Research Article

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Malnutrition-associated liver steatosis and ATP depletion is caused by peroxisomal and mitochondrial dysfunction

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Background & Aims: Severe malnutrition in young children is associated with signs of hepatic dysfunction such as steatosis and hypoalbuminemia, but its etiology is unknown. Peroxisomes and mitochondria play key roles in various hepatic metabolic functions including lipid metabolism and energy production. To investigate the involvement of these organelles in the mechanisms underlying malnutrition-induced hepatic dysfunction we developed a rat model of malnutrition.

Methods: Weanling rats were placed on a low protein or control diet (5% or 20% of calories from protein, respectively) for four weeks. Peroxisomal and mitochondrial structural features were characterized using immunofluorescence and electron microscopy. Mitochondrial function was assessed using high-resolution respirometry. A novel targeted quantitative proteomics method was applied to analyze 47 mitochondrial proteins involved in oxidative phosphorylation, tricarboxylic acid cycle and fatty acid β-oxidation pathways.

Results: Low protein diet-fed rats developed hypoalbuminemia and hepatic steatosis, consistent with the human phenotype. Hepatic peroxisome content was decreased and metabolomic analysis indicated peroxisomal dysfunction. This was followed by changes in mitochondrial ultrastructure and increased mitochondrial content. Mitochondrial function was impaired due to multiple defects affecting respiratory chain complex I and IV, pyruvate uptake and several β-oxidation enzymes, leading to strongly reduced hepatic ATP levels. Fenofibrate supplementation restored hepatic peroxisome abundance and increased mitochondrial β-oxidation capacity, resulting in reduced steatosis and normalization of ATP and plasma albumin levels.

Conclusions: Malnutrition leads to severe impairments in hepatic peroxisomal and mitochondrial function, and hepatic metabolic dysfunction. We discuss the potential future implications of our findings for the clinical management of malnourished children.

Lay summary: Severe malnutrition in children is associated with metabolic disturbances that are poorly understood. In order to study this further, we developed a malnutrition animal model and found that severe malnutrition leads to an impaired function

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Abbreviations: VLCFA, very long-chain fatty acids; FAO, fatty acid β-oxidation; TCA, tricarboxylic acid; LPD, low protein diet; QconCAT, concatamers; TG, triglycerides; ALT, alanine-aminotransferase; TBARS, thiobarbituric acid reactive substances; EM, electron microscopy; PMP70, peroxisomal membrane protein 70; DHCA, dihydroxycholestanoic acid; THCA, trihydroxycholestanoic acid; Hsp60, mitochondrial heat shock protein 60; Mfn2, mitofusin-2; PM, pyruvate plus malate; PCM, palmitoyl-CoA plus L-carnitine plus malate; SPM, succinate plus pyruvate plus malate; OXPHOS, oxidative phosphorylation; FA, fatty acid; AMPKα, adenosine monophosphate-activated protein kinase α subunit; Cpt, carnitine palmitoyltransferase; Acadm, medium-chain specific acyl-CoA dehydrogenase; Hadh, medium and short-chain L-3-hydroxyacyl-coenzyme A dehydrogenase; PPARα, peroxisome proliferator-activated receptor alpha; UCP, uncoupling protein.

of liver mitochondria which are essential for energy production and a loss of peroxisomes, which are important for normal liver metabolic function.

Introduction

Malnutrition still contributes to 45% of all global childhood deaths below the age of 5 years [1]. Despite great achievements in improved management of severely malnourished children, for example through development of a World Health Organization Guideline in 1999 [2], the condition remains difficult to treat and in hospital fatality rates can be more than 30% [3,4]. In fact, it is estimated that more than a half of a million children die each year from severe malnutrition [1].

Severe malnutrition is defined by wasting but can also present with more complex phenotypical characteristics, including edema, hair discoloration, and hepatomegaly. Although a deficiency of dietary protein and calories underlie the development of severe malnutrition [5], the metabolic disturbances at the cellular level remain an enigma. Children hospitalized for severe malnutrition are initially presented with profound electrolyte disturbances, increased oxidative stress, hepatic steatosis and decreased albumin synthesis (hypoalbuminemia) [6,7]. In addition, a recent study found an altered gut microbiome in children with severe malnutrition, further underscoring the complexity of this disease state [8].

