Biochimica et Biophysica Acta xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Peroxisomes compensate hepatic lipid overflow in mice with fatty liver

Birgit Knebel [a], Sonja Hartwig [a], Jutta Haas [b], Stefan Lehr [a], Simon Goeddeke [a], Franciscus Susanto [a], Lothar Bohne [a], Sylvia Jacob [a], Cornelia Koellmer [a], Ulrike Nitzgen [a], Dirk Müller-Wieland [b], Jorg Kotzka [a,*]

[a] Institute of Clinical Biochemistry and Pathobiochemistry, German Diabetes Center, Leibniz Center for Diabetes Research, Heinrich-Heine-University Duesseldorf, Aufm Hennekamp 65, Duesseldorf 40225, Germany

[b] Institute for Diabetes Research, Department of General Internal Medicine, Asklepios Clinic St. Georg, Medical Faculty of Semmelweis University, Asklepios Campus Hamburg, Lohmuehlen Str 5, Hamburg 20099, Germany

ARTICLE INFO

Article history:
Received 30 January 2015
Accepted 10 March 2015
Available online xxxx

Keywords:
Mitochondrial and peroxisomal protein profiles
Fatty acids
Cytokines
Dyslipidemia
NAFLD
Gene expression

ABSTRACT

Major causes of lipid accumulation in liver are increased import or synthesis or decreased catabolism of fatty acids. The latter is caused by dysfunction of cellular organelles controlling energy homeostasis, i.e., mitochondria. Peroxisomes also appear to be an important organelle in lipid metabolism of hepatocytes, but little is known about their role in the development of non-alcoholic fatty liver disease (NAFLD). To investigate the role of peroxisomes alongside mitochondria in excessive hepatic lipid accumulation, we used leptin-resistant db/db mice on C57BLKS background, a mouse model that develops hyperphagia-induced diabetes with obesity and NAFLD. Proteome and gene expression analyses along with lipid analyses in the liver revealed differential expression of genes related to lipid metabolism and β-oxidation, whereas genes for peroxisomal proteins were predominantly regulated.

Conclusion: Our investigations show that in fatty liver disease in combination with obesity and diabetes, the hepatocyte-protecting organelle peroxisome is altered. Hence, peroxisomes might indicate a stage of pre-NAFLD play a role in the early development of NAFLD and appear to be a potential target for treatment and prevention of NAFLD.

1. Introduction

Increased lipid accumulation in the liver is the clinical hallmark of non-alcoholic fatty liver disease (NAFLD) and is almost always found in patients with a combination of obesity and type 2 diabetes. The clinical spectrum of NAFLD ranges from fatty liver simply due to intracellular lipid accumulation, to fatty liver with inflammatory cell infiltration and signs of inflammation, e.g., steatohepatitis, which might progress to fibrosis and cirrhosis [1,2].

The liver is the key organ that regulates lipid metabolism, especially that of cholesterol and triglycerides. On a cellular level, lipid metabolism is a prominent example that compartmentation of cellular processes allows efficiency and tight control. The major subcellular compartments controlling intracellular lipid homeostasis are: cytosol for synthesis; lipid droplets for storage; and mitochondria for degradation, but peroxisomes also appear to be responsible. Although the exact mechanisms are unknown, hepatic lipid accumulation is the result of an imbalance between syntheses, storage and catabolism of fatty acids (FAs) [3]. One cause of the disturbance is the increased availability of free fatty acids (FFAs) in the serum, which can accumulate in the liver. This overflow causes alterations in de novo lipogenesis, export of lipids or fatty oxidation rates. The decreased oxidative phosphorylation in mitochondria has been shown to be associated with reduced insulin sensitivity and increased intracellular lipid accumulation in non-diabetic insulin-resistant individuals [3–5]. Together, any defect in these processes could result in an increased accumulation of lipids in hepatocytes, which can be either the cause or the result of fatty liver.

β-Oxidation of fatty acids in eukaryotes occurs mainly in mitochondria and, to a lesser extent, in peroxisomes. Although there are enzymatic and functional overlaps, both organelles differ in respect to substrate specificities, FA import systems, the amount of reactive oxygen species and net energy (i.e., adenosine triphosphate [ATP] production) from β-oxidation. Even though peroxisomes have specialized substrate specificity and an inefficient β-oxidation energy balance, in contrast to mitochondria, the lipid uptake is not restricted by a substrate-inhibited feedback mechanism. Therefore, it is tempting to speculate that peroxisomes appear to be designated to protect the liver from lipotoxicity.

