Research Article

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MicroRNA-29a-c decrease fasting blood glucose levels by negatively regulating hepatic gluconeogenesis

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Background & Aims: The expression levels of microRNA-29 (miR-29) family members (miR-29a, miR-29b, miR-29c, here denoted collectively as miR-29a-c) are increased in livers of Goto-Kakizaki diabetic rats and *db*/*db* diabetic mice. However, the functional consequences of miR-29a-c upregulation in diabetic livers are not explored. The objective of this study was to evaluate the roles of miR-29a-c in the regulation of hepatic glucose production and blood glucose levels using different mouse models.

Methods: *db*/m, *db*/*db* diabetic and diet-induced obese (DIO) mice were injected with adenovirus expressing miR-29a-c through the tail vein. Blood glucose levels were measured and glucose-tolerance tests and pyruvate-tolerance tests were performed. To explore the molecular mechanism by which miR-29a-c regulate hepatic glucose metabolism, gain or loss of miR-29a-c function studies were performed in primary mouse hepatocytes and the direct effectors of miR-29-mediated effects on glucose metabolism were identified.

Results: Adenovirus-mediated overexpression of miR-29a-c in the livers of *db*/m, *db*/*db*, and DIO mice decreased fasting blood glucose levels and improved glucose tolerance. Overexpression of miR-29a-c in primary hepatocytes and mouse livers decreased the protein levels of PGC-1α and G6Pase, the direct targets of miR-29a-c, thereby reducing cellular, and hepatic glucose production. In contrast, loss of miR-29a-c function in primary hepatocytes increased the protein levels of PGC-1α and G6Pase and increased cellular glucose production. Finally, enforced expression of PGC-1α increased miR-29a-c expression levels in primary hepatocytes, thus forming a negative feedback regulation loop.

Conclusions: miR-29a-c can regulate hepatic glucose production and glucose tolerance in mice.

Keywords: miR-29a-c; Diabetes; Gluconeogenesis; *PGC-1α*; Glucose tolerance.
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Abbreviations: miR-29, microRNA-29; DIO, diet-induced obese; PGC-1α, peroxisome proliferative activated receptor, gamma, co-activator 1 alpha; G6Pase, glucose-6-phosphatase, catalytic; Ad-GFP, adenovirus-containing green fluorescent protein; 3′UTR, 3′untranslated region; SE, standard error; Ad-sp-miR-29a-c, adenovirus expressing miR-29 sponges; GTT, glucose tolerance test; Ad-miR-29a-c, adenovirus expressing miR-29 family members; PTT, pyruvate tolerance test; Fsk/Dex, forskolin/dexamethasone; LPS, lipopolysaccharide.

Introduction

It is critical for the body to maintain its blood glucose level within a tight range. Red blood cells and the brain depend on glucose for metabolic fuel, and severe decreases in circulating glucose levels may result in seizures or death [1]. The liver is the major source of glucose when food supply is limited, through the mobilization of glycogen and hepatic gluconeogenesis. However, abnormal activation of hepatic gluconeogenesis contributes to hyperglycemia [2]. The expression of key gluconeogenic genes, including phosphoenolpyruvate carboxykinase (*PEPCK*) and glucose-6-phosphatase (*G6Pase*), is controlled by hormones, including glucagon, glucocorticoids, catecholamines, and insulin [3]. Several transcriptional factors, such as cAMP responsive element binding protein (CREB), forkhead box O1 (FOXO1), hepatocyte nuclear factor 4 (HNF4), and glucocorticoids receptor (GR), have been identified to regulate the expression of these enzymes. Notably, the transcriptional co-activator peroxisome proliferative activated receptor gamma co-activator 1 alpha (PGC-1α) is a master regulator of the gluconeogenic program in fasting and diabetic states by directly interacting with several of these transcriptional factors [4–8].

PGC-1α expression levels are elevated by a number of external stimuli, such as cold temperatures in brown adipose tissue, exercise, and decreased ATP levels in skeletal muscle and fasting in the liver [9]. In addition, PGC-1α protein activity can be

modulated by post-translational modification. PGC-1α can be phosphorylated by p38 MAPK, resulting in the release of inhibitory factors of PGC-1α and extending its half-life [9,10]. Insulin-stimulated *Akt* can also phosphorylate PGC-1α, subsequently preventing the recruitment of PGC-1α to cognate promoters and impairing its ability to promote gluconeogenesis [11]. Insulin-induced Cdc2-like kinase 2 directly phosphorylates the SR domain on PGC-1α, resulting in the repression of hepatic gluconeogenesis [12]. In addition to being phosphorylated, the PGC-1α protein is also acetylated by the GCN5 acetyltransferase complex and acetylation of PGC-1α decreases its activity [13]. In contrast, deacetylation of PGC-1α through Sirtuin 1 increases its activity, promoting gluconeogenesis in the liver [14]. Adenovirus-mediated knockdown of PGC-1α in *db*/*db* diabetic mouse livers decreases circulating glucose levels and improves glucose tolerance [15]. Global *PGC-1α* knockout mice also display fasting hypoglycemia, and glucose output from PGC-1α-deficient hepatocytes in response to hormones is impaired [16,17].