Peroxisomes and mitochondria play key roles in various hepatic metabolic functions including lipid metabolism and energy production, and dysfunction in these organelles are linked to various disorders affecting liver, e.g., Zellweger syndrome and non-alcoholic fatty liver disease [9]. Peroxisomes are single membrane bound organelles that are important for bile acid synthesis, β-oxidation of very long-chain fatty acids (VLCFA) and cellular redox homeostasis [10], as well as the maintenance of normal mitochondrial function [11]. In turn, mitochondria are essential for aerobic ATP production, fatty acid β-oxidation (FAO) (acyl-chain length of $\leqslant$C20), ketogenesis and gluconeogenesis from pyruvate and tricarboxylic acid (TCA) cycle intermediates. Defect in these two organelles have been shown to cause various degrees of liver dysfunction resulting in clinical phenotypes similar to those observed in severely malnourished individuals, e.g., increased oxidative stress and hepatic steatosis [12,13]. Furthermore, we recently provided some evidence for a relative decrease in hepatic mitochondrial function in severely malnourished children with signs of metabolic maladaptation [14].

To study the physiological and cellular changes that drive hepatic pathogenesis in malnutrition we placed weanling rats on a low protein diet (LPD) for four weeks. Using this animal model, we showed that a LPD led to early loss of hepatic peroxisomes followed by mitochondrial dysfunction with severe metabolic disturbances and energy depletion. Induction of peroxisomal biogenesis and boosting of mitochondrial FAO by fenofibrate treatment resulted in a profound amelioration of the metabolic phenotype, including normalization of serum albumin levels and restored hepatic energy status.

Materials and methods

Animals

Pregnant Wistar rats (Harlan Laboratories, Venray, The Netherlands) were housed in a temperature-controlled environment (21 °C) and 12 h, 12 h light:dark cycle with *ad libitum* access to regular chow and water. The offspring were kept with the mother until day 21 when male animals were placed on either a LPD (5% of calories from protein) or control diet (20% of calories from protein, as described in Supplementary Table 1) (Harlan, Madison, WI, USA). Animals were kept on the diet for 1 or 4 weeks as indicated. In a different set of experiments, the offspring were weaned to a 5% protein and after 2 weeks, half of the rats received the 5% protein diet supplemented with 0.1% (wt/wt) fenofibrate for another 2 weeks. The animal treatment conformed to the guidelines of The Institutional Animal Care and Use Committee of the University of Groningen and was in accordance with EC Directive 86/609/EEC for animal experiments.

Biochemical analyses

Plasma and hepatic metabolite analysis, enzyme activity assays and Western blot analysis were carried out as detailed in the Supplementary materials.

Histology and microscopy

Livers were immediately fixed after harvesting as detailed in the Supplementary materials followed by histological and ultrastructural analyses.

Isolation of liver mitochondria and high-resolution respirometry

Liver mitochondria were isolated by differential centrifugation and oxygen consumption rates were measured at 37 °C using a two-channel high-resolution Oroboros oxygraph-2 k (Oroboros, Innsbruck, Austria) as described in detail in Supplementary materials.

Targeted quantitative mitochondrial proteomics

Selected 47 mitochondrial proteins involved in substrate transport, FAO and TCA cycle were quantified in isolated mitochondria using isotopically labeled standards (^{13}C-labeled lysines and arginines), derived from synthetic protein concatemers (QconCAT) (PolyQuant GmbH, Bad Abbach, Germany) as described in Supplementary materials.

Transcriptomics

RNA expression profiling was performed using Affymetrix Gene chip Rat Gene 1.1 ST arrays according to standard Affymetrix protocols and data were deposited to Gene Expression Omnibus (NCBI, accession number GSE63096).

Statistical analysis

All values are reported as means ± S.D. Depending on the type of experiment, Student's *t* test or ANOVA was used for statistical evaluation of data as described in detail in Supplementary materials. All analyses were performed with IBM SPSS Statistics 22.0 (SPSS Inc., Chicago, IL, USA). Differences were considered significant at p <0.05.

Results

LPD leads to severe growth retardation and hepatic steatosis

We first aimed to determine the general phenotypic effects of a LPD diet. Fig. 1 shows basic animal characteristics after 4 weeks of LPD or control diet (5% and 20% of calories from protein, respectively). We aimed to keep the total caloric density and fat content equal between the diets. The LPD-fed rats had a