Recently, we have shown that the direct, parallel comparison of mitochondria and peroxisomes at a proteomic level allows the dissection of functional overlaps and specificities between both organelles [6]. Here, we wanted to test the hypothesis that in the leptin-resistant

Abbreviations: NAFLD, non-alcoholic fatty liver disease; FA, fatty acids; FFA, free fatty acids; TFA, total FFA; mtDNA, mitochondrial deoxyribonucleic acid; RT-PCR, real-time polymerase chain reaction; ANOVA, analysis of covariance; HOMA-IR, homeostatic model assessment of insulin resistance; PUFA, polyunsaturated FFAs; KEGG, Kyoto Encyclopedia of Genes and Genomes; NASH, non-alcoholic steatohepatitis

* Corresponding author. Tel.: +49 211 3382 531.
E-mail address: jkotzka@ddz.uni-duesseldorf.de (J. Kotzka).

http://dx.doi.org/10.1016/j.bbalip.2015.03.003
1388-1981/

diabetic (db/db) mouse model (BKS.Cg-Lepr^{db}) with obesity and diabetes, fatty liver is associated with alterations in both mitochondria and peroxisomes. This mouse model reflects a status of dyslipidemia due to increased lipid intake by hyperphagia and decreased lipid catabolism [7,8]. We compared db/db mice and their C57BLKs (BKS) littermates in terms of cytokine patterns, ectopic lipid accumulation in hepatocytes, lipid profiles and mitochondrial and peroxisomal protein or gene expression profiles in the liver. The results indicate that peroxisomes might play a role in the development of fatty liver disease.

2. Materials and Methods

2.1. Animals

C57BL/KS$^{lepr+/+}$ (BKS) and BKS.Cg-Lepr^{db} (db/db) mice were bred and maintained in a regular 12 h light/dark cycle under constant temperature and humidity (22 ± 1 °C, 50 ± 5% humidity). Genotyping was performed according to Horvat and Bunger [9]. At 6 weeks of age, male littermates of each genotype ($n = 20$ of each) were kept under standardized conditions with free access to water and standard laboratory food (Ssniff, Soest, Germany). Weight gain and food intake of male mice were measured once a week and monitored for an observation period of 8 weeks. Food uptake per body weight and weight gain per food uptake were determined in each group of mice as the mean of the observation period. Mice were sacrificed by CO_2 asphyxiation at 14 weeks of age. Blood samples were collected by left ventricular puncture, and organ samples were removed. The Animal Care Committee of the University Duesseldorf approved animal care and procedures (Approval#50.05-240-35/06).

2.2. Animal characterization

Phenotypical characterization, serum diagnostics of clinical parameters, lipid profiling in serum and liver tissue and determination of the cytokine profiles with low-density proteome arrays (Proteome Profiler™; R&D Systems, Abingdon, UK) were performed as previously described[10,11]. Triglycerides, cholesterol and liver enzymes (ALT, AST, GLDH) were determined on a Hitachie 912 laboratory automat (Roche Diagnostics, Mannheim, Germany). Leptin, insulin and PAI were determined by Multiplex immune assay (BioRad, Munich, Germany) Serum FFA and hepatic TFA content and specific fractional composition of FAs were determined by gas chromatography. FA data in the liver were further used to calculate the desaturase index (cC16:1/C16:0), DNL index (C16:0/cC18:2) and elongation index (C18:0/C16:0).

2.3. Subcellular fractionation and marker enzyme activity

Mitochondria and peroxisomes were prepared as formerly described from 1.5 g freshly isolated liver tissue, and the organelle quality of all preparation steps was monitored by assessing marker enzyme activity and electron microscopy [6].

2.4. 2D-DIGE™ and protein identification by MALDI-MS

2D-DIGE™ experiments of subcellular organelles and data analyses were performed as previously described [6]. Significantly altered protein spots had to be present in all replicate experiments. The analysis parameters were set to a standardized average spot volume ratio of 1.7-fold, $p < 0.01$ and a coefficient of variation (CV) of 20%. All selected protein spots were excised from four separate 2D-DIGE™ gels and analyzed by MALDI-MS in a time-of-flight Ultraflex-Tof/Tof (BrukerDaltoniks, Bremen, Germany) as previously described[8]. Further analyses for protein identification against the mouse sub-set of Swiss-Prot (Sprot_2014) non-redundant database and mitochondrial or peroxisomal reference maps from our database (http://www.diabesityprot.de/) were performed as described [6].

2.5. Quantification of mtDNA

Quantification of mouse mtDNA copy number was performed by quantitative PCR (qPCR) with primers and double-fluorescent probes (Eurogentec, Liège, Belgium) on an ABI Prism 7000 Sequence Detection System (Life Technologies, Darmstadt, Germany) with NADH dehydrogenase subunit 1 gene (*ND1*) for quantification of mtDNA (forward: 5′-CTACAACCATTTGCAGACGC 3′, reverse: 5′ GGAACTCATAGACTTA ATGC 3′, probe: 5′ CCAATACGCCCTTTAACAACCTC 3′) and lipoprotein lipase (*LPL*)) as nuclear target (forward: 5′ GGTTTGGATCCAGCTGGG CC 3′, reverse: 5′ GATTCCAATACTTCGACCAGG 3′, probe 5′ CTTTGAGT ATGCAGAAGCCC 3′. Gene copy numbers were determined in comparison to log–linear standard curves determined from both PCR products subcloned into TOP-TA-cloning vectors (Life Technologies, Darmstadt, Germany) to distinct copy numbers for both plasmids.

