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Hepatic NAD salvage pathway is enhanced in mice on a high-fat diet

Melanie Penke [a,b,*], Per S. Larsen [c], Susanne Schuster [a,b], Morten Dall [c], Benjamin A.H. Jensen [d], Theresa Gorski [a], Andrej Meusel [d,e], Sandy Richter [a], Sara G. Vienberg [c], Jonas T. Treebak [c], Wieland Kiess [a,b], Antje Garten [a]

[a] Center for Pediatric Research Leipzig (CPL), University Hospital for Children & Adolescents, University of Leipzig, Liebigstr. 21, 04103 Leipzig, Germany
[b] LIFE Leipzig Research Centre for Civilization Diseases, University of Leipzig, Philipp-Rosenthalstr. 27, D-04103 Leipzig, Germany
[c] The Novo Nordisk Foundation Center for Basic Metabolic Research, Section of Integrative Physiology, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark
[d] Department of Biology, Laboratory for Genomics and Molecular Biomedicine, Faculty of Science, University of Copenhagen, Copenhagen, Denmark
[e] Institut für Medizinische Physik und Biophysik, University of Leipzig, Härtelstr. 16-18, 04107 Leipzig, Germany

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ABSTRACT

Nicotinamide phosphoribosyltransferase (Nampt) is the rate-limiting enzyme for NAD salvage and the abundance of Nampt has been shown to be altered in non-alcoholic fatty liver disease. It is, however, unknown how hepatic Nampt is regulated in response to accumulation of lipids in the liver of mice fed a high-fat diet (HFD). HFD mice gained more weight, stored more hepatic lipids and had an impaired glucose tolerance compared with control mice. NAD levels as well as Nampt mRNA expression, protein abundance and activity were significantly increased in HFD mice. Enhanced NAD levels were associated with deacetylation of p53 and Nfκb indicating increased activation of Sirt1. Despite impaired glucose tolerance and increased hepatic lipid levels in HFD mice, NAD metabolism was significantly enhanced. Thus, improved NAD metabolism may be a compensatory mechanism to protect against negative impact of hepatic lipid accumulation.

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1. Introduction

More than 30% of Western adult population is affected by non-alcoholic fatty liver diseases (NAFLD) with a rapidly increasing number in obese and diabetic people. NAFLD covers a wide range of conditions from simple steatosis caused by accumulation of triglycerides to non-alcoholic steatohepatitis (NASH) which is associated with inflammatory processes. NASH is a risk factor for fibrosis and cirrhosis which are associated with a significant liver-related mortality and a median survival of 6 years (Tarantino and Finelli, 2013).

The pathological pathways leading to NASH are not yet fully understood. Interestingly, specific sirtuins (Sirts) seem to affect the development of NAFLD by regulating processes like hepatic gluconeogenesis, mitochondrial biogenesis and fatty acid synthesis. Liver-specific Sirt1 knockout mice develop hepatic steatosis and show impaired insulin signaling on a high-fat diet (HFD) (Purushotham et al., 2009), while global transgenic mice overexpressing Sirt1 are protected against the negative effects of a HFD such as inflammation, impaired glucose tolerance and hepatic steatosis (Pfluger et al., 2008). Additionally, whole-body Sirt3 knockout mice show enhanced storage of hepatic triglycerides and insulin resistance (Hirschey et al., 2010) although liver-specific Sirt3 knockout mice do not display an overt phenotype (Fernandez-Marcos et al., 2012).

Sirtuins are nicotinamide adenine dinucleotide (NAD) dependent in respect to their deacetylase activity. The key enzyme in the mammalian NAD salvage pathway starting from nicotinamide is nicotinamide phosphoribosyltransferase (Nampt) which converts nicotinamide to nicotinamide mononucleotide (NMN), an intermediate in NAD biosynthesis (Revollo et al., 2007a). Additionally, Nampt is secreted from adipocytes (Tanaka et al., 2007), hepatocytes (Garten et al., 2010), and leucocytes (Friebe et al., 2011). Nampt circulates in the blood where it acts as a NMN biosynthetic enzyme (Revollo et al., 2007b) and/or cytokine (Samal et al., 1994). Several human studies have investigated how Nampt protein levels in biopsies and blood are affected in individuals with steatosis and NASH, however, results are conflicting (Auguet et al., 2013; Dahl et al., 2010; Kukla et al., 2010).

