Biochimica et Biophysica Acta 1831 (2013) 1276–1286

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

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

Altered hepatic retinyl ester concentration and acyl composition in response to alcohol consumption

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ARTICLE INFO

Article history:
Received 4 January 2013
Received in revised form 2 April 2013
Accepted 5 April 2013
Available online 12 April 2013

Keywords:
Vitamin A
Retinyl ester
Phosphatidylcholine
Hepatic stellate cells
Liver metabolism
Alcoholic liver disease

ABSTRACT

Retinoids (vitamin A and its metabolites) are essential micronutrients that regulate many cellular processes. Greater than 70% of the body's retinoid reserves are stored in the liver as retinyl ester (RE). Chronic alcohol consumption induces depletion of hepatic retinoid stores, and the extent of this has been correlated with advancing stages of alcoholic liver disease. The goal of this study was to analyze the mechanisms responsible for depletion of hepatic RE stores by alcohol consumption. A change in the fatty-acyl composition of RE in alcohol-fed mice was observed within two weeks after the start of alcohol consumption. Specifically, alcohol-feeding was associated with a significant decline in hepatic retinyl palmitate levels; however, total RE levels were maintained by a compensatory increase in levels of usually minor RE species, particularly retinyl oleate. Our data suggests that alcohol feeding initially stimulates a futile cycle of RE hydrolysis and synthesis, and that the change in RE acyl composition is associated with a change in the acyl composition of hepatic phosphatidylcholine. The alcohol-induced change in RE acyl composition was specific to the liver, and was not seen in lung or white adipose tissue. This shift in hepatic RE fatty acyl composition is a sensitive indicator of alcohol consumption and may be an early biomarker for events associated with the development of alcoholic liver disease.

1. Introduction

Dietary retinoids (vitamin A and its metabolites) are required to maintain many important physiological processes [1]. The active retinoid metabolites include 11-*cis*-retinal, which is required for chromophore formation in vision [2], and all-*trans*- and 9-*cis*-retinoic acids, which are required for normal cell proliferation, differentiation, and apoptosis [1,3,4]. The liver is of central importance in whole body retinoid metabolism; it is the primary site of retinoid storage in the body and it also mediates distribution of stored retinoid to other tissues through the secretion of retinol bound to retinol-binding protein [5,6]. Chronic alcohol consumption decreases hepatic retinoid stores, as reviewed [7]. In a seminal study, Leo and Lieber demonstrated that alcoholics have decreased hepatic retinoid content and that as the severity of alcoholic liver disease (ALD) progresses, there is a progressive decline in hepatic retinoid levels [8]. Subsequent studies in animal models confirmed this observation and established that loss of hepatic retinoid is not due to dietary-insufficiency, but rather a direct effect of alcohol on hepatic retinoid homeostasis [7].

Approximately 70% of total retinoid present in the body is located in the liver. Within this tissue, greater than 90% of this retinoid is stored as retinyl ester (RE) within the lipid droplets of hepatic stellate cells (HSC) [5]. The synthesis of RE in HSCs is catalyzed by lecithin:retinol acyltransferase (LRAT), and the substrates for this reaction are retinol and the fatty acyl group present at the sn-1 position of phosphatidylcholine (PC) [9–11]. In addition to LRAT, a second RE synthesizing activity has been identified, termed acyl CoA:retinol acyltransferase (ARAT), which uses fatty acyl-CoA as a substrate for RE synthesis [12]. Diacylglycerol acyltransferase 1 (DGAT1) has been shown to act as an ARAT in the skin and intestine [13,14]. Although an ARAT activity was previously reported to be present in liver homogenates [15], the complete absence of hepatic RE stores in *Lrat*-null (*Lrat*−/−) mice establishes that LRAT is essential and solely responsible for RE synthesis in the liver under normal physiological conditions [16–18]. The enzyme responsible for the hydrolysis of HSC RE stores is less well understood; while several enzymes have been proposed to possess retinyl ester hydrolase (REH) activity, definitive identification of physiologically significant REHs in HSCs is lacking [19,20]. Nevertheless, it is clear that the central mechanism controlling HSC RE levels is the balance between RE synthesis and hydrolysis.