2.6. Gene expression analyses

RNA extraction (Qiagen, Hilden, Germany) of biopsies and RT-PCR with gene-specific probes and 18S RNA as internal standard (Assay on Demand™, Applied Biosystems, Darmstadt, Germany) to determine relative RNA amounts of the specific target was performed as described [12].

RNA samples ($n = 4$ per genotype) for genome-wide gene expression analyses were processed according to the GeneChip One-Cycle eukaryotic Target Labeling Assay manufacturer's recommendations. Synthesis steps were quality controlled and monitored with an RNA 6000 nano kit (Agilent, Taufkirchen, Germany). Complementary RNA (cRNA) samples were hybridized to Affymetrix GeneChip Mouse 430_2 (Affymetrix UK Ltd, Lahr, Germany). Detection of probe sets was performed using a GeneChip scanner 3000 7G (GDAS 1.4 package, Affymetrix). Data were analyzed with Genespring 12.0 (Agilent). Volcano

Table 1
Physiological parameters of BKS and db/db mice at 14 weeks of age.

	BKS ($n = 20$)	db/db ($n = 20$)
Body weight (g)	26.7 ± 1.8	46.2 ± 6.9*
Food uptake/bodyweight (kJ/g)	13.2 ± 0.5	19.4 ± 1.8*
Weight gain/food uptake (mg/kJ)	2.0 ± 0.6	3.1 ± 0.7*
Liver weight (g)	1.5 ± 0.2	3.2 ± 0.8*
Blood glucose (mmol/l)	7.0 ± 1.6	43.5 ± 12.6*
Insulin (μU/ml)	1.5 ± 1.1	2.9 ± 1.8*
HOMA-IR (mg μU/ml)	0.5 ± 0.2	5.7 ± 3.1*
Leptin (ng/ml)	2.0 ± 1.8	26.3 ± 8.1*
ALT (U/l)	45.6 ± 18.4	175.4 ± 44.3*
AST (U/l)	49.4 ± 18.3	257.9 ± 87.5*
GLDH (U/l)	14.2 ± 7.6	128.1 ± 62.7*
PAI (ng/ml)	2.9 ± 0.6	5.7 ± 1.7*
Cholesterol (mg/dl)	98.1 ± 14.8	127.0 ± 33.8
Triglyceride (mg/l)	22.7 ± 3.3	116.2 ± 27.7*
sICAM-1/CD54 (ng/ml)#	101.5 ± 5.5	23.7 ± 3.0***
CXCL1 (ng/ml)#	7.9 ± 0.4	2.6 ± 0.3**
CSF1 (ng/ml)#	66.6 ± 3.6	25.7 ± 3.6**
MCP-1 (ng/ml)#	13.7 ± 0.8	1.0 ± 0.1***
TIMP-1 (ng/ml)#	19.0 ± 1.0	7.6 ± 1.2**
TREM-1 (ng/ml)#	7.8 ± 0.5	1.4 ± 0.2***

Data are expressed as mean ± SD.
Abundances of CXCL13, CSF-2, CSF-3, CCL-1, CCL-11, IFN-γ, IL1-α, IL1-β, IL-1ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12-p70, IL-13, IL-16, IL-17, IL-23, IL-27, CXCL-10, CXCL-11, MCP-5, CXCL-9, CCR-1a, CCL-4, CCL-2, CCL-5, CXCL-12, CCL-17 or TNF-α were below the detection limit in serum for BKS and db/db mice.
ALT, alanine transaminase; AST, aspartate transaminase; CCL, chemokine (C-C motif) ligand; CSF, colony stimulating factor; CXCL, chemokine (C-X-C motif) ligand; GLDH, glutamate dehydrogenase; HOMA-IR, homeostatic model assessment of insulin resistance; IFN, interferon; IL, interleukin; MCP, monocyte chemo attractant protein; PAI, plasminogen activator inhibitor; sICAM, soluble intercellular adhesion molecule; TIMP, metalloproteinase; TNF, tumor necrosis factor; TREM, triggering receptor expressed on myeloid cells.
* $p < 0.01$ by Student's t test.
** $p < 0.01$.
*** $p < 0.001$ by 2-way ANOVA.
\# In six mice of each genotype.

Please cite this article as: B. Knebel, et al., Peroxisomes compensate hepatic lipid overflow in mice with fatty liver, Biochim. Biophys. Acta (2015), http://dx.doi.org/10.1016/j.bbalip.2015.03.003