Research Article

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Intestinal GATA4 deficiency protects from diet-induced hepatic steatosis

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Background & Aims: GATA4, a zinc finger domain transcription factor, is critical for jejunal identity. Mice with an intestine-specific GATA4 deficiency (*GATA4*iKO) are resistant to diet-induced obesity and insulin resistance. Although they have decreased intestinal lipid absorption, hepatic *de novo* lipogenesis is inhibited. Here, we investigated dietary lipid-dependent and independent effects on the development of steatosis and fibrosis in *GATA4*iKO mice.

Methods: *GATA4*iKO and control mice were fed a Western-type diet (WTD) or a methionine and choline-deficient diet (MCDD) for 20 and 3 weeks, respectively. Functional effects of *GATA4*iKO on diet-induced liver steatosis were investigated.

Results: WTD-but not MCDD-fed *GATA4*iKO mice showed lower hepatic concentrations of triglycerides, free fatty acids, and thiobarbituric acid reactive species and had reduced expression of lipogenic as well as fibrotic genes compared with controls. Reduced nuclear sterol regulatory element-binding protein-1c protein levels were accompanied by lower lipogenic gene expression. Oil red O and Sirius Red staining of liver sections confirmed the observed reduction in hepatic lipid accumulation and fibrosis. Immunohistochemical staining revealed an increased number of jejunal glucagon-like peptide 1 (GLP-1) positive cells in *GATA4*iKO mice. Consequently, we found enhanced phosphorylation of hepatic AMP-activated protein kinase and acetyl-CoA carboxylase alpha.

Conclusions: Our results provide strong indications for a protective effect of intestinal GATA4 deficiency on the development of hepatic steatosis and fibrosis via GLP-1, thereby blocking hepatic *de novo* lipogenesis.

Keywords: *GATA4*; Non-alcoholic fatty liver disease; GLP-1; Ileal interposition surgery; *De novo* lipogenesis.

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Abbreviations: DNL, *de novo* lipogenesis; TG, triglycerides; NAFLD, non-alcoholic fatty liver disease; WTD, Western-type diet; ACC, acetyl-CoA carboxylase alpha; MCDD, methionine and choline-deficient diet; GLP-1, glucagon-like peptide-1; IIS, ileal interposition surgery; *GATA4*iKO, intestine-specific *GATA4* deficiency; ALT, alanine aminotransferase; AST, aspartate transaminase; LDH, lactate dehydrogenase; FFA, free fatty acids; TBARS, thiobarbituric acid reactive substances; AMPK, AMP-activated protein kinase; p, phosphorylated; p38, p38 mitogen-activated protein kinase; Pparg, peroxisome proliferator-activated receptor gamma; α-SMA, alpha-smooth muscle actin; SREBP-1c, sterol regulatory element-binding protein-1c; HSC, hepatic stellate cells.

Introduction

Metabolic syndrome is a state of metabolic deregulation characterized by obesity, hyperlipidemia, atherosclerosis, glucose intolerance, and hepatic steatosis [1]. A key mechanism contributing to the development of metabolic syndrome is the rate of hepatic *de novo* lipogenesis (DNL) [2]. Hepatic DNL contributes only 5% of liver triglycerides (TG) under healthy circumstances but up to 30% in case of non-alcoholic fatty liver disease (NAFLD) [3]. The derangement of DNL in murine models of high fat diet-induced hepatic steatosis underscores the intestinal contribution in regulating hepatic lipid metabolism. Western-type diet (WTD), which is generally used to induce atherosclerosis, insulin resistance, and metabolic syndrome in mice, also causes non-alcoholic steatohepatitis [4,5]. WTD contains 43% of calories in the form of carbohydrates and dietary carbohydrates are known to activate hepatic DNL via acetyl-CoA carboxylase (ACC) [6]. Methionine and choline-deficient diet (MCDD) is widely used as a model of hepatic steatosis and fibrosis by inhibiting the release of very low density lipoproteins and by decreasing mitochondrial fatty acid oxidation [7].

