Glucose-regulated insulin production in the liver improves glycemic control in type 1 diabetic mice

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ABSTRACT

Objective: Type 1 diabetes results from autoimmune destruction of beta-cells in the pancreas. Our objective is to reconstitute a glucose-responsive system in the liver to regulate hepatic insulin production for improving glycemic control in type 1 diabetes.

Methods: We have cloned the glucose-responsive element (GRE) from the promoter of acetyl-CoA carboxylase (ACC), an enzyme that catalyzes the rate-limiting step in fatty acid synthesis in the liver in response to glucose. To increase the amplitude of glucose induction, we quadruplicated the GRE DNA by gene duplication. The resulting GRE multimer (4 × GRE) was tested for its ability to drive rat proinsulin cDNA expression in hepatocytes and insulin-deficient diabetic mice.

Results: We showed that this GRE multimer-directed glucose-responsive system produced insulin in hepatocytes in a glucose-dependent manner. When delivered into the liver by adenovirus-mediated gene transfer, this glucose-responsive insulin production system was able to reverse hyperglycemia to a normal range without causing hypoglycemia after glucose challenge or overnight fasting. Insulin vector-treated diabetic mice exhibited significantly improved blood glucose profiles in response to glucose tolerance, correlating with insulin production in the liver. We recapitulated these findings in streptozotocin-induced diabetic CD1 mice and autoimmune non-obese diabetic mice.

Conclusion: Our data characterized the GRE motif from the ACC promoter as a potent glucose-responsive element, and provided proof-of-concept that the 4 × GRE-mediated hepatic insulin production is capable of correcting insulin deficiency and improving glycemic control in type 1 diabetes.

Keywords Insulin; Liver; Diabetes; NOD mice

1. INTRODUCTION

Type 1 diabetes results from autoimmune destruction of β-cells in the pancreas [1,2]. It usually strikes children and adolescents with no established cure. As a result, patients with type 1 diabetes must live with a compromised life-style in compliance with daily vigilance of blood sugar and insulin injection. Although the use of different formulations of insulin in combination has improved the quality of glycemic control for patients with type 1 diabetes [3,4], exogenous insulin injection multiple times daily has proven inadequate in providing physiological control of blood sugar, nor is it effective in preventing diabetic complications [5]. Tight glycemic control through intensive insulin therapy is associated with hypoglycemic episodes and undue weight gain [6,7].

To restore endogenous insulin production, islet transplantation along with immunotherapy is developed [8]. Although this protocol is able to achieve insulin independence with near normal glycemic control in recipients with type 1 diabetes, its widespread clinical application is limited by the scarcity of cadaver pancreata. Aside from relatively poor revascularization [9–12], transplanted islets suffer from autoimmune destruction and allograft rejection, contributing to graft failure [13–23]. While efforts have been made to derive islet cells from pluripotent stem cells, it remains challenging to differentiate stem cells in large quantities into characteristic β-cell types that secrete insulin in a glucose-responsive manner [24–31].

As an alternative approach, the liver is being sought as a surrogate organ for restoring endogenous insulin production. Unlike transplanted islets that succumb to autoimmune destruction, insulin-producing hepatocytes are of non-β-cell type and are refractory to autoimmune destruction and allograft rejection, contributing to graft failure [13–23]. Similar to β-cells, hepatocytes possess the glucose-sensing mechanism due to the expression of glucokinase (GK) and glucose transporter 2 (Glut2) [33]. Indeed, hepatic insulin production has been shown to prevent ketoadiposis, restore hepatic glycogen storage, abate body weight loss, and reverse insulin deficiency in animal models of type 1 diabetes [34,35]. It remains a major hurdle to achieve glucose-regulated hepatic insulin secretion [5,36,37].

*This project was supported by American Diabetes Association.

