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Adipose triglyceride lipase is involved in the mobilization of triglyceride and retinoid stores of hepatic stellate cells

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ABSTRACT

Hepatic stellate cells (HSCs) store triglycerides (TGs) and retinyl ester (RE) in cytosolic lipid droplets. RE stores are degraded following retinoid starvation or in response to pathogenic stimuli resulting in HSC activation. At present, the major enzymes catalyzing lipid degradation in HSCs are unknown. In this study, we investigated whether adipose triglyceride lipase (ATGL) is involved in RE catabolism of HSCs. Additionally, we compared the effects of ATGL deficiency and hormone-sensitive lipase (HSL) deficiency, a known RE hydrolase (REH), on RE stores in liver and adipose tissue. We show that ATGL degrades RE even in the presence of TGs, implicating that these substrates compete for ATGL binding. REH activity was stimulated and inhibited by comparative gene identification-58 and G0/G1 switch gene-2, respectively, the physiological regulators of ATGL activity. In cultured primary murine HSCs, pharmacological inhibition of ATGL, but not HSL, increased RE accumulation. In mice globally lacking ATGL or HSL, RE contents in white adipose tissue were decreased or increased, respectively, while plasma retinol and liver RE levels remained unchanged. In conclusion, our study shows that ATGL acts as REH in HSCs promoting the degradation of RE stores in addition to its established function as TG lipase. HSL is the predominant REH in adipocytes but does not affect lipid mobilization in HSCs.

1. Introduction

Retinoids (vitamin A) are fat-soluble micronutrients with numerous important functions, including a role in vision, reproduction, immunity, and the development and maintenance of differentiated tissues [1]. Dietary retinol (ROH) is esterified with long-chain fatty acids (FAs), preferentially palmitic acid (PA), and stored in form of retinyl ester (RE). Although most tissues of the body contain trace amounts of RE, they are predominantly (up to 80%) stored in the liver where they are deposited in lipid droplets (LDs) of hepatic stellate cells (HSCs) [2]. Healthy individuals contain vitamin A reserves sufficient for an adequate supply of the body for up to several months. In times of insufficient vitamin A uptake, RE stores are mobilized to ensure a constant concentration of ~1 μM ROH in the circulation which is essential for normal body function [3]. In the circulation, ROH is attached to retinol-binding protein 4 (RBP4) and is transported to target tissues where it can be re-esterified or converted into its bioactive metabolites, which possess essential roles in vision and gene regulation. 11-*cis* retinaldehyde functions as the active chromophore in rhodopsin. All-*trans* and 9-*cis* retinoic acid interact with a number of nuclear receptors of the retinoic acid receptor (RAR) and the retinoid X receptor (RXR) family. These receptors function as ligand-activated transcription factors and regulate the expression of numerous genes [4,5].

The availability of ROH from endogenous stores is determined by the synthesis and hydrolysis of RE. In HSCs, ROH is esterified by the action of lecithin:retinol acyltransferase (LRAT). LRAT-deficient mice possess only trace amounts of RE in HSCs and are more susceptible to develop retinoid deficiency [6], demonstrating that this enzyme is required for efficient RE storage. Yet these mice exhibit elevated RE levels in white adipose tissue (WAT) and are capable of esterifying ROH in other tissues in an acyl-CoA-dependent reaction. Presumably, this reaction [acyl-CoA:retinol acyltransferase activity] is mediated by acyl-CoA:diacylglycerol acyltransferase 1, an enzyme with broad substrate specificity catalyzing the synthesis of triglycerides (TGs), diglycerides (DGs), and waxes [7]. The mobilization of ROH from RE stores requires the activity of enzymes possessing RE hydrolase (REH) activity. To date, very little is known about lipases involved in RE mobilization in

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HSCs and the molecular mechanisms regulating lipolysis in this cell type [7,8]. Yet it is reasonable to assume that RE mobilization is a tightly regulated process, since the liver is capable of keeping constant plasma ROH levels despite strong variations in dietary retinoid uptake.