Ileum of the small intestine is an important endocrine organ, which signals the dietary status to other organs including the liver by the release of hormones such as glucagon-like peptide-1 (GLP-1) [8,9]. A recent meta-analysis of 15 studies has investigated the effect of various bariatric procedures on NAFLD. The study has concluded that bariatric surgery ameliorates steatosis in 92%, steatohepatitis in 81% and leads to complete resolution in 70% of patients [10]. Ileal interposition surgery (IIS) is one of the bariatric procedures that mitigate the metabolic syndrome [11]. IIS in rats improves glucose tolerance and increases synthesis and release of GLP-1 and peptide YY [12,13]. Kohli *et al.* have recently reported that the cycling of bile acids is increased in rats that have undergone IIS and that these mice are protected from

obesity-associated co-morbidities [14]. These studies provide evidence that postsurgical changes in intestinal anatomy and function, especially earlier exposure of the ileum to nutrients together with alterations in the secretion of enteric hormones, contribute to improve glucose tolerance and lipid homeostasis after IIS. However, the long-term effects this surgical procedure may exert on hepatic lipid metabolism, steatosis, fibrosis, and inflammation are still unknown.

GATA4, a zinc finger domain transcription factor expressed throughout the small intestine except the distal ileum, plays an important role in maintaining jejuno-ileal differences in absorptive enterocyte gene expression [15,16]. Using a Villin-Cre approach, Battle *et al.* generated intestine-specific *GATA4* knockout (*GATA4*iKO) mice and showed that 47% of ileal genes were ectopically expressed in the jejunum of these mice [15]. This jejuno-ileal transition resembles IIS. We have previously demonstrated that *GATA4*iKO mice were resistant to diet-induced obesity and insulin resistance owing to reduced intestinal lipid absorption and increased GLP-1 release [17].

In the present study, we have investigated the effect of intestinal GATA4 deficiency on hepatic steatosis and fibrosis. Using two dietary conditions, we provide evidence that decreased hepatic DNL leads to protection from diet-induced NAFLD in *GATA4*iKO mice.

Materials and methods

Animals and diets

Generation of *Gata4*$^{loxP/null}$*Villin-Cre* knockout and *Gata4*$^{loxP/+}$ *Villin-Cre* control mice have previously been described [15]. All experiments were performed using male mice. Mice had free access to food and water under a 12 h light/12 h dark cycle in a temperature-controlled environment. Individually housed knockout and control littermates were fed a normal chow diet (11.9% caloric intake from fat, Ssniff®, Soest, Germany) or switched to WTD (TD88137 mod.; Ssniff®, Soest, Germany) for 20 or MCDD (A02082002B, Research Diets, Inc. New Brunswick, NJ) for 3 weeks. WTD contained 21% (weight/weight) crude fat and 0.15% (weight/weight) cholesterol with ≈ 4.53 kcal/g (42% of calories from crude fat, 15% from protein, and 43% from carbohydrate). Features of metabolic syndrome along with steatohepatitis are reported to develop after long-term WTD feeding [4]. MCDD is reported to induce severe steatohepatitis in a relatively shorter time period from 3 to 6 weeks [7]. Appropriate feeding regimens were chosen accordingly. Animal experiments were performed in accordance with the standards established by the Austrian Federal Ministry of Science and Research, Division of Genetic Engineering and Animal Experiments (Vienna, Austria).

Biochemical analyses

Blood was drawn via retro-orbital puncture and plasma was isolated for analysis. Levels of alanine aminotransferase (ALT), aspartate transaminase (AST), and lactate dehydrogenase (LDH) were routinely measured in 4 mice per genotype.

Oral fat tolerance test

Blood was drawn via retro-orbital puncture before and after an oral gavage of 200 µl of corn oil at pre-established time points and plasma was isolated. Plasma TG (n = 8/group) was estimated according to manufacturer's protocol (DiaSys, Holzheim, Germany).

Hepatic TG and free fatty acid (FFA) estimation

Hepatic TG and FFA concentrations were determined from Folch extracts using enzymatic kits according to manufacturer's protocols (DiaSys, Holzheim, Germany; Wako Chemicals GmbH, Neuss, Germany). Briefly, liver samples from *GATA4*iKO and control mice fed WTD (n = 5/group), MCDD (n = 4/group) or chow diet (n = 5/group) were weighed and homogenized, and lipids were extracted in chloroform:methanol (2:1) in a volume 20 times the weight of the sample. Lipid extracts were then solubilized in freshly prepared 0.2% Triton X-100 in chloroform, dried under a gentle stream of nitrogen, and resuspended in 200 µl sterile phosphate-buffered saline.