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Received September 23, 2014 • Revision received October 21, 2014 • Accepted October 26, 2014 • Available online 1 November 2014

http://dx.doi.org/10.1016/j.molmet.2014.10.005
In this study, we harnessed the glucose-responsive element (GRE) for regulating insulin production in the liver. GRE is a cis-acting DNA motif that is conserved in the promoter of glucose-inducible genes, such as the liver-type pyruvate kinase (L-PK), S14 and acetyl-CoA carboxylase (ACC) [38–40]. GRE comprises two tandem repeats of 5′-CACGTG-3′, known as E-box, separated by a 5-bp spacer [40]. When bound by carbohydrate responsive element binding protein (ChREBP), GRE mediates glucose-dependent induction of hepatic enzymes in the post-prandial phase. We compared the relative strength of GRE derived from the ACC and L-PK promoter, using the luciferase reporter system. We showed that both the L-PK-GRE and ACC-GRE DNA motifs were able to drive luciferase expression in cultured hepatocytes in a glucose-dependent manner, whereas the ACC-GRE was 3- to 4-fold stronger than the L-PK-GRE in response to glucose. We then used the ACC-GRE for driving insulin production in the liver. To increase the amplitude of glucose inducibility, we placed the insulin cDNA under the control of multiple copies of the ACC-GRE DNA motif. As plasma insulin has a relatively short-life (~8 min) [41], we cloned a liver-specific enhancer from hepatic aldolase B gene into the glucose-regulated insulin expression system to enhance hepatic insulin production. We showed that such an augmented glucose-responsive system was able to produce insulin in hepatocytes in a glucose-dependent manner. Using adenovirus-mediated gene transfer approach, we delivered this augmented glucose-responsive insulin production system into the liver of diabetic mice. We showed that hepatic insulin production was able to reverse hyperglycemia to a normal range without experiencing hypoglycemia after overnight fasting or after glucose challenge. We recapitulated these findings in streptozotocin (STZ)-induced diabetic CD1 mice and autoimmune non-obese diabetic (NOD) mice. Furthermore, insulin vector-treated diabetic mice exhibited significantly improved blood glucose profiles in response to glucose tolerance, correlating with insulin production in the liver. In contrast, glucose intolerance and hyperglycemia secondary to insulin deficiency persisted in control vector-treated diabetic mice. These data provided the feasibility for using the liver-specific GRE motif to achieve glucose-regulated insulin production in hepatocytes. Glucose-regulated hepatic insulin production is able to correct insulin deficiency and improve glycemic control in type 1 diabetes.

2. MATERIALS AND METHODS

Cell culture and adenovirus: HepG2 cells were purchased from ATCC and were cultured, as described in Ref. [42]. Rat primary hepatocytes were obtained from Cambrex Corporate (East Rutherford, New Jersey). To transduce cells, we added to cell culture 100 μl of adenoviral vectors encoding the engineered rat proinsulin-1 cDNA at a defined multiplicity of infection (MOI). Such a genetically modified rat preproinsulin contains the furin consensus sequence (Arg-X-Arg/Lys-Arg) between the B/C and C/A junctions to allow processing of preproinsulin to mature insulin by furin, a proprotein convertase that is abundantly expressed in the liver [43,44]. The control adenoviral vector Adv-Empty has been reported [45]. After 24-h incubation, cells and medium were collected for analysis. Both control and insulin adenoviral vectors were produced in HEK293 cells and purified, as described in Ref. [42].

Animal studies: CD1 and NOD mice (male, 10-week old) were purchased from the Jackson Laboratory and kept in isolator cages in a barrier animal facility with a 12-h light/dark cycle. Mice were fed ad libitum with a regular diet. Mice were rendered diabetic by intraperitoneal injection of streptozotocin (STZ, 160 mg/kg). Blood glucose levels were determined with a Glucometer Elite (Bayer, Elkhart, IN). Adenoviral vectors (1.5 × 10^9 plaque-forming units) were intravenously injected via tail vein to individual diabetic mice, after confirming diabetes 5 days post STZ administration. For determining plasma insulin levels, blood was collected from tail vein into capillary tubes pre-coated with potassium-EDTA (Sarstedt, Nümbrecht, Germany) and subjected to the ultrasensitive mouse insulin enzyme-linked immunosorbent assay (ALPCO, Windham, NH). All procedures were approved by the Institutional Animal Care and Use Committee of University of Pittsburgh.

Glucose tolerance test: Mice were fasted for 5 h, followed by intraperitoneal injection of glucose (2 g/kg body weight), as described in Ref. [46].

