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Lipidomic analysis of the liver from high-fat diet induced obese mice identifies changes in multiple lipid classes

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ABSTRACT

Fatty liver is closely associated with obesity and sensitizes the liver to further insults. The aims of the current study are 1) to identify lipid species changed in rodent fatty liver, 2) to analyze for possible associations of these lipids with triglycerides, cholesterol or CXCL8 which is elevated in the steatotic liver and 3) to find out whether systemic levels of these lipids are concordantly altered. Lipidomic analysis has confirmed an already reported reduction of phosphatidylcholine in the steatotic liver. Phosphatidylserine is lower and phosphatidylethanolamine tends to be diminished. Sphingomyelin levels are normal while monounsaturated ceramides and hexosylceramides are reduced. Sixteen of the 20 fatty acid species measured in the total lipid fraction are elevated while α-linolenic acid is diminished. Of note, medium chain saturated fatty acids are markedly decreased. Plasmalogen 18:0 and 18:1 species are strongly increased in the steatotic liver. None of the markedly changed individual lipid species strongly correlates with hepatic CXCL8 mRNA, triglycerides or cholesterol. About 60% of the lipids altered in fatty liver are congruently altered in serum. These data show that there are multiple changes in lipid composition in fatty liver and part of the lipids may be monitored by serum analysis.

Introduction

The incidence of overweight and obesity has dramatically increased over the past decades and is closely associated with the rising prevalence of non-alcoholic fatty liver disease (NAFLD). More than 50% of obese people have liver steatosis which may progress to non-alcoholic steatohepatitis (NASH) and liver cirrhosis. Inappropriate storage of triglycerides in adipose tissues, increased dietary fat intake and de novo lipogenesis contribute to the accumulation of triglycerides in the liver (Bass, 2010; Donnelly et al., 2005). Liver steatosis is thought to sensitize the liver to the harmful effects of further insults (Bass, 2010; Buechler et al., 2011; Donnelly et al., 2005).

Recent studies have applied lipidomic techniques for in-depth analysis of hepatic lipids in NAFLD. Numerous changes in the hepatic lipid composition have been identified. In human fatty liver total triglycerides are markedly increased. Within triglycerides levels of saturated fatty acids (SFAs) are not altered while monounsaturated fatty acids (MUFAs) are elevated and polyunsaturated fatty acids (PUFAs) are decreased (Puri et al., 2007). Stearoyl-coenzyme A desaturase-1 (SCD1) converts the SFA palmitic acid and stearic acid to their MUFA derivatives palmitoleic acid and oleic acid, and is raised in murine fatty liver (Li et al., 2009).

In human steatotic liver free fatty acids, free cholesterol and cholesteryl ester (CE) are similar to non-steatotic organs. Unexpectedly, SFA CEs are reduced, MUFA CEs are not changed and PUFA CEs are even increased (Puri et al., 2007). Lecithin–cholesterol acyltransferase (LCAT) in serum forms the PUFA CEs 20:4, 22:5 and 22:6 and acyl CoA:cholesterol acyltransferase (ACAT) the MUFA CE 18:1 (Lee et al., 2004) suggesting that LCAT activity may be induced in patients with liver steatosis.

Fatty acid composition of phosphatidylcholine (PC) is not changed in the steatotic liver but total PC and phosphatidylethanolamine (PE) are decreased. Total phosphatidylserine, lysophosphatidylcholine (LPC) and sphingomyelin levels are not altered (Puri et al., 2007). Choline is reduced in fatty livers of rats and this may limit synthesis of PC (An et al., 2013). Reduced PC in human NAFLD has been confirmed in a second study while total PE is not changed in this cohort (Wattacheril et al., 2013). Total PCs are nevertheless increased in liver of mice fed a high-fat diet (HFD) (Kim et al., 2011). Further, LPC species 20:4 and 22:6 are raised and LPCs 14:0, 16:0 and 16:1 are reduced (Kim et al., 2011).

Ceramides are not affected in the liver of rats fed a high-fat diet (Ichi et al., 2007; Longato et al., 2012) while ceramide species 16:0 and 18:0 are even diminished when diet has been supplemented with cholesterol (Ichi et al., 2007). Ceramide and sphingomyelin are nevertheless found

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increased in the fatty liver of rats and blockage of ceramide synthesis ameliorates liver steatosis (Kurek et al., 2013). Therefore, data on lipid species altered in steatotic liver and possibly having a function in pathogenesis of further liver injury are not concordant. The intention of the current study was to measure various lipid species in the liver of male mice fed a standard chow or a high-fat diet for 14 weeks to identify and/or validate lipids associated with fatty liver disease.

Materials and methods

Materials

Triglyceride concentrations were measured using GPO-PAP microtest (purchased from Roche, Mannheim, Germany). Oligonucleotides were synthesized by Metabion (Planegg-Martinsried, Germany). LightCycler® 480 SYBR Green I Master was purchased from Roche (Mannheim, Germany). GAPDH antibody was from New England Biolabs GmbH (Frankfurt, Germany). ABCA1 antibody was from Abcam (Cambridge, UK) and SR-BI antibody was from US Biological (Massachusetts, MA, USA).

Animal model

Mice used herein have been recently described (Eisinger et al., 2014). The mice were ordered from The Jackson Laboratory (Bar Harbor, USA) and housed in a 21 ± 1 °C controlled room under a 12 h light–dark cycle. Animals had free access to food and water and were housed with 3 to 5 mice per cage. Blood was drawn after fasting overnight. Rising concentrations of CO_2 were used to produce loss of consciousness followed by cervical dislocation. Procedures were approved by the University of Regensburg Laboratory Animal Committee and complied with the German Law on Animal Protection.