Abbreviations: ARAT, Acyl:CoA retinol acyltransferase; ALD, alcoholic liver disease; GPAT, glycerol-3-phosphate acyltransferase; LRAT, lecithin:retinol acyltransferase; PC, phosphatidylcholine; RE, retinyl ester; VAD, vitamin A-deficient; VAS, vitamin A-sufficient; WAT, white adipose tissue

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http://dx.doi.org/10.1016/j.bbalip.2013.04.006

During our studies of the pathogenesis of ALD in experimental mice, we observed that alcohol consumption is associated with a marked alteration in the fatty acyl composition of hepatic RE stores, which precedes their loss from the liver. The data reported in this manuscript examines the effect that alcohol consumption has on hepatic RE, and includes a series of follow-up studies addressing the potential mechanism(s) mediating this change. We interpret these data to indicate that alcohol stimulates a futile cycle of HSC RE synthesis and hydrolysis that starts during the initial phase of chronic alcohol consumption. We propose that the observed change in fatty acyl composition of RE has the potential to serve as an early biomarker of alcohol consumption. Our data further suggest that this change reflects an underlying switch in the fatty acyl composition of hepatic PC, which we believe has implications for the development of ALD.

2. Methods

2.1. Animal husbandry and alcohol feeding protocol

All animal experiments were performed according to the criteria established by the National Academy of Science as described in the "Guide for the Care and use of Laboratory Animals", and were approved by the Institutional Animal Care and Use Committee at Columbia University. The majority of our experiments were carried out using 3 month old male C57BL/6J mice. The generation of *Lrat*−/− and *Dgat1* null (*Dgat1*−/−) mice has been previously described [16,21], and these mice have been previously used in our laboratory to study retinoid physiology [13]. Both *Lrat*−/− and *Dgat1*−/− mice were in congenic C57BL/6J backgrounds and were bred and maintained in our colony at Columbia University. Note that the *Dgat1*−/− mice used in these studies were 6 months old, which explains the higher baseline levels of hepatic RE in these mice. All mice were maintained in an environmentally controlled animal facility with a 12-h light:dark cycle. Prior to the start of our alcohol feeding studies, mice were maintained on a standard rodent chow diet. At the start of each alcohol feeding experiment, mice were randomized into groups receiving control or alcohol-containing Lieber–DeCarli liquid diets (control: F5937SP, alcohol: F5938SP; Bio-Serv, Frenchtown, NJ). This alcohol feeding paradigm is well established in the literature and has previously been employed by our laboratory to study the effect of alcohol on hepatic lipid metabolism [22,23]. In the vitamin A-deficient (VAD) diet study, custom diets were identical to the vitamin A-sufficient (VAS) control and alcohol-containing liquid diets, except that they were devoid of any source of vitamin A (VAD control: F6360SP, VAD alcohol: F6361SP; Bio-Serv). All experimental mice were individually housed and diet consumption in the alcohol-fed mice was measured to allow pair-feeding of control animals. Our alcohol feeding protocol is shown schematically in Fig. 1A. In brief, mice underwent an alcohol adaptation period that included 1 week consuming the control diet, followed by 1 week of 2.1% v/v alcohol and 1 week of 4.2% v/v alcohol. Following the adaptation period, mice were fed 6.4% v/v alcohol for up to 4 weeks. At the end of the alcohol feeding experiments, plasma, liver, lung and epigonadal white adipose tissue (WAT) samples were collected and snap frozen in liquid N_2, and then stored at −80 °C prior to analysis. Additional alcohol feeding protocols using the low-fat formulation of the Lieber–DeCarli liquid diet and ethanol added to the drinking water of mice have been previously described [24,25].