RNA isolation and RT-PCR: Total RNA was prepared from HepG2 cells, using the RNeasy Mini Kit (QIAGEN, Valencia, CA). Aliquots of RNA (250 ng) were subjected to RT-PCR analysis for detecting insulin mRNA levels in HepG2 cells that were pre-transduced with Adv-Empty and Adv-INS vectors. The insulin primers were INS-5′ (5′-GCCCTGTTGATGCGCTTC-3′) and INS-3′ (5′-GTTCGAGTATTTTCACGTG-3′). The control β-actin primers were described in Ref. [42].

Luciferase assay: HepG2 cells were transfected with plasmid encoding the luciferase gene under the control of ACC-GRE or L-PK-GRE regulated system, using the Lipofectamine 2000 and plus reagents (Invitrogen). In each transfection, plasmid pCMV-LacZ was included and the amount of β-galactosidase activity was used as a control to normalize transfection efficiency. After 16-h incubation, cells were subjected to luciferase or β-galactosidase activity assays, as described in Ref. [45].

Immunohistochemistry: Liver tissues from euthanized mice were fixed in 4% paraformaldehyde for 4 h, followed by incubation in 30% sucrose at 4 °C overnight. Cryo-sections (8 μm) were cut and subjected to immunohistochemistry using rabbit anti-insulin antibody (1:200 dilution, sc-9168, Santa Cruz Biotechnology, Santa Cruz, CA). The secondary antibody was Cy3-conjugated goat anti-rabbit IgG (1:500 dilution, Jackson Immunoresearch Laboratories, West Grove, PA). Hepatocytes with positive insulin immunostaining were visualized in the Aivoert 200 fluorescent microscope (Zeiss, Oberkochen, Germany).

Statistics: Statistical analyses of data were performed by analysis of variance (ANOVA) using StatView software (Abacus Concepts, CA). Data were expressed as the mean ± SEM. P-values <0.05 were considered statistically significant.

3. RESULTS

3.1. Characterization of GRE for mediating glucose-inducible gene expression

To achieve glucose-inducible insulin production in liver, we harnessed the liver-specific glucose-responsive element (GRE), a cis-acting DNA motif that is present in the promoter of glucose-inducible genes, such as liver-type pyruvate kinase (L-PK) and acetyl-CoA carboxylase (ACC) [38–40]. To determine the ability of GRE to mediate glucose-inducible gene expression, we cloned GRE from the rat ACC promoter (~126/−102 nt) or L-PK promoter (~171/−147 nt) upstream of the minimum L-PK promoter (~40/−1 nt)-directed luciferase expression system (Figure 1A,B). The resulting constructs were transfected into HepG2 cells. After 24-h incubation, we shifted culture media from low to higher glucose concentrations for 16 h, followed by the determination of luciferase activity. We showed that the GRE derived from both L-PK and ACC promoters was capable of conferring upon the minimum L-PK promoter glucose inducibility, as evidenced by glucose dose-dependent induction of luciferase activity in HepG2 cells (Figure 1C,D). Of particular significance is that the ACC-GRE is 3- to 4-fold stronger than the L-PK-GRE in mediating glucose-dependent
induction of luciferase activity. We chose the ACC-GRE for driving insulin expression.

3.2. GRE-mediated induction of insulin production in hepatocytes

To test the GRE from the ACC promoter for its ability to regulate insulin production, we cloned the ACC-GRE DNA upstream of the rat proinsulin cDNA that is genetically modified for its processing to mature insulin by hepatic proprotein convertase furin [34,35]. The resulting construct was converted to an adenoviral vector encoding the ACC-GRE-directed insulin expression, followed by intravenous administration into STZ-induced diabetic mice. This approach has been shown to result in transgene expression predominantly in liver with little transduction of cells in extrahepatic tissue [46,47]. We showed that the ACC-GRE-directed insulin expression was able to abate weight loss and improve blood glucose levels in STZ-induced diabetic mice. Nonetheless, due to relatively lower plasma insulin levels, the ACC-GRE-directed insulin production in the liver was insufficient to correct hyperglycemia secondary to insulin deficiency in STZ-induced diabetic mice (Supplemental Figure 1).

To overcome this limitation, we took two additional measures. First, we cloned four copies of GRE (4×GRE) upstream of the preproinsulin cDNA to increase the amplitude of glucose induction. Second, we cloned the enhancer element from the liver-specific aldolase B gene. Based on two independent studies [48,49], this aldolase B enhancer (435 nt in length) is responsible for binding hepatocyte nuclear factor HNF1 and HNF4 to significantly enhance transgene expression, when cloned upstream of a heterologous promoter in liver cell types. We hypothesized that hepatic insulin production from such an enhanced glucose-responsive system would be induced under hyperglycemic conditions for correcting insulin-deficient diabetes.

