Research Article

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Sortilin deficiency improves the metabolic phenotype and reduces hepatic steatosis of mice subjected to diet-induced obesity

Liane Rabinowich[1,†], Sigal Fishman[1,†], Einav Hubel[1], Tamar Thurm[2], Woo-Jae Park[3,4], Yael Pewzner-Jung[3], Ashish Saroha[3], Noam Erez[1], Zamir Halpern[1], Anthony H. Futerman[3], Isabel Zvibel[1,*]

[1]*The Research Center for Digestive Tract and Liver Diseases, Tel Aviv Sourasky Medical Center and the Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel;* [2]*Internal Medicine D, Tel Aviv Sourasky Medical Center, Tel Aviv, Israel;* [3]*Department of Biological Chemistry, Weizmann Institute of Science, Rehovot, Israel;* [4]*Department of Biochemistry, School of Medicine, Gachon University, Incheon 406-799, Republic of Korea*

Background & Aims: Sortilin traffics newly synthesized molecules from the trans-Golgi apparatus along secretory pathways to endosomes, lysosomes or to the cell surface. Sortilin trafficking of acid sphingomyelinase (aSMase) may regulate ceramide levels, a major modulator of insulin signalling. We therefore tested whether sortilin deficiency reduces hepatic and adipose tissue aSMase activity, improving insulin sensitivity in diet-induced obesity (DIO).

Methods: DIO in C57BL/6 (WT) and sortilin$^{-/-}$ mice was induced by high-fat diet feeding for 10 weeks.

Results: Sortilin$^{-/-}$ mice gained less body weight and less visceral fat, despite similar food intake compared to WT type mice and had enhanced glucose uptake in insulin tolerance tests, which was further corroborated by enhanced hepatic pAkt expression. Sortilin deficiency led to attenuated hepatic steatosis, reduced expression of genes involved in lipogenesis, ceramide synthesis and inflammatory cytokine production and reduced activity of ceramide synthase 5/6 (CerS5/6). Sortilin$^{-/-}$ mice had reduced hepatic aSMase activity under both steady-state and DIO. Likewise, sortilin$^{-/-}$ hepatocytes displayed hypersensitivity to insulin, due to enhanced insulin receptor downstream signalling. In adipose tissue, sortilin$^{-/-}$ mice exhibited lower expression of inflammatory cytokines and lower expression and activity of CerS5/6. As in liver, adipose tissue displayed increased insulin signalling, accompanied by attenuated aSMase activity.

Conclusions: Sortilin deficiency induces a beneficial metabolic phenotype in liver and adipose tissue upon DIO, mediated in part by reduced aSMase activity.

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Keywords: Hepatic steatosis; Sortilin; Insulin resistance; Acid sphingomyelinase; Ceramide synthesis.

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* Corresponding author. Address: Tel Aviv Sourasky Medical Center, Department of Gastroenterology, Weizman 6, Tel Aviv, Israel. Tel.: +972 3 697 4481; fax: +972 3 697 4622.

E-mail address: isab@tlvmc.gov.il (I. Zvibel).

† These authors contributed equally to this work.

Abbreviations: DIO, diet-induced obesity; aSMase, acid sphingomyelinase; NAFLD, non-alcoholic fatty liver disease; TGN, trans-Golgi network; TGs, triglycerides; LDL, low-density lipoprotein; GWAS, genome wide association studies; VPS10P, vacuolar protein sorting 10 protein; SAP, sphingolipid activation protein; IR, insulin resistance; HFD, high fat diet.

Introduction

Obesity has become a world-wide epidemic in western countries, leading to insulin resistance, heart disease, type 2 diabetes and non-alcoholic fatty liver disease (NAFLD). NAFLD is commonly associated with insulin resistance and characterized by hepatic accumulation of triglycerides (TGs) [1].

Sortilin is a member of the vacuolar protein sorting 10 protein (VPS10P)-domain receptor family. Sortilin functions both as a co-receptor and as a trafficking molecule that binds and directs newly synthesized molecules from the trans-Golgi network (TGN) to regulated secretion pathways, including endosomes, lysosomes or to the cell surface [2]. Recent studies have implicated sortilin in several metabolic processes in the liver and adipose tissue [2–4]. Human genome wide association studies (GWAS) indicate a strong association of a specific SNP in the *SORT1* gene with decreased serum levels of low-density lipoprotein (LDL) cholesterol and myocardial infarction [3]. Following this discovery, sortilin was shown to control plasma LDL by two possible mechanisms. In the first, sortilin modulates LDL secretion by binding to ApoB in the Golgi and directing it towards the cell surface and secretion, or towards a degradation pathway [3–4]. In the second, sortilin regulates LDL catabolism by uptake of circulating serum LDL and transferring it to lysosomes for degradation [3–6]. Recently, sortilin was shown to influence sphingolipid metabolism by trafficking acid sphingomyelinase (aSMase) to the lysosome or cell surface, as well as by participating in the trafficking of sphingolipid activation proteins (SAPs) [7–9].