It has been shown that rats fed a HFD develop fatty liver and hepatic insulin resistance already after 3 days of HFD feeding (Samuel et al., 2004). Moreover, another study in mice showed that hepatic insulin resistance is present already after 3 days on a HFD before insulin resistance develops in muscle and adipose tissue (Turner et al., 2013). Thus, hepatic insulin resistance develops very rapidly, but whether NAD metabolism is also affected in the early stages of the development

* Corresponding author. Center for Pediatric Research Leipzig (CPL), University Hospital for Children & Adolescents, University of Leipzig, Liebigstraße 21, 04103 Leipzig, Germany. Tel.: +49/341 97 26513; fax: +49/341 97 26069.
E-mail address: melanie.penke@medizin.uni-leipzig.de (M. Penke).

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of NAFLD is unknown. We hypothesize a link between the development of NAFLD and the ability to maintain NAD levels. To define the effect of lipid accumulation on hepatic NAD metabolism, we fed mice a HFD for 11 weeks and examined the effect on hepatic NAD metabolism. Despite hepatic lipid accumulation and impaired whole body glucose tolerance in mice fed a HFD, we found an improved hepatic NAD metabolism suggesting that this may be a compensatory mechanism to protect against the negative impact of hepatic lipid accumulation on inflammation and apoptosis.

2. Methods and procedures

2.1. Mice and Ethical approval

All experiments were approved by the Danish Animal Experimental Inspectorate and complied with the EU convention for protection of vertebra animals used for scientific purposes (Council of Europe 123, Strasbourg, France, 1985). Twenty-four male mice were obtained from Taconic when they were 10 weeks of age (C57BL/6Ntac, #DIO-B6). At the time of arrival in the laboratory, mice had been on a HFD (Research Diets Industry, D12492) or corresponding chow diet (Ctl) for 4 weeks. Mice were kept in a 12:12-h light:dark cycle and kept on the diet for additional 7 weeks. Water was given ad libitum. Mice were either single- (s) or group-housed (gr). To confirm that housing conditions (i.e., single- vs. group-housing) did not affect NAD metabolism, we measured Nampt protein abundance and NAD levels in a separate cohort of mice. In this cohort we applied 26 carefully weight matched male mice (C57BL/6JBomTac) at 7 weeks of age. Upon arrival, the mice were acclimatized on a standard chow diet for 10 days before they were randomly assigned to the experimental groups and fed either a HFD (D12492) or a corresponding low fat diet (D12450J) for 11 weeks. The mice were either single-housed (5 mice per group per diet) or group-housed (8 mice per group per diet, 4 mice per cage) from the point of arrival to the day of termination. Housing conditions did not affect hepatic Nampt protein and NAD levels (Fig. S1A,B). After 11 weeks on their respective diets, mice were anesthetized (Pentobarbital, 100 mg/kg body weight) and livers were carefully dissected, frozen in liquid nitrogen, and stored at –80 °C until further analysis. Mice were all sacrificed at the same time point.

2.2. Metabolic characterization

Animals were weighed and body composition was assessed by MR scanning (EchoMRI™, USA). At the age of 14 and 15 weeks an oral glucose tolerance test (OGTT) and an insulin tolerance test (ITT) were performed, respectively. Both tests were performed after a 6-hour fast and blood glucose measurements were done from the tail vein. For the OGTT a bolus of glucose (25% glucose/saline solution, 2 g glucose/kg lean body mass) was delivered by oral gavage, and tail vein blood glucose was measured just before and at 20, 40, 60, 90 and 120-minutes after the glucose bolus. The ITT was performed following 8 days of recovery after the OGTT. Insulin (100 U/ml) was diluted in gelofusine (B. Braun, Denmark) for a working solution of 0.333 U/ml, and administered by intraperitoneal injection (0.75 U/kg lean body mass). Blood glucose in the tail blood was measured before and at 15, 30, 45, 60, 90 and 120-minutes after the injection. The mice were conscious, and placed in their cages during both tests. A week before collecting tissues, 200 µl of blood was drawn from the mandible into EDTA coated tubes, left to clot at room temperature for 30-minutes and spun down at 2000 × *g* at 4 °C for 10-minutes. Supernatants were transferred to clean tubes and stored at –80 °C.