To test this hypothesis, we first characterized the Adv-INS vector encoding the glucose-regulated insulin expression system under the control of 4×GRE and aldolase B enhancer in vitro. We transduced HepG2 cells with Adv-INS or control Adv-Empty vector, followed by the determination of insulin concentrations in conditioned medium. We showed that transduction of Adv-INS resulted in vector dose-dependent increases in medium insulin levels (Figure 2B), correlating with the induction of insulin mRNA levels in HepG2 cells (Figure 2C). To determine the effect of glucose on insulin production, we transduced rat primary hepatocytes with Adv-INS or Adv-Empty vector. After incubation for 16 h at low glucose conditions (final conc. 2.8 mM), cells were induced by shifting culture medium from low to higher glucose conditions (5, 10, 20, and 30 mM), followed by the determination of insulin levels in conditioned medium. The Adv-INS vector exhibited glucose-dose dependent production of insulin in rat primary hepatocytes (Figure 2D).

3.3. GRE-mediated induction of insulin production in STZ-induced diabetic mice

To test the hypothesis that hepatic insulin production from the enhanced glucose-responsive expression system would reverse hyperglycemia in diabetic mice, we rendered CD1 mice (male, 18-week-old) hyperglycemia by a single i.p. dose of STZ (160 mg/kg). Diabetic CD1 mice were stratified by body weight and degree of hyperglycemia to ensure a similar mean level of body weight (31 ± 1 g) and blood glucose (460 ± 42 mg/dL) in two groups (n = 9–14), which were intravenously injected with Adv-INS or Adv-Empty vector at a predefined dose (1.5 × 10⁸ pfu/g). We showed that insulin vector administration resulted in the remission of STZ-induced diabetes for about one month (Figure 3A). Furthermore, insulin vector-treated diabetic mice exhibited significantly improved blood glucose profiles in response to glucose challenge (Figure 3B), consistent with higher plasma insulin levels in the Adv-INS vector group (Figure 3C). In contrast, hyperglycemia persisted in control vector-treated diabetic mice (Figure 3A). This effect was accompanied by glucose intolerance (Figure 3B) and severe insulin deficiency (Figure 3C) in the control group. To address the potential episodes of fasting hypoglycemia in insulin vector-treated diabetic mice, we determined blood glucose levels up to 5 h after glucose tolerance
under fasting conditions by removing food and allowing free access to water. No fasting hypoglycemia (blood glucose < 50 mg/dL) was detected in insulin vector-treated diabetic mice. Likewise, we performed overnight fasting and did not detect hypoglycemic episodes in insulin vector-treated diabetic mice. Consistent with improved glycemic control, insulin vector-treated diabetic mice, as opposed to control vector-treated diabetic mice, were able to maintain weight gain (Figure 3D).

To confirm hepatic insulin production, diabetic mice treated with insulin and control vectors were sacrificed and liver tissues were subjected to insulin immunohistochemistry. Livers from control vector-treated mice were negative for insulin immunostaining (Figure 3E), correlating with insulin deficiency and hyperglycemia in the control group. In contrast, livers from insulin vector-treated mice were positive for insulin immunostaining (Figure 3F, G), consistent with increased plasma insulin levels and improved glucose profiles in the insulin vector group.

3.4. GRE-mediated induction of insulin production in diabetic NOD mice

To corroborate the above findings, we determined hepatic insulin production and blood glucose control in non-obese diabetic (NOD) mice (male, 18-week-old). NOD mice spontaneously develop diabetes with variable times and variable blood glucose levels. To remove those confounding factors, we generated diabetic NOD mice with a single i.p. dose of STZ (160 mg/kg) to obtain simultaneous onset of diabetes. We stratified diabetic NOD mice by body weight and degree of hyperglycemia to ensure a similar mean body weight (25 ± 2 g) and blood glucose level (481 ± 52 mg/dL) in two groups (n = 9), followed by intravenous injection of Adv-INS or Adv-Empty vector at a predefined dose (1.5 × 10^6 pfu/g). As shown in Figure 4, hyperglycemia was reduced to a normal range (80–120 mg/dL), with concomitant improvement in glucose tolerance and recovery of insulin deficiency at day 7 after insulin vector delivery into diabetic NOD mice. This beneficial effect lasted from day 7 to 26 post vector administration. As a result, insulin vector-treated diabetic NOD mice were able to maintain body weight. In contrast, control vector-treated diabetic NOD mice manifested severe hyperglycemia and glucose intolerance, accompanied by progressive weight loss secondary to insulin deficiency.