Although a number of enzymes have been reported to hydrolyze RE *in vitro* [7], hormone-sensitive lipase (HSL) and retinal pigment epithelium 65 (RPE65) are the only known enzymes, which have been shown to affect retinoid metabolism *in vivo* [9,10]. However, HSL is hardly detectable in HSCs [11], and RPE65 is specifically expressed in retinal pigment epithelium, a specialized cell type controlling retinoid metabolism in the eye [12]. In the search for enzymes involved in HSCs lipid catabolism, we found that adipose triglyceride lipase [ATGL [13], identical to desnutrin and patatin-like phospholipase domain containing 2 (PNPLA2)] can hydrolyze RE in the presence of its co-activator comparative gene identification-58 [CGI-58, identical to α/β-hydrolase domain containing-5 [14]]. Our findings indicate that ATGL functions as REH in HSCs in addition to its established role in TG catabolism.

2. Materials and methods

2.1. Materials

All-*trans*-retinol (ROH), retinyl acetate (RAc), retinyl palmitate (RP), and fatty acid (FA)-free BSA were from Sigma-Aldrich (Taufkirchen, Germany).

2.2. Animals

Mice were maintained on a regular light–dark cycle (12 h light, 12 h dark) and kept *ad libitum* on a standard laboratory chow diet (Ssniff Spezialdiaeten, Soest, Germany, Vitamin A ~15,000 IU/kg) or on a Vitamin A-deficient diet (Ssniff, Vitamin A < 120 IU/kg). ATGL-ko and HSL-ko mice were generated by targeted homologous recombination as described previously [15,16]. Animals were 8–12 weeks of age. Female mice were used for all studies. Non-fasted animals were anesthetized with IsoFlo/Isoflurane (Abbott, Animal Health, Queenborough, Kent, UK) and euthanized by cervical dislocation. The study was approved by the ethics committee of the University of Graz and is in accordance with the Council of Europe Convention (ETS 123).

2.3. cDNA cloning of recombinant tagged proteins

The open reading frames (ORF) of murine PNPLA1, ATGL, and adiponutrin were cloned into pcDNA4™/HisMaxC (Invitrogen; Life Technologies, Carlsbad, USA), as described previously [17,18], and transfected into COS-7 cells (ATCC CRL-1651) using Metafectene (Biontex GmbH, Munich, Germany). PNPLA1 was cloned using the following primers: fw-5′-AAGAATTCGAACAGGTGTTCAAAGGAG-3′ and rev-5′-AACTCGAGTTAGGAGTTCTGGCCACTCACT-3′: for the expression of murine ATGL and CGI-58 in *Escherichia coli*, sequences encoding the ORF of ATGL and CGI-58 were inserted into the target vector pASK-IBA5plus (IBA, Goettingen, Germany) and transformed into *E. coli* as described [17].

2.4. Immunoblotting

Proteins of cell lysates or tissue homogenates were separated by SDS–PAGE according to their molecular weight using Tris/glycine as electrophoresis buffer and were transferred onto polyvinylidene fluoride membranes (Carl Roth GmbH, Karlsruhe, Germany) using CAPS buffer. After blocking, membranes were hybridized with respective primary antibodies. Membranes were washed, incubated with respective secondary horseradish-peroxidase (HRP)-conjugated antibody and detected using ECL2 Western blotting substrate (Thermo Scientific, Waltham, MA). Antibodies used were rabbit anti-HSL, rabbit anti-phospho-HSL (S660), and rabbit anti-GAPDH (all from Cell Signaling, Danvers, MA, USA), rabbit anti-CGI-58 (Abnova, Heidelberg), mouse anti-β-Actin (Santa Cruz, Santa Cruz, CA, USA), rabbit anti-alpha smooth muscle actin (α-SMA, Pierce, Thermo Scientific), HRP-linked sheep-anti mouse antibody (GE Healthcare Amersham, Buckinghamshire, UK), and HRP-linked rat-anti rabbit antibody (Dako, Glostrup, Denmark).