Assay for hepatic lipid peroxidation levels

Hepatic levels of thiobarbituric acid reactive substances (TBARS) were determined using the Cayman TBARS Assay kit (Cayman Chemicals, Ann Arbor, MI). Briefly, 25 mg of liver tissue from WTD-fed control and *GATA4*iKO mice (n = 5/group) was homogenized in 250 µl of radio-immunoprecipitation assay buffer supplemented with protease inhibitors (Roche, Indianapolis, IN) and sonicated on ice for 15 s at medium power. Supernatants were separated by centrifugation at 3500 rpm for 10 min at 4 °C. One hundred µl of each sample and standard was mixed with 4 ml color reagent containing 100 µl of sodium dodecyl sulfate solution. Samples were boiled for 1 h, and the reaction was stopped by placing samples on ice for 10 min. Thereafter, samples were centrifuged at 3500 rpm for 10 min at 4 °C. The absorbance was measured at 530 nm. Results were expressed as µM of malondialdehyde-adduct per g liver weight.

Assay for hepatic 4-hydroxyproline levels

Hepatic levels of 4-hydroxyproline were measured as previously described [18]. Briefly, liver tissues were homogenized in 2 N NaOH and autoclaved at 120 °C for 25 min for alkaline hydrolysis. The hydrolysates were cooled to RT and filtered through Whatmann filter paper No. 1 to remove particulate matter. Standard dilutions of 4-hydroxyproline (Sigma Aldrich, GmbH, Germany), samples and blanks in acetate-citrate buffer pH 6.5 were mixed in freshly prepared chloramine T and allowed to react at room temperature for 25 min. Color development was achieved by the addition of fresh Ehrlich's aldehyde reagent and incubation at 65 °C for 20 min. Absorbances were measured at 550 nm. Results were plotted as mg collagen/g liver using the following formula:

$$\text{g collagen} = (\text{g 4-hydroxyproline/ml}) * \text{dilution factor} * \text{conversion factor}(7.5)$$

Histochemistry and immunohistochemistry

For conventional light microscopy, livers of WTD-fed control and *GATA4*iKO mice (n = 3/group) and MCDD-fed control and *GATA4*iKO mice (n = 4/group) were fixed in 4% neutral buffered formaldehyde solution for 24 h and embedded in paraffin. Five µm sections were deparaffinized and subjected to routine haematoxylin and eosin, periodic acid Schiff and Sirius Red staining. For histochemical staining with Oil Red O, 7 µm cryosections were prepared and stained according to protocol; nuclei were counter-staining with Mayer's haematoxylin. For the detection of GLP-1 positive cells in the jejunum of control and *GATA4*iKO mice, harvested jejunum was longitudinally exposed and luminal contents were washed in PBS. The tissue was immediately fixed in 10% formaldehyde for 24 h and embedded in paraffin. Five µm sections were processed for staining. Sections were deparaffinized and blocked in 2% pre-immune goat serum. Sections were incubated with anti-GLP-1 antibody (1:500; Phoenix Pharmaceuticals Inc., Burlingame, CA) overnight at 4 °C, washed, incubated with biotinylated goat anti-rabbit secondary antibody followed by signal enhancement using the ABC reagent (Vector Laboratories Inc., Burlingame, CA). Horse radish peroxidase was detected using the 3,3′-diaminobenzidine with metal enhancer reagent (Sigma Aldrich, St. Louis, MO). Images were acquired using an Olympus FSX100 at indicated magnifications.

RNA isolation and quantitative real-time PCR

Total RNA from tissues of mice was isolated using the TriFast reagent according to the manufacturer's protocol (Peqlab Biotechnologies GmbH, Erlangen, Germany). Two µg of total RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was performed on a Roche LightCycler 480 instrument (Roche Diagnostics, Palo Alto, CA) using the QuantiFast™ SYBR® Green PCR Kit (Qiagen, Valencia, CA). Samples were measured in triplicate for each experiment and normalized to *cyclophilin A* mRNA expression as an internal control. Expression profiles and associated statistical parameters were analyzed using the public domain program Relative Expression Software Tool – REST 2010 (http://www.gene-quantification.com/download.html) [19]. Primer sequences are available upon request.