Fourteen week old male C57BL/6 mice were kept on a high-fat diet (HFD) or standard chow (SD) for 14 weeks. Gross energy of SD (ssniff® EF acc. D12450B (I) mod.) was 17.8 MJ/kg, 70% of kJ was from carbohydrate, 20% from protein and 10% from fat. Gross energy of HFD (ssniff® EF R/M acc. D12451 (II) mod.) was 22.1 MJ/kg, 35% of kJ was from carbohydrate, 20% from protein and 45% from fat (Ssniff, Soest, Germany). Mice on a high-fat diet had increased body weight, fasting glucose and HOMA index. Serum triglycerides were not induced and serum cholesterol showed a trend to be higher in HFD (Eisinger et al., 2014).

Quantification of lipids

Lipids were quantified by direct flow injection electrospray ionization tandem mass spectrometry (ESI–MS/MS) in positive ion mode using the analytical setup and strategy described previously (Liebisch et al., 2004). A precursor ion of m/z 184 was used for phosphatidylcholine (PC) (Liebisch et al., 2004). A neutral loss of 141 and 277 Da was used for phosphatidylethanolamine (PE) and phosphatidylinositol (PI) (Matyash et al., 2008), respectively. Sphingosine based ceramides (Cer) and hexosylceramide (HexCer) were analyzed using a fragment ion of m/z 264 (Liebisch et al., 1999). Free cholesterol (FC) and cholesteryl ester (CE) were quantified using a fragment ion of m/z 369 after selective derivatization of FC (Liebisch et al., 2006). Total fatty acid composition was analyzed by gas chromatography coupled to mass spectrometry (GC–MS) (Ecker et al., 2012). Lipid species were annotated according to the recently published proposal for shorthand notation of lipid structures that are derived from mass spectrometry (Liebisch et al., 2013). Glycerophospholipid annotation is based on the assumption of even numbered carbon chains only. SM species annotation is based on the assumption that a sphingoid base d18:1 is present. In case the fatty acid composition was not determined, annotation represents the total number of carbons and double bonds. For example, PC 36:4 comprises species like PC 16:0/20:4 or 18:2/18:2. Liver lipids are given as nmol/mg wet weight.

Monitoring of gene expression by real-time RT-PCR

The mRNA expression was investigated by semiquantitative real-time PCR using SYBR Green. Total cellular RNA was isolated with TRIzol reagent from Life Technologies (Darmstadt, Germany) and 1 μg RNA was reverse transcribed using the reverse transcription system (Promega, Madison, USA) in a volume of 40 μl; 2 μl of the cDNA was used for amplification in LightCycler 480 system (Roche, Mannheim, Germany). Murine SCD1 was amplified with the primers 5′ ccg gga gaa tat cct ggt tt 3′ and 5′ cac ccc gat agc aat atc ca 3′, CYP7A1 with 5′ cac ata aag ccc ggg aaa g 3′ and 5′ ggc tgc ttt cat tgc ttc a 3′, CYP27 with 5′ gag atg caa ctg atg ctg tca 3′ and 5′ ttg tgc cag aca ttt ggt gt 3′, CXCL8 with 5′ ctt gaa ggt gtt gcc ctc ag 3′ and 5′ tgg gga cac ctt tta gca tc 3′, and 18S rRNA with 5′ gat tga tag ctc ttt ctc gat tcc 3′ and 5′ cat cta agg gca tca cag acc 3′. Real-time RT-PCR was performed using the LightCycler® 480 SYBR Green I Master (Roche, Mannheim, Germany) and the specificity of the PCRs was confirmed by sequencing of the amplified DNA fragments (Geneart, Regensburg, Germany). For quantification of the results RNA of respective liver samples was reverse transcribed, and cDNA was serially diluted and used to create a standard curve for each of the genes analyzed. The second derivative maximum method was used for quantification with the LightCycler software. Results were normalized to the housekeeping gene 18S rRNA.

SDS-PAGE and immunoblotting

Proteins (10–20 μg) were separated by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes (Bio-Rad, Munich, Germany). Incubations with antibodies were performed in 1.5% BSA in TBS, and 0.1% Tween. Detection of the immune complexes was carried out with the ECL Western blot detection system (Amersham Pharmacia, Deisenhofen, Germany). Quantification was done using an ImageJ software (Schneider et al., 2012).

Statistical analysis

Data are presented as median values and range of the values. Statistical differences were analyzed by two-tailed Mann–Whitney U test (SPSS Statistics 19.0 program, IBM, Leibniz Rechenzentrum, München, Germany) and a value of $p < 0.05$ was regarded as significant. Spearman correlations (IBM SPSS Statistics 19.0 program) were calculated. Correlations of lipid species with CXCL8, triglycerides or cholesterol were regarded as relevant when $p < 0.05$ and $R^2 > 0.5$ after adjusting for body weight.

Results

Fatty acid profile

Hepatic steatosis in the mice on a high-fat diet (HFD) was confirmed by histology (Fig. 1A) and elevated hepatic triglycerides (Fig. 1B). Liver weight to body weight ratio was not higher in the HFD fed animals (Fig. 1C).

CXCL8 (mouse homolog KC) was increased in the liver indicating mild inflammation (Fig. 1D). As expected, total fatty acids (FAs) were markedly elevated in the fatty liver (Fig. 1E), and concentrations of total saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) FAs were increased (data not shown). Analysis of single FA species showed that 16 of the 20 different fatty acid species measured were elevated (data not shown). Of note, linoleic acid tended to be decreased ($p = 0.065$, data not shown) and α-linolenic acid was significantly diminished (Fig. 1F). Concentration of all n-3 PUFA species measured was, however, similar in liver of SD and HFD fed mice. The content of n-6 PUFAs and subsequently n-6/n-3 ratio was not changed (data not shown). Interestingly, capric acid and lauric acid were