Eicosanoid synthesis typically starts with the release of AA from the sn-2 position of PLs by specific Ca^{2+}-dependent phospholipases in response to hormones/cytokines [12]. Subsequently AA is converted to various eicosanoids, like prostaglandins, thromboxanes or leukotrienes, depending on the relative activity of their respective synthases.

Here, we report that ACSL4 upregulation is critically involved in the increase in TAG-PUFA formation in activated HSCs, which may serve as a storage pool for eicosanoid production.

2. Materials and methods

2.1. Reagents

AA-d8 was purchased from Cayman Chemical (Ann Arbor, MI, USA). 15-deoxy-Δ12,14 prostaglandin J2 (15-dPGJ2) was from Tocris Bioscience (United Kingdom). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin were obtained from Gibco (Paisley, UK). Bovine Serum Albumin (BSA) fraction V was obtained from PAA (Pasching, Austria). Rosiglitazone was obtained from Enzo Life Sciences (Belgium). Collagenase (Clostridium histolyticum Type I) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Anti-rabbit IgG ACSL4 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody against alpha-smooth muscle actin (α-SMA) was from Thermo Scientific (Waltham, MA, USA), and mouse monoclonal anti-tubulin antibody from Sigma-Aldrich (St. Louis, MO, USA). Lipid droplet staining dye LD540 was kindly donated by Dr. C. Thiele, Bonn, Germany. Hoechst 33342 was obtained from Molecular Probes (Paisley, UK), paraformaldehyde (PF) (8%) was obtained from Electron Microscopy Sciences (Hatfield, PA, USA). FluorSave was obtained from Calbiochem (Billerica, MA, USA), all HPLC-MS solvents were from Biosolve (Valkenswaard, the Netherlands) with exception of chloroform (Carl Roth, Karlsruhe, Germany) and were of HPLC grade. Silica-G (0.063–0.200 mm) was purchased from Merck (Darmstadt, Germany).

2.2. Animals

Procedures of rat care and handling were in accordance with governmental and international guidelines on animal experimentation, and were approved by the Animal Experimentation Committee (Dierexperimentencommissie; DEC) of Utrecht University (DEC-numbers: 2010.III.09.110 and 2012. III.10.100).

2.3. Cell line

Human hepatic stellate cell line (LX-2) was kindly donated by Dr. Friedman (New York, NY, USA). LX-2 cells were cultured in DMEM medium supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin and cells were maintained in a humidified 5% CO_2 incubator at 37 °C.

2.4. Rat HSC isolation and in vitro primary cell culture

Adult male Wistar rats (300–400 g) were used in all experiments. Stellate cells were isolated from rat liver by collagenase digestion followed by differential centrifugation [13]. After isolation, HSCs were plated in 24, 12 or 6 well plates at a density of 2×10^4, 5×10^4 or 1×10^5 cells/well, respectively. Cells were maintained in DMEM supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin and 4 μl/ml Fungizone and cells were maintained in a humidified 5% CO_2 incubator at 37 °C. Medium was changed every 3 days. Purity of the HSC preparation as determined by desmin staining was 80–90%.

2.5. Gene silencing of Acsl4

Gene-silencing experiments were performed using small interfering RNA (siRNA) for *Acsl4* or non-targeting siRNA as a control (ON-TARGETplus SMARTpool of 4 siRNAs, Thermo-Scientific, Rochester, NY, USA) according to the manufacturer's instructions. Briefly, two days after plating, the cells were treated with 40 nM siRNA using 5 μl/ml RNAiMAX (Invitrogen, Breda, the Netherlands) in antibiotic free complete media (with FBS). After 6 h of transfection, media was changed to standard culturing conditions up to day 7.

2.6. RNA isolation, cDNA synthesis and QPCR

Total RNA was isolated from rat HSCs grown in a 24-well plate using RNeasy Mini Kit (Qiagen, Venlo, the Netherlands) including the optional on-column DNase digestion (Qiagen RNase-free DNase kit). RNA was dissolved in 30 μl of RNase free water and was quantified spectrophotometerically using a Nanodrop ND-1000 (Isogen Life Science, IJsselstein, the Netherlands). An iScript cDNA Synthesis Kit (Bio-Rad, Veenendaal, the Netherlands) was used to synthesize cDNA. Primer design and qPCR conditions were as described previously [14]. Briefly, qPCR reactions were performed in duplicate using Bio-Rad detection system. Amplifications were carried out in a volume of 25 μl containing 12.5 μl of 2xSYBR green supermix (BioRad), 1 μl of forward and reverse primer and 1 μl cDNA. Cycling conditions were as follows: initial denaturation at 95 °C for 3-minute, followed by 45 cycles of denaturation (95 °C for 10 s), annealing temperature (see Supplementary Table S1) for 30 s, and elongation (72 °C for 30 s). A melting curve analysis was performed for every reaction. To determine relative expression of a gene, a 4-fold dilution series from a pool of all samples were used. IQ5 Real-Time PCR detection system software (BioRad) was used for data analysis. Expression levels were normalized by using the average relative amount of the housekeeping genes. Housekeeping genes used for normalization are, based on their stable expression in stellate cells, namely, *tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta (Ywhaz)*, *hypoxanthine phosphoribosyl transferase (Hprt)*, and *hydroxylmethylbilane synthase (Hmbs)*. Primers for housekeeping genes and genes of interest are listed in Supplementary Table S1.

2.7. Western blot analysis

Cell homogenates were collected and equal amounts of proteins were heated to 95 °C for 5 min in loading buffer and then separated on 10% SDS-polyacrylamide gel and blotted onto polyvinylidene difluoride membranes (GE Healthcare Europe GmbH, Belgium). The membranes were incubated with 5% BSA in Tris-buffered saline (TBS) for 30 min at room temperature. The incubation of the primary antibody was performed at 4 °C overnight for all antibodies (see Supplementary Table S2) in TBS with 0.1% Tween-20 (Boom B.V., Meppel, the Netherlands) and 1% BSA. After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Nordic Immunology, the Netherlands) at room temperature for 1 h. Blots