ASMase and neutral-sphingomyelinase 2 (nSMase) regulate the turnover of sphingomyelin (SM), to produce ceramide in

Table 1. Body and serum parameters of WT and sortilin$^{-/-}$ mice after 10 weeks of HFD (60% kcal from fat).

	WT	*Sort*$^{-/-}$
Body weight (g)	39.1 ± 1.2	30.1 ± 1.1*
Food intake (kcal/mouse/day)	11.8 ± 1.8	12 ± 3.2
Liver weight (g)	1.5 ± 0.1	1.1 ± 0.01*
Epididymal fat (g)	2.0 ± 0.04	0.9 ± 0.1*
Perinephric fat (g)	1.0 ± 0.05	0.3 ± 0.1*
Total fat (g)	3.0 ± 0.1	1.2 ± 0.2*
% fat/body weight	7.6 ± 0.1	4 ± 0.5*
AST (U/L)	322 ± 74	390 ± 106
ALT (U/L)	221 ± 42	275 ± 77
Serum triglycerides (mg/dl)	103 ± 12	103 ± 16
Serum cholesterol (mg/dl)	149 ± 13	120 ± 11
Serum progranulin (ng/ml)	2.9 ± 0.7	15.3 ± 5.1*
Hepatic TG (mg/g tissue)	60 ± 3.2	20.9 ± 3.9*
Hepatic cholesterol (mg/g tissue)	2.6 ± 0.2	2.2 ± 0.1

*p <0.05 (n = 7).

the lysosome or at the cell surface [10–11]. Ceramide contributes to insulin resistance and lipotoxicity by suppressing IRS-1 phosphorylation, thereby reducing insulin signalling [12–14]. In addition to its role in the regulation of ceramide levels, aSMase modulates the partitioning of diet-derived palmitate into two competitive and inversely correlated lipid pools, sphingolipids (SLs) and TGs [15]. Interestingly, *aSMase*$^{-/-}$/*LDLR*$^{-/-}$ mice show attenuated diet-induced hepatic TG accumulation and improved insulin sensitivity compared to *LDLR*$^{-/-}$ mice [15].

We hypothesized that sortilin deficiency, which results in reduced activity of lysosomal and/or cell surface hepatic and adipose tissue aSMase, and thus reduced ceramide levels, may lead to improved insulin sensitivity and reduced hepatic TG accumulation in diet-induced obesity (DIO). Thus, we investigated the connection between sortilin deficiency, aSMase activity, insulin sensitivity and hepatic steatosis in DIO.

Materials and methods

Animals and diets

Wild type (WT) male C57BL/6 mice (Harlan) and sortilin$^{-/-}$ male mice, obtained by introduction of the *neo* gene into exon 14 and the following intron of the sortilin gene [16] (a generous gift of Prof. A. Nykjaer, Aarhus University, Denmark) were fed *ad libitum* with regular rat chow (RC) (64% carbohydrate, 30% protein, and 6% fat) or a rodent high fat diet (HFD) from Research Diets (New Brunswick, NJ) with 60% kcal from fat (#D12492, 26% protein, 26% carbohydrate and 36% fat). Thirty min before sacrifice, mice were injected i.p. with 0.75 U/kg body weight insulin. Serum was taken for the measurement of metabolic and adipokine analysis. Liver and epididymal adipose tissue were frozen in liquid nitrogen and kept at −80 °C for biochemical and molecular analysis. Liver and adipose tissue were fixed in formaldehyde for histochemical and morphological assays. The studies were approved by the Animal Care and Use Committee of Tel Aviv Sourasky Medical Center.

Insulin tolerance test (ITT)

Mice were fasted for 4 h, and basal glucose was measured using the AccuCheck sensor blood glucometer. Insulin (0.75 U/kg body weight) was administered by i.p. injection, followed by serum glucose measurements 30, 60, and 120 min later.

Assays and analytical procedures

Hepatic triglycerides (TGs) and hepatic cholesterol and serum ALT, AST, TGs and cholesterol were measured using a Hitachi 747 Automatic Analyzer.

