Research Article

JOURNAL OF HEPATOLOGY

MicroRNA-206 prevents hepatosteatosis and hyperglycemia by facilitating insulin signaling and impairing lipogenesis

Heng Wu[1], Tianpeng Zhang[1], Fei Pan[1], Clifford J. Steer[1,2], Zhuoyu Li[3], Xin Chen[4], Guisheng Song[1,2,*]

[1]*Department of Medicine, University of Minnesota Medical School, Minneapolis, MN 55455, USA;* [2]*Department of Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN 55455, USA;* [3]*Institute of Biotechnology, Shanxi University, Taiyuan City 030006, China;* [4]*Department of Bioengineering and Therapeutic Sciences, University of California, San Francisco, CA 94143, USA*

Background & Aims: The paradox of selective hepatic insulin resistance, wherein the insulin-resistant liver fails to suppress glucose production but continues to produce lipids, has been central to the pathophysiology of hepatosteatosis and hyperglycemia. Our study was designed to investigate the mechanism(s) by which microRNA-206 alleviates the pathogenesis of hepatosteatosis and hyperglycemia.

Methods: Dietary obese mice induced by a high fat diet were used to study the role of microRNA-206 in the pathogenesis of hepatosteatosis and hyperglycemia. A mini-circle vector was used to deliver microRNA-206 into the livers of mice.

Results: Lipid accumulation impaired biogenesis of microRNA-206 in fatty livers of dietary obese mice and human hepatocytes (p <0.01). Delivery of microRNA-206 into the livers of dietary obese mice resulted in the strong therapeutic effects on hepatosteatosis and hyperglycemia. Mechanistically, miR-206 interacted with the 3′ untranslated region of *PTPN1* (protein tyrosine phosphatase, non-receptor type 1) and induced its degradation. By inhibiting *PTPN1* expression, microRNA-206 facilitated insulin signaling by promoting phosphorylation of INSR (insulin receptor) and impaired hepatic lipogenesis by inhibiting *Srebp1c* transcription. By simultaneously modulating lipogenesis and insulin signaling, microRNA-206 reduced lipid (p = 0.006) and glucose (p = 0.018) production in human hepatocytes and livers of dietary obese mice (p <0.001 and p <0.01 respectively). Re-introduction of *Ptpn1* into livers offset the inhibitory effects of microRNA-206, indicating that *PTPN1* mediates the inhibitory effects of microRNA-206 on both hepatosteatosis and hyperglycemia.

Conclusions: MicroRNA-206 is a potent inhibitor of lipid and glucose production by simultaneously facilitating insulin signaling and impairing hepatic lipogenesis. Our findings potentially provide a novel therapeutic agent for both hepatosteatosis and hyperglycemia.

Lay summary: The epidemic of obesity is causing a sharp rise in the incidence of insulin resistance and its major complications, type 2 diabetes and non-alcoholic fatty liver disease (NAFLD). However, there are no effective treatments because the mechanisms underlying both disorders are not well described. We identified microRNA-206 as a novel and effective inhibitor for both glucose and lipid production in liver and potentially provide a unique therapeutic drug for both hepatosteatosis and hyperglycemia.

Introduction

Results from the American Heart Association showed that nearly 73% of U.S adults are overweight or obese. Obesity and its associated co-morbidities are among the most prevalent and challenging conditions confronting the medical profession in the past century. A major metabolic consequence of obesity is insulin resistance, which is a major risk factor of type 2 diabetes (T2D) and non-alcoholic fatty liver disease (NAFLD) [1]. NAFLD is the accumulation of excessive amounts of lipids within hepatocytes that is not caused by alcohol. It is estimated that 90% of obese patients have some form of fatty liver, ranging from hepatosteatosis to more severe forms of NASH (non-alcoholic steatohepatitis), which can give rise to fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) [2]. Until recently, T2D was a disease that primarily afflicted adults. However, there is now a growing number of children who are being diagnosed with obesity-related T2D [1]. Although the pathogenesis of NAFLD and T2D has been studied extensively, the molecular mechanisms underlying these two co-morbid disorders are still under investigation. A better understanding of their pathogenesis and the development of new therapeutic strategies will be required to combat the epidemic of both disorders [2].

