Original Article

MOLECULAR METABOLISM

Inhibition of citrate cotransporter Slc13a5/mINDY by RNAi improves hepatic insulin sensitivity and prevents diet-induced non-alcoholic fatty liver disease in mice

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ABSTRACT

Objective: Non-alcoholic fatty liver disease is a world-wide health concern and risk factor for cardio-metabolic diseases. Citrate uptake modifies intracellular hepatic energy metabolism and is controlled by the conserved sodium-dicarboxylate cotransporter solute carrier family 13 member 5 (SLC13A5, mammalian homolog of INDY: mINDY). In *Drosophila melanogaster* and *Caenorhabditis elegans* INDY reduction decreased whole-body lipid accumulation. Genetic deletion of *Slc13a5* in mice protected from diet-induced adiposity and insulin resistance. We hypothesized that inducible hepatic mINDY inhibition should prevent the development of fatty liver and hepatic insulin resistance.

Methods: Adult C57BL/6J mice were fed a Western diet (60% kcal from fat, 21% kcal from carbohydrate) ad libitum. Knockdown of mINDY was induced by weekly injection of a chemically modified, liver-selective siRNA for 8 weeks. Mice were metabolically characterized and the effect of mINDY suppression on glucose tolerance as well as insulin sensitivity was assessed with an ipGTT and a hyperinsulinemic-euglycemic clamp. Hepatic lipid accumulation was determined by biochemical measurements and histochemistry.

Results: Within the 8 week intervention, hepatic mINDY expression was suppressed by a liver-selective siRNA by over 60%. mINDY knockdown improved hepatic insulin sensitivity (i.e. insulin-induced suppression of endogenous glucose production) of C57BL/6J mice in the hyperinsulinemic-euglycemic clamp. Moreover, the siRNA-mediated mINDY inhibition prevented neutral lipid storage and triglyceride accumulation in the liver, while we found no effect on body weight.

Conclusions: We show that inducible mINDY inhibition improved hepatic insulin sensitivity and prevented diet-induced non-alcoholic fatty liver disease in adult C57BL6/J mice. These effects did not depend on changes of body weight or body composition.

Keywords INDY/Slc13a5; siRNA; Insulin resistance; Steatosis; Citrate transport; Lipid accumulation

1. INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a global health concern. Estimates suggest approximately 3.4 million deaths/year from obesity-associated diseases such as cardiovascular disease, type-2 diabetes (T2D), and certain types of cancer [1–4]. NAFLD has been recognized as one of the leading causes of liver cirrhosis today [5,6]. It is present in up to 30% of Americans and is involved in the development of insulin resistance (IR) and T2D [5]. Available treatment options for NAFLD are sparse.

Pathophysiological accumulation of intracellular hepatic lipids results from increased energy supply and de-novo-lipogenesis together with

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Abbreviations: 2-DG, 2-Deoxy-D-glucose; e, epididymal; p, perirenal; s, subcutaneous; WAT, white adipose tissue; EE, energy expenditure; EGP, endogenous glucose production; FA, fatty acids; FLD, fatty liver disease; GIR, glucose infusion rate; HE clamp, hyperinsulinemic-euglycemic clamp; HFD, high-fat diet; IEX, anion-exchange high-performance liquid chromatography; INDY, 'I'm not dead Yet'; KO, knockout; mINDY, Slc13a5/SLC13A5; solute carrier family 13, member 5; ORO, oil red O; RER, respiratory exchange ratio; SCR, non-silencing scrambled control siRNA; siINDY, mINDY-specific siRNA; SKM, skeletal muscle; T2D, type-2 diabetes; TCA, tricarboxylic acid; WD, western diet

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insufficient fatty acid (FA) oxidation and secretion from liver [7]. Hepatic fat accumulation has been linked to IR via specific lipid species such as diacylglycerols and ceramides [8].

Cytosolic citrate is a precursor for FA synthesis in the liver, and consequently triacylglycerols and cholesterol. Moreover, citrate has signaling properties modifying the activity of FA synthesis pathways by an allosteric activation of acetyl-CoA carboxylase 2 [9]. The resulting malonyl-CoA from this reaction inhibits FA oxidation. Moreover, citrate has been shown to reduce the flux of glucose through glycolysis in human cells [10]. In liver and adipose tissue, the rate of FA synthesis appears to correlate with the cytosolic citrate concentration and this is partially depending on direct transport of citrate into the cell by solute carrier family 13, member 5 (SLC13A5) [10].

The *SLC13A5* gene is the mammalian ortholog of the '*I'm not dead, Yet*' (*INDY*) gene in *Drosophila melanogaster* where its mutation is linked to a life-span extension [11]. The human *SLC13A5* gene encodes a transmembrane sodium-dependent dicarboxylate cotransporter protein [12]. In vertebrates, it is referred to as mammalian INDY homolog (*mINDY*), *Nact*, or *NaC2/NaCT*. *mINDY* is highly expressed in liver and moderately in brain, testis, and kidney, where it transports TCA cycle intermediates, including succinate and citrate, but also malate, fumarate and 2-oxo-glutarate [12–16]. The transport process for cellular uptake of citrate is several times more selective than for other TCA cycle intermediates [17]. The Na^+-dicarboxylate cotransport process is electrogenic, coupling three to four Na^+ ions to the transport of each divalent anion substrate [12,17–19]. In *D. melanogaster* and *Caenorhabditis elegans*, down-regulation of INDY facilitated longevity similar to caloric restriction and resulted in reduced fat storage in flies [11,20,21]. We have recently shown in *C. elegans* that longevity induced by INDY knockdown depends on AMPK [22]. Genetic whole-body deletion of mINDY in mice also led to striking similarities to caloric restriction and protected mice from high-fat diet (HFD)-induced obesity, steatosis hepatis, and IR by increasing energy expenditure, improving hepatic mitochondrial biogenesis, enhancing hepatic FA oxidation, and reducing hepatic lipogenesis [16]. In primary rat hepatocytes, mINDY expression and citrate uptake could be induced by physiological concentrations of glucagon via a cAMP-dependent and cAMP-responsive element-binding protein (CREB)-dependent mechanism at a confirmed CREB-binding site within the mINDY promoter [23]. In line with these observations, the siRNA-mediated knockdown of mINDY in human HepG2 cells reduced their lipid content [24].

