Research Article

JOURNAL OF HEPATOLOGY

Hepatic ceramides dissociate steatosis and insulin resistance in patients with non-alcoholic fatty liver disease

Panu K. Luukkonen[1,2,*,†], You Zhou[1,†], Sanja Sädevirta[1,2], Marja Leivonen[3], Johanna Arola[4], Matej Orešič[5], Tuulia Hyötyläinen[5], Hannele Yki-Järvinen[1,2]

[1]*Minerva Foundation Institute for Medical Research, Helsinki, Finland;* [2]*Department of Medicine, University of Helsinki and Helsinki University Hospital, Helsinki, Finland;* [3]*Department of Surgery, University of Helsinki and Helsinki University Hospital, Helsinki, Finland;* [4]*Department of Pathology, University of Helsinki and Helsinki University Hospital, Helsinki, Finland;* [5]*Steno Diabetes Center, Gentofte, Denmark*

Background & Aims: Recent data in mice have identified *de novo* ceramide synthesis as the key mediator of hepatic insulin resistance (IR) that in humans characterizes increases in liver fat due to IR ('Metabolic NAFLD' but not that due to the I148M gene variant in *PNPLA3* ('*PNPLA3* NAFLD'). We determined which bioactive lipids co-segregate with IR in the human liver.

Methods: Liver lipidome was profiled in liver biopsies from 125 subjects that were divided into equally sized groups based on median HOMA-IR ('High and Low HOMA-IR', n = 62 and n = 63) or *PNPLA3* genotype (*PNPLA3*$^{148MM/MI}$, n = 61 *vs.* *PNPLA3*^{148II}, n = 64). The subjects were also divided into 4 groups who had either IR, the I148M gene variant, both of the risk factors or neither.

Results: Steatosis and NASH prevalence were similarly increased in 'High HOMA-IR' and *PNPLA3*$^{148MM/MI}$ groups compared to their respective control groups. The 'High HOMA-IR' but not the *PNPLA3*$^{148MM/MI}$ group had features of IR. The liver in 'High HOMA-IR' *vs.* 'Low HOMA-IR' was markedly enriched in saturated and monounsaturated triacylglycerols and free fatty acids, dihydroceramides (markers of *de novo* ceramide synthesis) and ceramides. Markers of other ceramide synthetic pathways were unchanged. In *PNPLA3*$^{148MM/MI}$ *vs.* *PNPLA3*^{148II}, the increase in liver fat was due to polyunsaturated triacylglycerols while other lipids were unchanged. Similar changes were observed when data were analyzed using the 4 subgroups.

Conclusions: Similar increases in liver fat and NASH are associated with a metabolically harmful saturated, ceramide-enriched liver lipidome in 'Metabolic NAFLD' but not in '*PNPLA3* NAFLD'. This difference may explain why metabolic but not *PNPLA3* NAFLD increases the risk of type 2 diabetes and cardiovascular disease.

Keywords: Patatin-like phospholipase domain containing protein 3; Non-alcoholic fatty liver disease; Insulin resistance; Non-alcoholic steatohepatitis; Ceramides; Free fatty acids; *PNPLA3*; Dihydroceramides.

Received 28 July 2015; received in revised form 5 November 2015; accepted 4 January 2016; available online 11 January 2016

* Corresponding author. Address: Tukholmankatu 8, 00290 Helsinki, Finland. Tel.: +358 294125708.

E-mail address: panu.luukkonen@fimnet.fi (P.K. Luukkonen).

† These authors contributed equally to this work.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CoA, coenzyme A; DAG, diacylglycerol; DNL, *de novo* lipogenesis; FFA, free fatty acid; HDL, high density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; IR, insulin resistance; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; OGTT, oral glucose tolerance test; *PNPLA3*, patatin-like phospholipase domain containing protein 3; TAG, triacylglycerol.

Introduction

Both features of the metabolic syndrome and non-alcoholic fatty liver disease (NAFLD) predict type 2 diabetes and cardiovascular disease even independent of obesity in several prospective studies [1]. Such a form of NAFLD ('Metabolic NAFLD') is characterized by hepatic insulin resistance (IR) [2,3], increased serum triacylglycerols (TAG), low high density lipoprotein (HDL) cholesterol and low serum adiponectin concentrations [4]. The latter may reflect adipose tissue inflammation [5,6]. 'Metabolic NAFLD' precedes and predicts type 2 diabetes and cardiovascular disease [1].

