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Alpha-syntrophin null mice are protected from non-alcoholic steatohepatitis in the methionine-choline-deficient diet model but not the atherogenic diet model

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ABSTRACT

Adipose tissue dysfunction contributes to the pathogenesis of non-alcoholic steatohepatitis (NASH). The adapter protein alpha-syntrophin (SNTA) is expressed in adipocytes. Knock-down of SNTA increases preadipocyte proliferation and formation of small lipid droplets, which are both characteristics of healthy adipose tissue. To elucidate a potential protective role of SNTA in NASH, SNTA null mice were fed a methionine-choline-deficient (MCD) diet or an atherogenic diet which are widely used as preclinical NASH models. MCD diet mediated loss of fat mass was largely improved in SNTA −/− mice compared to the respective wild type animals. Hepatic lipids were mostly unchanged while the oxidative stress marker malondialdehyde was only induced in the wild type mice. The expression of inflammatory markers and macrophage immigration into the liver were reduced in SNTA −/− animals. This protective function of SNTA loss was absent in atherogenic diet induced NASH. Here, hepatic expression of inflammatory and fibrotic genes was similar in both genotypes though mutant mice gained less body fat during feeding. Hepatic cholesterol and ceramide were strongly induced in both strains upon feeding the atherogenic diet, while hepatic sphingomyelin, phosphatidylserine and phosphatidylethanolamine levels were suppressed.

SNTA deficient mice are protected from fat loss and NASH in the experimental MCD model. NASH induced by an atherogenic diet is not influenced by loss of SNTA. The present study suggests the use of different experimental NASH models to study the pathophysiological role of proteins like SNTA in NASH.

1. Introduction

Obesity is a major risk factor for non-alcoholic fatty liver disease (NAFLD) and its progressive form, non-alcoholic steatohepatitis (NASH) [1,2]. Deposition of triglycerides in the liver is a characteristic feature of NAFLD, and may be accompanied by lipotoxicity [1,2]. Physiologically, triglycerides are mostly stored in adipose tissues and fatty acid mobilization is a tightly controlled process [3]. Insufficient lipid storage capacity of fat in obesity is a major cause for lipid deposition in peripheral organs [1,4]. Liver steatosis is also commonly diagnosed in patients with lipodystrophies [5] demonstrating that an appropriate amount of body fat is essential for metabolic health.

The degree of liver steatosis does not predict disease progression in NASH [6]. Indeed, inhibition of diacylglycerol acyltransferase 2 which catalyzes the final step in the biosynthesis of triglycerides improves liver steatosis but increases hepatic oxidative stress, inflammation and fibrosis [7]. The pathogenesis of NAFLD involves immune cell immigration, inflammation, oxidative stress and impaired gut function which all contribute to disease progression and severity [1,8]. Metabolomic analysis has differentiated two subtypes of NAFLD patients [9] and this may reflect variances in disease pathogenesis. So far it has not been resolved why a subgroup of patients with liver steatosis develops progressive liver disease. Accordingly, there are no biomarkers for a timely identification of those patients [1].

Methionine-choline-deficient (MCD) diet is a widely used preclinical model to study NASH. Feeding this diet shortly results in liver steatosis, inflammation and eventually fibrosis. Dietary deficiency of methionine contributes to weight loss, oxidative stress, inflammation and fibrosis, while lack of choline is mostly responsible for liver steatosis [10]. MCD fed animals rapidly lose adipose tissue mass [11]. In the fat tissues the activities of adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL) are induced and this contributes to increased fatty acid flux

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to the liver [12]. While CC-Chemokine-Ligand 2 (CCL2) and Tumor-Necrosis-Factor (TNF) expression is enhanced in the liver, their mRNA levels are unchanged in white adipose tissues [12]. Teufel et al. have recently shown that hepatic gene expression of MCD fed mice is more similar to human NAFLD when compared to other preclinical models [13]. NAFLD is mostly diagnosed in the obese but also develops in non-obese patients with visceral adiposity, upon rapid weight loss and in lipodystrophic patients [5,14,15]. This highlights the central role of appropriate adipose tissue function in the pathogenesis of NAFLD [1,5,14,15].

Feeding mice an atherogenic diet is a further model to induce NASH. This diet is enriched in cholesterol and cholic acid to produce dyslipidemia and oxidative stress [16]. Animals are described to lose or to gain body weight and this may depend on the exact composition of the chow and eventually duration of feeding [16,17].

Excess dietary fat is stored in growing adipose tissue to minimize ectopic lipid deposition [18]. Adipose tissue enlargement may be achieved by cell hypertrophy, a well described feature of dysfunctional adipocytes. Enhanced proliferation of preadipocytes which differentiate to mature cells is a further possibility to store excess lipids. This hyperplastic expansion of fat is metabolically more favorable [18].

Recently, our group has shown enhanced preadipocyte proliferation in 3T3-L1 cells with low alpha-syntrophin (SNTA). These cells differentiate to adipocytes with normal expression of various proteins including ATGL and HSL. Noteworthy, adipocytes with low SNTA have smaller lipid droplets while their capacity to store additional lipids is improved [19].

SNTA is well studied as a component of the dystrophin complex in skeletal muscle [20]. Though this protein is expressed in various cells and tissues its possible role in metabolic diseases has not been described in detail [21].