2.3. Triacylglycerol measurement of liver tissue

The triacylglycerol (TAG) content of the liver tissue was quantified by ^{1}H-high resolution magic-angle spinning–nuclear magnetic resonance spectroscopy. Five to fifteen milligrams of intact liver tissue was transferred into a 4 mm zirconia HR-MAS rotor with a volume of 15 µl. Additionally, 5 µl of a 100 µM solution of trisodium phosphate was added as reference. The NMR spectra were recorded using a 600 MHz Bruker Avance III NMR spectrometer (Bruker, Rheinstetten, Germany). All measurements were conducted under MAS at a frequency of 9 kHz and a temperature of 25 °C. The water signal was suppressed by presaturation (1 mW power for 3 s). NMR spectra were excited by a $\pi/2$ pulse of 4 µs duration. To allow for complete relaxation, the delay time between successive scans was 30 s. The amount of TAG was determined by comparing the integrals of the trisodium phosphate signal with the glycerol backbone signal at 4.29 ppm which is exclusively found in TAG. All spectra were corrected for baseline and phase distortions using Spinworks (University of Manitoba). Deconvolution of the signals was performed using an in-house written SciPy script. The peaks were fitted to a Voigt-Profile applying a constrained least-squares approach based on the L-BGFGS optimization algorithm (Zhu et al., 1997). The TAG content is expressed per gram liver tissue.

2.4. Protein extraction, western blot analyses and immunoprecipitation

Approximately 10 mg liver tissue was lysed in modified RIPA buffer as previously described (Schuster et al., 2014). Protein concentration was determined using Pierce BCA protein assay (Thermo Scientific) and equal amounts of protein were separated by SDS–PAGE and transferred using a semi-dry transfer apparatus to nitrocellulose membranes. Next, membranes were blocked in 5% non-fat dry milk in TBS buffer containing 0.1% Tween 20. Applied antibodies are listed in Supplementary Table S1. Detection of proteins was carried out using Luminata Classico Western HRP Substrate (Merck Millipore) or Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare). Gapdh immunoblotting was performed as a loading control. For immunoprecipitation livers were incubated with anti-acetyl lysine antibody conjugated to µMACS Protein A Micro Beads (Miltenyi Biotec) overnight and eluted with 1× SDS sample buffer. After 5 min heating, samples were analyzed by Western blotting with an anti-Srebp1 antibody (H-160, Santa Cruz Biotechnology).

2.5. Total RNA extraction and realtime qPCR

Total RNA of liver tissue was extracted by TRIzol® Reagent (Life Technologies) according to manufacturer's protocol. One microgram of total RNA was transcribed into cDNA by M-MLV Reverse Transcriptase (Invitrogen). Quantitative PCR analyses were performed using the qPCR Master Mix Plus Low ROX (Eurogentec) or Absolute qPCR SYBR Green Low ROX Mix (Thermo Scientific) and the Applied Biosystems 7500 Real Time PCR System. Primer sequences are summarized in Supplementary Table S2.

2.6. NAD measurement

NAD was measured by EnzyChrom™ NAD^+/NADH Assay Kit (E2ND-100, Biotrend) according to manufacturer's protocol or reversed-phase HPLC using the Chromaster Purospher STAR RP-18 endcapped 3 µm Hibar RT 150-3 HPLC column (Merck). Ten milligrams of frozen liver tissue was sonicated in 100 µl 1 M perchloric acid. After a 10-minute incubation period on ice samples were centrifuged and the supernatant was neutralized with 3 M potassium carbonate. After repeated centrifugation samples were loaded onto the column as previously described (Schuster et al., 2014).

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