In insulin resistant 'Metabolic NAFLD', studies that trace pathways contributing to intrahepatocellular TAGs have shown that increased hepatic *de novo* lipogenesis (DNL) is the major cause for increased intrahepatocellular TAG content in 'Metabolic NAFLD' [7]. DNL produces exclusively saturated fatty acids, which can be desaturated to form monounsaturated fatty acids by SCD-1 [8,9]. A hepatic venous catheterization study showed that fatty liver overproduces saturated TAGs [10].

At least two classes of bioactive lipids, ceramides and diacylglycerols (DAGs), have been suggested to mediate IR. The 'ceramide-centric' view postulates both saturated fat from DNL or from the diet and adiponectin deficiency induce IR via increasing ceramide synthesis [11]. Ceramides also induce ER stress and mitochondrial dysfunction, which characterize human NAFLD [12]. The immediate precursors of TAGs, DAGs, induce IR by inhibiting PI3-kinase and Akt/protein kinase B activation via stimulation of protein kinase C isoforms [13].

Ceramides can be synthesized via the *de novo* synthetic pathway from palmitate, via sphingomyelin hydrolysis and via the salvage pathway, which uses hexosylceramides as its substrate [11]. Two very recent studies in mice identified the same

sphingolipid species, C16:0-ceramide, formed via ceramide synthase 6, in the *de novo* ceramide synthetic pathway as the principal mediator of obesity-related IR [14–16]. There are no human data to examine which, if any, of these pathways contributes to changes in ceramide concentrations in the human liver.

A common I148M variant in patatin-like phospholipase domain containing protein 3 (*PNPLA3*) at rs738409 increases liver fat content [17]. Depending on ethnicity, 20–50% of all subjects carry this gene variant [17]. *In vitro*, this gene variant inhibits lipolysis of TAGs in the liver [18] and acts as a gain-of-function mutation to increase TAG synthesis by acting as a lysophosphatidic acyltransferase (LPAAT) converting lysophosphatidic acid into phosphatidic acid [19]. Monounsaturated fatty acid containing acyl CoAs such as oleyl CoA are preferred substrates for the former activity while polyunsaturated fatty acyl CoAs such as linoleoyl and arachidonoyl CoA are preferred substrates for the latter activity [18,19]. In contrast to the metabolic abnormalities observed in 'Metabolic NAFLD', NAFLD due to the I148M variant ('*PNPLA3* NAFLD') is not characterized by features of IR, such as adipose tissue inflammation, adiponectin deficiency or an increased risk of type 2 diabetes and cardiovascular disease [20,21].

'Metabolic NAFLD' and '*PNPLA3* NAFLD' provide models to characterize how IR and steatosis dissociate in the human liver. Given the high prevalence of both the metabolic syndrome and the I148M *PNPLA3* gene variant, some individuals will have both of these risk factors for NAFLD. In the present study, we hypothesized that the liver in 'Metabolic NAFLD' might be characterized by increased concentrations of saturated/monounsaturated TAGs, free fatty acids (FFA) and IR-inducing bioactive lipids, while such metabolically harmful lipids may not be found in '*PNPLA3* NAFLD'. To this end, we analyzed the human liver lipidome in 125 liver biopsy samples using ultra high performance liquid chromatography (UHPLC) and gas chromatography combined with mass spectrometry (MS). This was done in groups divided based on median homeostasis model assessment of insulin resistance (HOMA-IR) into those with 'High HOMA-IR' (a model for 'Metabolic NAFLD') and 'Low HOMA-IR', and based on genotyping at rs738409 into carriers (*PNPLA3*$^{148MM/MI}$) (a model for '*PNPLA3* NAFLD') and non-carriers (*PNPLA3*^{148II}) of the *PNPLA3* I148M gene variant. We also compared liver lipidomes in 4 groups of subjects who had both IR and carried the I148M gene variant ('double trouble'), either ('single trouble') or neither risk factor.

Materials and methods

Study subjects

A total of 125 subjects were recruited amongst those undergoing laparoscopic bariatric surgery. Subjects were eligible if they met the following criteria: (a) age 18 to 75 years; (b) no known acute or chronic disease except for obesity or type 2 diabetes or hypertension on the basis of medical history, physical examination and standard laboratory tests (complete blood count, serum creatinine, electrolyte concentrations); (c) alcohol consumption less than 20 g per day for women and less than 30 g per day for men; (d) no clinical or biochemical evidence of other liver disease, or clinical signs or symptoms of inborn errors of metabolism; (e) no history of use of toxins or drugs associated with liver steatosis. Elevated liver enzymes (alanine aminotransferase [ALT] and aspartate aminotransferase [AST]) were not exclusion criteria. The study protocol was approved by the ethics committee of the Hospital District of Helsinki and Uusimaa. The study was conducted in accordance with the Declaration of Helsinki. Each participant provided written informed consent after being explained the nature and potential risks of the study.