Initial *in vitro* studies suggest a function of SNTA in reverse cholesterol transport through stabilizing ATP-binding cassette transporter A1 (ABCA1) [22]. Cholesterol and phospholipid efflux is, however, normal in syntrophin deficient macrophages [23] and hepatic ABCA1 protein is unchanged in SNTA and beta 2 syntrophin (SNTB2) null mice [24]. These animals nevertheless have low concentrations of hepatic cholesterol and sphingomyelin, and display strongly reduced protein levels of the scavenger receptor-BI (SR-BI) which mediates selective cholesterol uptake of liver cells [24,25].

Previous studies demonstrate a function of SNTA in adipocyte growth and lipid metabolism [19,24], and this led us to investigate its possible role in experimental NASH models.

2. Materials and methods

2.1. Animal handling

The male mice were from the Jackson Laboratory (Bar Harbor, USA). Mice (14 weeks old) were fed an atherogenic diet (S7803-E012) or the appropriate control diet (S7803-E000, Ssniff, Soest, Germany) for 12 weeks or the methionine-choline-deficient diet (E15653-94, Ssniff, Soest, Germany) or the respective control chow (E15654–04) for 2 weeks. Composition of the diets is specified in Supplementary Table 1. Rising levels of carbon dioxide led to loss of consciousness and animals were killed by cervical dislocation. Tissues of the overnight fasted mice were excised, immediately frozen in liquid nitrogen and maintained at −80 °C or incubated overnight in 4% formaldehyde solution for paraffin embedding.

Procedures were approved by the University of Regensburg Laboratory Animal Committee. They complied with the German Law on Animal Protection and the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals, 1999. Experiments were conducted according to institutional and governmental regulations for animal use (Government of the Oberpfalz and Unterfranken).

2.2. Immunohistochemistry

Immunohistochemical studies for the expression of CD163 were performed with an antibody ordered from Bio-Rad AbD Serotec GmbH (Puchheim, Germany) and utilized the EnVision+ Kit (Dako, Glostrup, Denmark) based on a HRP labeled polymer which is conjugated with a secondary antibody. Three micrometer sections were cut from formalin-fixed and paraffin-embedded mouse liver tissues and stained according to a previously described protocol [26].

2.3. Analysis of adipocyte size and liver fibrosis

The adipocyte size distribution was determined in formalin-fixed paraffin-embedded tissues. Tissues were cut into 5 µm sections and stained with hematoxylin and eosin. Analysis was done with the AxioVision Rel. 4.8 software (Carl Zeiss Microscopy GmbH, Oberkochen, Germany). Liver tissues were fixed with formalin and embedded in paraffin. Liver was cut into 3 µm sections. Liver fibrosis was assessed by Sirius Red staining.

2.4. Quantification of lipids

Lipid quantification was done by direct flow injection electrospray ionization tandem mass spectrometry (ESI–MS/MS) in positive ion mode. The analytical setup and strategy has been described [27]. Shortly, liver homogenates were prepared by the method described by Bligh and Dyer [28] and non-naturally occurring lipid species were added as internal standards. Analysis employed the Quattro Ultima triple-quadrupole mass spectrometer (Micromass, Manchester, UK) with an autosampler (HTS PAL, Zwingen, Switzerland) and a binary pump (Model 1100, Agilent, Waldbronn, Germany). A precursor ion of m/z 184 was used for phosphatidylcholine (PC), sphingomyelin (SM) [27] and lysophosphatidylcholine (LPC) [29]. A neutral loss of 141 Da and 277 Da was used for phosphatidylethanolamine (PE) and phosphatidylinositol (PI) [30], respectively. Neutral loss scan of m/z 185 was used for phosphatidylserine. Sphingosine based ceramides (Cer d18:1) were quantified by a fragment ion of m/z 264 [31,32]. Free cholesterol (FC) and cholesteryl ester (CE) were measured using a fragment ion of m/z 369 (after selective derivatization of free cholesterol) [33]. Correction of isotopic overlap of lipid species and data analysis for all lipid classes were performed by self-programed Excel Macros. Lipid species were annotated in compliance with the proposed shorthand notation of lipid structures analyzed by mass spectrometry [34]. Glycerophospholipid annotation assumed even numbered carbon chains only. Sphingomyelin species annotation assumed that a sphingoid base d18:1 was present. Liver lipids are all given as nmol/mg wet weight.

2.5. SDS-PAGE and immunoblotting

SDS-PAGE and immunoblotting were performed as described [35,36]. SNTA and SNTB2 antibodies were kindly provided by Prof. Marvin Adams (University of Washington) [21,37]. Antibodies for ATGL, FAS, GAPDH, HSL, phosphorylated HSL (pHSL) and SCD1 were from New England Biolabs GmbH (Frankfurt am Main, Germany). SREBP1c antibody was from Thermo Fisher Scientific (Schwerte, Germany). SR-BI antibody was from Biomol (Hamburg, Germany). PGC1alpha antibody and myostatin antibody were from Abcam (Cambridge, United Kingdom), and an aliquot of the latter was a kind gift from Dr. Oliver Felthaus (University Hospital of Regensburg).

2.6. Semiquantitative real-time PCR

Semiquantitative real-time PCR using the LightCycler® FastStart DNA *Master* SYBR Green I Kit from Roche (Mannheim, Germany) was performed as described [35]. Primers to amplify CTGF, F4/80, TGF beta