It is widely accepted that insulin resistance is central to the pathogenesis of NAFLD and T2D [3]. The principal function of insulin in the liver is to suppress glucose production when blood glucose concentrations increase. This process is impaired in hepatic insulin resistance and contributes to hyperglycemia. In the

Keywords: Insulin signaling; Lipogenesis; Hyperglycemia; NAFLD; MicroRNAs; Phosphorylation; Diet, high-fat.

Received 8 August 2016; received in revised form 23 November 2016; accepted 15 December 2016; available online 23 December 2016

* Corresponding author. Address: Department of Medicine, Division of Gastroenterology, Hepatology and Nutrition, University of Minnesota Medical School, MMC 36, VFW Cancer Research Center, V354, 406 Harvard Street SE, Minneapolis, MN 55455, USA.

E-mail address: gsong@umn.edu (G. Song).

normal state, insulin can potently induce *de novo* lipogenesis by modulating SREBPs (sterol regulatory element–binding protein) at multiple levels, including *SREBP1c* mRNA, the proteolytic processing of SREBP1c, and the stability and abundance of nuclear SREBP1c [4]. In the insulin-resistant state, compensatory hyperinsulinemia is postulated to activate *SREBP1c* transcription and cleavage, thereby increasing expression of lipogenic genes, enhancing fatty acid synthesis, and promoting triglyceride accumulation in the liver [5]. Based on the role of insulin in promoting lipogenesis, it is hard to use insulin resistance to explain hepatic lipid accumulation, and the molecular mechanisms regarding this observance have remained elusive.

MicroRNAs (miRNAs) are naturally-occurring small non-coding RNAs that function by binding to the 3′-untranslated regions (3′UTR) of specific mRNAs, which leads to either mRNA or translational pausing [6]. Given their critical role in lipid metabolism and carcinogenesis [7,8], miRNAs now represent novel therapeutic agents for human cancers and metabolic diseases. Many dysregulated miRNAs have been identified to modulate the pathogenesis of NAFLD, hyperlipidemia and T2D [7,9,10]. However, the specific miRNAs that have the capacity to prevent both NAFLD and hyperglycemia remain unknown. In this study, we investigated the underlying mechanisms by which miRNAs inhibit hepatic lipogenesis and glucose production, in part to explain the insulin resistance paradox.

Materials and methods

Establishment of dietary obese mice

Eight-week-old wild-type male C57Bl/6 mice (Jackson Laboratory, n = 6) were maintained on either a normal chow diet (Open Source D12450B: 10% kCal fat) or a high fat diet (HFD) (Open Source D12492: 60% kCal fat) for 8 weeks as described [11]. After 8 weeks of HFD administration, livers were collected for miRNA and gene expression analysis.

Preparation of mini-circle expression vectors for miR-206 and Ptpn1

We generated an *in vivo* expression vector of miR-206 by cloning human miR-206 precursor into mini-circle vectors purchased from System Biosciences (Cat. MN511A-1). A transthyretin gene (*TTR*) promoter was inserted into the upstream of miR-206 precursor to ensure liver-specific expression of miR-206 [12]. To rule out non-specific effects of the plasmid, we generated a miR-206 mismatched-expression vector by mutating the seed region of miR-206, termed MC-*TTR*-miR-206-MM. We inserted the coding region of mouse *Ptpn1* into a mini-circle vector and the *TTR* promoter was used to ensure hepatic expression of *Ptpn1*. This vector was referred as to MC-*TTR*-*Ptpn1*. Parental MC-*TTR*-miR-206 or MC-*TTR*-*Ptpn1* vector was transformed into a special host *E. coli* bacterial strain ZYCY10P3S2T (System Biosciences, Cat: MN900A-1). Mini-circles were generated based on the manufacturer's instructions.

MC-TTR-miR-206 treatment of dietary obese mice

Two-month-old wild-type C57Bl/6 mice were kept on HFD for 8 weeks. At 16 weeks of age, mice were divided into two groups: one group (n = 10) treated with MC-*TTR*-miR-206 and the other with MC-*TTR*-miR-206-MM (control, n = 10). Mice received a dose of 1.5 μg/g MC-*TTR*-miR-206 or MC-*TTR*-miR-206-MM complexed with *in vivo*-jetPEI® (Polyplus Transfection, Strasbourg, France) weekly for eight weeks via tail vein injection. At that time point, the mice were anesthetized, and blood was collected by cardiac puncture. The livers were harvested and immediately frozen in liquid nitrogen for gene expression and histological analysis.