2.5. Isolation of HSCs

HSCs were isolated according to the method of Blomhoff et al. [19] with some modifications. Briefly, livers of anaesthetized mice were perfused via the portal vein using Krebs–Henseleit buffer (KHB, without Ca^{2+} and SO_4^{2-}) followed by perfusion with KHB containing 0.15 mg/ml collagenase type II (Worthington Biochemical Corporation, Lakewood, NJ), 0.1 mg/ml Pronase E (Merck, Darmstadt, Germany), 2% BSA, and 0.1 mM $CaCl_2$. Thereafter, livers were excised, disrupted, passed through a metal sieve, and filtered through a 70 μm nylon cell strainer (BD Biosciences, San Jose, CA). Parenchymal cells were separated from non-parenchymal cells (NPCs) by centrifugation (50 ×*g*, 3 min, 4 °C). The supernatant was used for the isolation of HSCs using OptiPrep™ self-forming density gradient solutions (Axis-Shield PoC AS, Rodeløkka, Norway) as described previously [20]. After centrifugation, HSCs were collected, washed with PBS, and used for Western blotting analyses. For cell culture experiments, total NPCs were seeded into cell culture dishes in Dulbecco's modified Eagle medium (DMEM) containing 20% fetal calf serum (FCS), 100 μg/ml primocin, and antibiotics. After 2 days in culture, cells were trypsinized and seeded again [selective detachment according to Trøen et al. [21]]. After 10 days in culture, ~90% of cells stained positive for α-smooth muscle actin (α-SMA), indicating the presence of activated stellate cells. A representative stain for α-SMA is shown in Fig. S1.

2.6. Lipid accumulation in primary HSCs

HSCs were seeded into 6-well plates and cultured for 10 days in DMEM media containing 20% FCS, 100 μg/ml primocin, and antibiotics. ROH (5 μM) was added to the medium to prevent complete loss of RE stores [21]. Cells were loaded for 24 h with 20 μM ROH and 50 μM palmitic acid (PA) complexed to BSA. Thereafter, cells were starved in DMEM media containing 2% FA-free BSA. At indicated time points, lipids were extracted with hexane:isopropanol (3:2; 0.5 mM butylated hydroxytoluene (BHT)).

2.7. Analysis of tissue and plasma ROH and RE by HPLC

Retinoids of liver, WAT, and plasma samples were extracted with ice-cold hexane:methanol:PBS (5:1:1; 0.5 mM BHT and 100pmol RAc/sample as internal standard). Hexane phase was dried, and lipids were dissolved in methanol and subjected to HPLC analysis. Retinoids were separated on a reverse phase pro-C18 column (250 × 4.6 mm; 12 nm, S-5 μm; YMC Europe, Dinslaken, Germany) using a gradient of methanol:toluene at a flow rate of 1.0 ml/min. The HPLC system consisted of Waters e2695 Separation Module, UV/VIS 2489 Detector, and a Multi-λ Fluorescence 2475 Detector (Waters, Millford, MA). For the detection of ROH and RE, excitation was set at 325 nm and emission at 490 nm. ROH eluted at a retention time of 5.3 min, whereas RE eluted as a single peak at a retention time of 12.2 min. ROH and RE were quantified by comparison with the internal standard.

2.8. Determination of enzyme activities

The determination of TG hydrolase activities was performed using [9,10-^{3}H]-triolein (TO) (PerkinElmer Life Sciences) as radioactive tracer as described [22]. The substrate for the determination of REH activities consisted of RP and phosphatidylcholine (molar ratio 1:0.9) in 0.1 M potassium phosphate buffer (pH 7.0). Substrate was prepared by sonication (Virsonic 475, Virtis, Gardiner, NJ) on ice and adjusted to 5% FA-