Based on the predominant expression in liver, its relative selectivity for citrate, and the promising data from existing studies including the whole-body knockout (KO) experiments, mINDY is a potential therapeutic target to treat NAFLD and metabolic disorders in humans [14–16,24].

We hypothesized that inducible hepatic mINDY inhibition should prevent the development of fatty liver and hepatic IR. Therefore, we investigated the effects of an inducible liver-specific, siRNA-based mINDY knockdown in adult C57BL/6J mice.

2. MATERIAL AND METHODS

2.1. Materials

All chemicals were purchased from Sigma-Aldrich (Munich, Germany), Merck (Darmstadt, Germany), or Roth (Karlsruhe, Germany), reagents for RNA, cDNA, and qPCR were purchased from Thermo Scientific (Schwerte, Germany), and radioactive labeled substances were purchased from Hartman Analytic (Braunschweig, Germany) unless stated otherwise.

2.2. Animal studies

All animal experiments were performed under the terms of the German Animal Protection Law, and according to the EU Directive 2010/63/EU for animal experiments as well as international animal welfare legislation and rules. Additionally, mice studies were conducted according to institutional ethical guidelines of the Charité Berlin, Germany, and were approved by the Landesamt für Gesundheit und Soziales (approval number G 0331/13, LAGeSo Berlin, Germany) and comply with the ARRIVE guidelines [25].

8 week-old male C57BL6/J mice (Forschungseinrichtungen für experimentelle Medizin, Charité Berlin, Germany) were fed ad libitum a Western diet (WD) consisting of a HFD (60% kcal from fat, D12492 (I), Ssniff Spezialdiäten, Soest, Germany; Supplemental Table A1) supplemented with 6% sucrose in the water. All mice were maintained in individually ventilated cages (4/cage) in an environmentally-controlled room with a 12 h light–dark cycle. Body weight was monitored weekly. For siRNA intervention mice were divided into two groups according to equal fat mass. 1 mg/kg liver-selective mINDY-specific (siINDY) or unspecific scrambled control siRNA (SCR) in PBS was weekly injected via tail vein for 8 weeks. Body composition of mice was assessed by ^{1}H-magnetic resonance spectroscopy using a Minispec LF50 Body Composition Analyzer (Bruker BioSpin, Billerica, USA). Isoflurane inhalation (1.5%) was used for anesthesia during operation, and mice were sacrificed by cervical dislocation.

2.2.1. Activity and calorimetry analysis

Basal metabolic parameters were analyzed in a TSE LabMaster System (TSE Systems, Bad Homburg, Germany). Mice were acclimated to the metabolic cages individually housed 16 h before starting and supplied with WD. Data on gas exchanges, activity, and food/liquid intake was collected for 48 h and analyzed as 24 h average. Calorimetry was performed with a computer-controlled open circuit calorimetry system composed of 10 metabolic cages. Each cage was equipped with a special water bottle and a food tray connected to a balance as well as an activity monitor. Parameters were measured for each mouse at 3 min intervals and respiratory quotient (RQ) was calculated as the ratio of CO_2 production to O_2 consumption. O_2 consumption, CO_2 production, energy expenditure (EE), and caloric intake were adjusted for lean body mass. Data were analyzed as described [26]. Additionally taken in consideration that fat mass is metabolic active, there were no differences in lean body or fat mass between siINDY and control animals.

2.2.2. Glucose tolerance test (ipGTT)

Mice were fasted overnight and basal blood glucose was measured before injecting an intraperitoneal glucose bolus of 1 g/kg body weight. Blood glucose levels were measured subsequently at 0, 15, 30, 60 and 120 min and the corresponding insulin levels were determined.

2.2.3. Hyperinsulinemic-euglycemic clamp studies

Hyperinsulinemic-euglycemic (HE) clamp studies were performed in conscious, restrained mice with 3 mU/kg/min insulin in a body weight matched fashion and a 2-Deoxy-D-glucose (2-DG) bolus injection to measure tissue-specific glucose uptake as described [16,27,28]. Indwelling jugular venous catheters were implanted, and mice recovered for 7 days. Following an overnight fast, whole-body glucose turnover was estimated under basal conditions with a 0.05 μCi/min infusion of [2-^{3}H]-D-glucose (HPLC purified) for 2 h. Afterwards, mice were primed with human insulin (21.43 mU/kg, INSUMAN rapid; Sanofi-Aventis, Frankfurt, Deutschland) and [2-^{3}H]-D-glucose (0.72 μCi) over 3 min, followed by HE conditions for 140 min with a