Murine hepatocyte isolation and culture

Livers of male C57BL/6 mice (WT) and male sortilin$^{-/-}$ mice were perfused with 0.5 mM EDTA, then with a solution containing 25 mg/ml collagenase type II (Worthington). Hepatocytes were plated on collagen I-coated plates in DMEM, supplemented with 10% FCS. After 4 h, the medium was changed to hormonally defined medium (HDM) without insulin for 16 h. The composition of the HDM was as described by Block *et al.*, [17] with modifications: DMEM supplemented with 100 μg/ml penicillin and streptomycin, 2 mg/ml bovine serum albumin, 610 μg/ml nicotinamide, 740 ng/ml $ZnSO_4$:$7H_2O$, 20 ng/ml $CuSO_4$:$5H_2O$, 5 mM glutamine, 5 μg/ml iron-saturated transferrin, 5 ng/ml selenious acid and 10^{-7} M dexamethasone. The next day, hepatocytes were treated with 100 nM insulin for 7 and 20 min and protein was extracted.

Western blots

Protein from liver or adipose tissue was extracted by homogenization in ice-cold RIPA buffer (PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, aprotinin 5 μg/ml, leupeptin 10 μg/ml, pepstatin A 1 μg/ml and phosphatase inhibitor cocktail). Homogenates were centrifuged for 25 min at 13,000 g, supernatants were collected and protein concentration was determined. Total protein from adipose tissue explants was extracted by incubation for 30 min on ice in lysis buffer (250 mmol/L sucrose, 5 mmol/L $MgCl_2$, 10 mmol/L Tris pH 8.0, 0.5% Triton X-100, protease and phosphatase inhibitors). Proteins were separated by SDS-PAGE, blotted onto Hybond C extra and blocked overnight in 5% milk. Blots were incubated with antibodies to pAkt (dilution 1:1000, Cell Signaling Technology, Danvers, MA) and Akt1/2 (dilution 1:1000), fatty acid synthase (FAS) (dilution 1:1000) or β-actin (dilution 1:5000) (Santa Cruz Biotechnology, Santa Cruz, CA), then incubated with horseradish peroxidase-conjugated secondary antibodies and subjected to chemiluminescent detection.

aSMase activity assays

aSMase activity from liver and adipose tissue extracts was assessed as described [18–19]. Briefly, liver or adipose tissue was homogenated in sodium acetate buffer (50 mM sodium acetate, pH 4.5) with protease inhibitor (Sigma). Protein concentration was measured using a BCA protein assay kit (Pierce Chemical Co.), 50 μg of protein was used for the assay. The assay was started by addition of 1 nmol C6–NBD-SM (Avanti Polar Lipids) into 0.5 ml of sodium acetate buffer (including 50 μg of protein), incubated at 37 °C for 15 min and stopped by the addition of 1.5 ml of chloroform/methanol (1:2; v/v). Lipids were separated by thin layer chromatography (TLC) using chloroform/methanol: 9.8 mM $CaCl_2$ (60:35:8; v/v/v). TLCs were scanned by a Typhon scanner (GE Life Sciences) and quantified using ImageQuant software (GE Life Sciences).

CerS activity assay

Liver and adipose tissues were homogenized in 20 mM Hepes (pH 7.2), 25 mM KCl, 250 mM sucrose, and 2 mM $MgCl_2$ containing a protease inhibitor mixture (Sigma). Protein was determined using the BCA reagent (Pierce). Homogenates (12 μg protein) were incubated with 15 μM N-(7-nitro-2-1,3-benzoxadiazole-4-yl) (2S, 3R)-2-aminooctadecane-1,3-diol (NBD-sphinganine [Avanti Polar Lipids]), 20 μM defatted BSA (Sigma), and 50 μM C16-CoA (Avanti Polar Lipids) for 15 min at 37 °C. Lipids were extracted and separated by TLC using chloroform/methanol/water and 2 M NH_4OH (40:10:0.75:0.25; vol/vol/vol) as the developing solvent. NBD-labelled lipids were visualized using the Typhoon 9410 variable mode imager and quantified by ImageQuant TL software (GE Healthcare).

Quantitative real time RT-PCR

Total RNA was extracted from livers using the EZ-RNA kit (Biological Industries, Bet Haemek, Israel) and from adipose tissue using the RNeasy lipid tissue kit (Qiagen) and 2.5 μg total RNA was reverse-transcribed using the reverse transcriptase enzyme MMLV (Promega, Madison, WI). Real time RT-PCR (qRT-PCR)

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