MicroRNAs are small, endogenous, non-coding RNAs that regulate expression of target genes through translational repression or degradation of target mRNAs [18]. Recent studies have suggested that the deregulation of microRNAs contributes to various diseases, including type 2 diabetes [19–21]. We have previously demonstrated that expression levels of miR-29 family members are upregulated in the liver, fat, and muscle tissues of Goto-Kakizaki diabetic rats [22]. However, the functional consequences of miR-29a-c upregulation in diabetic livers were not explored.

In this study, we show that levels of miR-29a-c are elevated in livers of *db*/*db* diabetic mice. Adenovirus-mediated overexpression of miR-29a-c in livers of both *db*/*db* diabetic and high-fat diet-induced obese (DIO) mice alleviated hyperglycemia and insulin resistance. Forced expression of miR-29a-c in livers of *db*/m control mice also lowered blood glucose levels. In addition, we discovered that miR-29a-c dampened hepatic glucose production by directly targeting *PGC-1α* and *G6Pase*.

Materials and methods

Animal treatments

C57BL/6J male mice and 8- to 10-week-old male *db*/*db* mice and heterozygous control *db*/m mice were purchased from Model Animal Research Center of Nanjing University (Nanjing, China). A diet-induced obese model was established as described in Supplementary Materials. Mice were injected intravenously through the tail vein with adenovirus encoding green fluorescent protein (Ad-GFP), miR-29a-c or miR-29 sponges at a dose of 1×10^9 plaque-forming units in 0.2 ml PBS. Mice were killed for real-time PCR and Western blotting analysis on day 9 after adenovirus injection. The mouse protocol was approved by the Animal Research Committee at the Institute of Laboratory Animals, Chinese Academy of Medical Science and Peking Union Medical College.

Preparation of recombinant adenovirus

Adenovirus expressing GFP or miR-29a-c was generated as previously described [22].

Construction of miR-29 sponges

Six bulged miR-29 binding sites (5′-TAACCGATTTTCTTGGTGCTA-3′) were inserted into the 3′untranslated region (3′UTR) of a *GFP* reporter gene controlled by the CMV promoter, and recombinant adenovirus was prepared as described previously [23].

Cells, constructs, and luciferase assays

293A cells were cultured as described [24]. Luciferase reporter vectors containing the 3'UTR of *PGC-1α* and *G6Pase* were generated as described in Supplementary Materials. Transfection was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. Luciferase reporter assays were performed using the Dual Luciferase Reporter Assay System (Promega).

RNA isolation, Northern blotting, and Western blotting

Total RNA was extracted from cells and tissues using the Trizol agent (Invitrogen). Northern blotting analyses of microRNAs were performed as described previously [22], primers are listed in Supplementary Table 1. Western blotting was performed as described previously [25].

Glucose- and pyruvate-tolerance tests

Mice fasted for 16 h were injected intraperitoneally with D-glucose (2 g/kg), or pyruvate (2 g/kg). Blood glucose levels were measured from the tail vein at indicated times using a glucometer (One Touch Ultra; LifeScan Inc.)

Real-time PCR

MicroRNAs were prepared using mirVana miRNA Isolation Kit (Ambion Inc.). MicroRNA levels were analyzed using TaqMan microRNA Assays (Applied Biosystems); expression of the microRNAs was normalized to that of the U6 snRNA.

The total RNA was isolated from cells or livers using the Trizol agent (Invitrogen). For gene expression analysis, real-time PCR was performed using a BioRad IQ5 instrument and SYBR mix (Promega). As an internal control, 36B4 was used. The sequences of the primers are presented in Supplementary Table 1.

Glucose production assay

Primary hepatocytes were prepared from livers of male C57BL/6J mice as described previously. Cells were cultured in RPMI1640 with 10% fetal bovine serum and then infected with adenovirus for 24 h. Cells were washed five times with PBS and then stimulated with Fsk/Dex (forskolin/dexamethasone) for 18 h in glucose-free DMEM containing 2 mM sodium pyruvate. Glucose concentrations in the medium were measured with a glucose assay kit (Sigma).

Antibodies and chemicals

The antibody against GAPDH (#2118) was purchased from Cell Signaling Technology. The antibody against PGC-1α (AB3242) was from Millipore. The antibody against G6Pase (sc-25840) was from Santa Cruz. Insulin, dexamethasone, and forskolin were from Sigma.

Statistical analysis

The data shown represent the mean ± standard error (SE) values of three independent duplicate experiments. Significance was tested using Student's *t* test ($^*p$ <0.05; $^{**}p$ <0.01).

Results

Hepatic miR-29a-c expression is upregulated in diabetic mice and induced by fasting in normal mice

We previously showed that the expression levels of miR-29 family members were upregulated in the livers of Goto-Kakizaki diabetic rats [22]. We hypothesized that an upregulation of miR-29a-c might also occur in the livers of diabetic mice. Our data indicated that the expression levels of miR-29b in *db*/*db* mouse livers are approximately twofold higher than those in the control *db*/m mice (Fig. 1A and Supplementary Fig. 1). The