2.2. Measurement of tissue retinoid content

Tissue RE levels were measured by reverse phase HPLC. This protocol is well established in our laboratory and has been previously described [13,26,27]. In brief, retinoids present in tissue homogenates were mixed with an equal volume of ethanol containing a known amount of retinyl acetate (Sigma-Aldrich Co., St Louis, MO) as an internal standard, and then extracted using hexane that was then evaporated under a stream of N_2. Extracted retinoids were then dissolved in benzene and separated using a 4.6 × 250 mm Waters Symmetry C18 column (Waters Corp., Milford, MA); the mobile phase was 70% acetonitrile, 15% methanol, and 15% methylene chloride, running at a flow rate of 1.8 ml/min. Absorption was measured at 325 nm, and retinoids present in tissue extracts were identified by comparing the retention time and spectral data of HPLC chromatograms with those of purified standards. Quantification of tissue retinoid levels were calculated using integrated peak areas and were adjusted relative to the recovery of the internal standard.

Highly sensitive ultra performance liquid chromatography mass spectrometry (LC/MS) was used to measure plasma RE levels. Samples were extracted using 100 μl of serum diluted in 400 μl of PBS in a clean glass tube. Five hundred microliters of absolute ethanol containing 100 pmol of retinyl acetate (Sigma-Aldrich Co.) was then added as an internal standard. Subsequently, 4 ml of hexane was added, the samples were thoroughly mixed, and then centrifuged for 10 min at 2000 g. The organic upper phase was then transferred into a clean glass tube and was evaporated under a gentle stream of N_2. The dried lipid was resuspended in 50 μl of methanol and transferred to an autosampler vial (Waters Corp.) for analysis.

All LC/MS analyses were carried out using a Waters Xevo TQ MS Acquity UPLC system (Waters Corp.), controlled by MassLynx software V4.1. Prior to injection, samples were maintained at 4 °C in the autosampler. Samples were injected into an Acquity UPLC BEH Phenyl column (Waters Corp., 3.0 mm inner diameter × 100 mm; with 1.7 μm particles). Throughout sample analysis, the column was maintained at 40 °C. The flow rate was 300 μl/min in a binary gradient mode, with the following mobile phase gradient: initiated with 5% mobile phase A (water containing 0.2% formic acid and 1 mM ammonium formate) and 95% mobile phase B (methanol containing 0.2% formic acid and 1 mM ammonium formate). The gradient for mobile phase B was increased linearly to 97.5% for 5 min. The column was then washed with 99% methanol for 1 min and the next sample was injected. Positive ESI-MS mass spectrometry with selected ion recording mode was performed using the following parameters: capillary voltage −4.0 kV, source temperature 150 °C, desolvation temperature 500 °C, and desolvation gas flow 1000 l/h. Retinol and RE were detected using m/z 269.25 (retinol anhydrate [M + H]+) and identified by comparing the retention times of experimental compounds with those of authentic standards. Concentrations of RE in the serum were quantitated by comparing integrated peak areas for those of each retinoid against those of known amounts of purified standards. Loss during extraction was accounted for by adjusting for the recovery of the internal standard added before extraction.

2.3. Measurement of hepatic phosphatidylcholine composition

Liquid chromatography tandem mass spectrometry (LC/MS/MS) was used to measure hepatic PCs. Samples for PC analysis were extracted using 2:1 (v/v) chloroform/methanol. Briefly, approximately 100 mg of liver was homogenized in 2 ml of PBS using a Tissue-Tearor (BioSpec Products, OK, USA), then 20 μl of the tissue homogenate was transferred to a clean glass tube and mixed with 2 ml chloroform and 1 ml methanol, containing 2 nmol 17:0/17:0 PC as an internal standard. After thorough mixing, 0.5 ml of PBS was added to allow for phase separation. The mixture was thoroughly mixed a second time and then centrifuged at 3000 *g* for 10 min to separate phases. Following centrifugation, the lower organic phase was transferred to a clean glass tube and 2 ml of chloroform was added to the residual aqueous phase, which was then vigorously mixed and centrifuged at 3000 *g* for 10 min, to extract any remaining lipids. The lower organic phases were pooled and evaporated to dryness under N_2. The extracted lipids were reconstituted in 1.5 ml of acetonitrile/methanol