Metabolic study

The subjects were invited to a separate clinical visit one week prior to surgery for detailed metabolic characterization. The subjects came to the clinical research center after an overnight fast. Body weight, height and waist circumference were measured as described [21]. An intravenous cannula was inserted in the antecubital vein for withdrawal of blood for measurement of HbA_{1c}, serum insulin and adiponectin, plasma glucose, low density lipoprotein (LDL) and HDL cholesterol, triglyceride, albumin, AST, ALT, alkaline phosphatase and gamma-glutamyl transferase concentrations and for genotyping as described [21]. Plasma albumin was measured using a photometric method on an autoanalyzer (Modular Analytics EVO; Hitachi High-Technologies Corporation, Tokyo, Japan). *PNPLA3* at rs739409 was genotyped as previously described [22]. After basal blood sampling and anthropometric measurements, an oral glucose (75 g) tolerance test (OGTT) was performed [23]. HOMA-IR was used as a proxy for IR by using the formula: HOMA-IR = fS-insulin (mU/L) × fP-glucose (mmol/L)/22.5 [24]. Matsuda insulin sensitivity index was used as another measure of insulin sensitivity. This measure was calculated from insulin and glucose concentrations measured at 0, 30 and 120 minutes during the OGTT [25]. Body weight of the subjects was similar at the time of the metabolic study and surgery (131.1 ± 2.0 and 130.1 ± 2.1 kg, n.s.).

The subjects (n = 125) were divided into two groups based on IR, as defined by median HOMA-IR. Because of a lack of universally accepted consensus regarding the cut-off threshold of HOMA-IR between the insulin resistant and sensitive subjects, median HOMA-IR was used to divide the subjects into 'High HOMA-IR' (HOMA-IR >3.19) and 'Low HOMA-IR' (HOMA-IR ⩽3.19) groups. In retrospect, this cut-off was very similar to the HOMA-IR cut-off differentiating subjects with and without NASH (3.38) (see Supplementary Material). However, as the primary aim was to examine how risk factors (IR or the *PNPLA3* gene variant) influenced the liver lipidome as well as other features including liver histology, we did not *a priori* divide the subjects into groups based on NASH.

Since some patients are both insulin resistant and carry the *PNPLA3* gene variant, we also divided the subjects 2 × 2 based on both of these characteristics into 4 groups (Venn diagram as Supplementary Fig. 1 and other data as Supplementary Material p. 10–12, Supplementary Figs. 6–8 and Supplementary Table 2).

Liver biopsies and liver histology

Immediately at the beginning of the surgery, wedge biopsies of the liver were obtained. Part of the biopsy was sent to the pathologist for histological assessment, and the rest was snap-frozen in liquid nitrogen for subsequent analysis of molecular lipids. The time from obtaining the biopsy until freezing the sample in liquid nitrogen was approximately one minute. Liver histology was analyzed by an experienced liver pathologist (J.A.) in a blinded fashion as proposed by Brunt *et al.* [26].

Lipidomic analysis

The lipidome was analyzed using UHPLC-MS as described in Supplementary Material. The analyses covered most of the main molecular lipids, including ceramides, dihydroceramides, TAGs, DAGs, sphingomyelins, hexosylceramides, phosphatidylcholines (PC), phosphatidylethanolamines (PE), phosphatidylserines (PS), and lysophosphatidylcholines. The lipid identification was based on an internal library, which had been constructed based on accurate mass measurements in combination with tandem mass measurements. For specific lipids, the composition of fatty acid chains had been determined with separate measurements, and for those the fatty acid composition was specified, e.g. TAG (14:0/16:0/18:0).

Cluster analysis of lipids

We performed Bayesian model-based clustering analysis to identify the groups of lipids with similar profiles across all the samples as described in the Supplementary Material.

Analysis of pathways of ceramide synthesis

The first step of the *de novo* ceramide synthetic pathway converts palmitate and serine to 3-ketosphinganine and ultimately to dihydroceramides prior to formation of ceramides (Fig. 5). The sphingomyelin hydrolysis pathway results in formation of ceramides via hydrolysis of sphingomyelins. The salvage pathway generates ceramides by breakdown of complex sphingolipids, such as hexosylceramides (Fig. 5) [11]. In the present study, we analyzed the concentrations of dihydroceramides, sphingomyelins and hexosylceramides as markers of these pathways, respectively.