To examine whether *Ptpn1* mediates the inhibitory effect of miR-206 on NAFLD and hyperglycemia, two-month old wild-type C57Bl/6 mice were maintained on the HFD (Open Source D12492: 60% kCal fat) for 8 weeks. At 16 weeks of age, mice were divided into three groups, in which group I (control group, n = 10) was injected with a combination of 1.5 μg/g MC-*TTR*-miR-206-MM; group II (n = 10) was injected with 1.5 μg/g MC-*TTR*-miR-206; and group III (n = 10) received a combination of 1.5 μg/g MC-*TTR*-miR-206 and 1.5 μg/g MC-*TTR*-*Ptpn1*. Group III was also used to determine whether additional treatment of *Ptpn1* could reverse the inhibitory effects of miR-206 on the development of NAFLD and hyperglycemia. All mice were maintained on the HFD as described above. Mice were housed, fed, and monitored in accordance with protocols approved by the committee for animal research at the University of Minnesota.

Fatty acid treatment of HepG2 cells and primary human hepatocytes

Human hepatocytes were purchased from Invitrogen. Sodium oleate was obtained from Sigma-Aldrich and was dissolved in DMEM medium with 1% fatty acid free bovine serum albumin (BSA) (Sigma). Oleate treatment of HepG2 cells was carried out as previously described with minor revision [13,7]. Specifically, HepG2 cells or human hepatocytes were plated in 4-well chamber slides with DMEM medium supplemented with 10% FBS (Invitrogen). After 24 h, cells were treated with either control medium (DMEM supplemented with 1% fatty acid free BSA), or medium containing oleate (0.5 mM). The cells were cultured for another 24 h, after which lipid accumulation and miR-206 expression were determined by Oil-Red O staining (Sigma-Aldrich) and qRT-PCR, respectively.

To determine whether *PTPN1* mediates the effect of miR-206 on lipogenesis, HepG2 cells cultured in the DMEM containing 0.5 mM oleate were transfected with MC-*TTR*-miR-206 (200 ng in 4-well chamber slides), or a combination of MC-*TTR*-miR-206 and *PTPN1* Target Protector (TP) (20 nM in 4-well chamber slides). The *PTPN1* TP and control TP were designed and generated by Exiqon. Lipofectamine 2000 was used for transfection of MC-*TTR*-miR-206 and *PTPN1* TP. After another 24 h of culture, lipid accumulation was assessed by Oil-Red O staining, microfluorimetry or imaging.

Gluconeogenic assay

HepG2 cells were maintained in the minimum Eagle's medium with low glucose (Invitrogen) in 24 wells plate overnight. 12 h later, cells were washed three times using cold PBS to remove glucose and incubated for 16 h in a 0.5 ml of glucose production medium (phenol and glucose free DMEM containing gluconeogenic substrates including 20 mM sodium lactate and 2 mM sodium pyruvate). Insulin was added into medium within the last 3 h; and glucose levels measured with an Amplex Red Glucose/Glucose Oxidase Assay Kit (Invitrogen). Glucose concentrations in the media were normalized with cellular protein concentrations [14].

Statistical analysis

Statistical analysis was performed using GraphPad Prism Software®. Data derived from cell-line experiments were presented as mean ± SEM and assessed by a two-tailed Student's *t* test. Mann-Whitney *U* test was used to evaluate the statistical significance for mouse experiments. All experiments were repeated at least three times. $p < 0.05$ was considered to be statistically significant.

Full details of these and other methods can be found in the Supplementary materials and methods.

Results

Lipid accumulation impaired biogenesis of miR-206 in livers of dietary obese mice and human hepatocytes

We measured expression of miR-206 in livers of wild-type mice on HFD, which led to increased hepatic lipid accumulation and blood glucose (Supplementary Fig. 1A–D). qRT-PCR revealed that miR-206 was significantly reduced in fatty livers of dietary obese mice compared to mice treated with standard diet (Fig. 1A). We then determined whether intracellular lipid accumulation can decrease expression of miR-206 in primary human hepatocytes and HepG2 cells [15]. Oleic acids are the most abundant fatty unsaturated acids in liver triglycerides in both normal subjects and patients with NAFLD. As expected, oleate treatment signifi-