As control, we subjected liver tissues of control and insulin vector-treated NOD mice to insulin immunohistochemistry (Supplemental Figure 2). Insulin positively stained hepatocytes were detectable in the liver of insulin vector-treated NOD mice. In contrast, livers from control vector-treated NOD mice were negative for insulin immunostaining.

4. DISCUSSION

Type 1 diabetes results from insulin deficiency secondary to autoimmune destruction of β-cells in the pancreas. An innovative approach for correcting insulin deficiency is to restore insulin production in the liver via hepatic insulin gene transfer. The liver is chosen as an insulin-producing surrogate for two prominent reasons. First, the liver expresses GK and Glut2, two key components of the glucose-sensing mechanism [33]. As such, hepatocytes retain the same characteristic ability to respond to changes in blood glucose levels. Second, hepatocytes are of non-beta cell types that do not succumb to autoimmune attack. Hepatic insulin gene transfer has the potential of restoring endogenous insulin production for long-term glycemic control without eliciting recurrent autoimmunity against insulin-producing hepatocytes [32]. However, achieving adequately regulated hepatic insulin production in coupling with blood glucose remains a major hurdle, although progress has been made to regulate insulin production in coupling with blood glucose.
deciency and reverse hyperglycemia in STZ-induced diabetic CD1 mice. Diabetic mice displayed significantly improved blood glucose profiles after insulin vector administration and during glucose tolerance. We reproduced these findings in diabetic NOD mice. Our data indicate that hepatic insulin production from such an enhanced glucose-responsive system is capable of correcting insulin deficiency and reversing hyperglycemia in type 1 diabetic mice.

In addition, we determined blood glucose levels in the postinduction phase (up to 5 h after glucose tolerance) as well as after overnight fasting. No fasting hypoglycemic episodes were detected in insulin vector-treated diabetic mice. It is plausible that this effect is due in part to rapid clearance of insulin (plasma half-life, ~8 min) [41]. In addition, the GRE activity becomes attenuated in response to blood glucose decline, thereby limiting hepatic insulin production. Indeed, ChREBP, the transcriptional factor responsible for binding to the GRE and stimulating GRE activity, remains inactive in the cytoplasm at basal glucose conditions [53,54]. In response to postprandial blood glucose elevation, ChREBP is translocated into the nucleus for facilitating glucose-stimulated gene expression in the liver [53,54].

We would like to acknowledge the limitation in using adenoviral vectors in this study. Although this approach results in transgene expression mainly in the liver, adenovirus-mediated gene transfer is associated with short-term transgene expression in immune competent hosts. It is noteworthy that our objective in this study was not to advance gene vector development. Rather we utilized adenoviral vectors as a vehicle for transferring insulin cDNA into the liver. Likewise, we used STZ-induced diabetic NOD mice for recapitulating our findings made in diabetic CD-1 mice. In our previous studies [34], hepatic insulin

Figure 3: Hepatic insulin production improved glycemic control in STZ-induced diabetic mice. CD1 mice (male, 18-week-old) were rendered diabetic by intraperitoneal injection of STZ (160 mg/kg). Diabetic CD1 mice were stratified by the degree of hyperglycemia and randomly assigned to two groups to ensure similar mean blood glucose levels per group. Diabetic mice were intravenously injected via tail vein with Adv-Empty (ctrl, n = 9) or Adv-INS (INS, n = 14) vector. Blood glucose levels were measured at ad libitum conditions in the morning (A). Blood glucose levels in control vector-treated mice exceeded the upper limit (600 mg/dL) of the Glucometer from day 10. Glucose tolerance test was performed at day 15 after vector administration (B). Blood glucose levels exceed the upper limit of Glucometer in the control group after glucose challenge. Plasma insulin levels were determined at day 18 post vector administration (C). (D) Body weight. Finally, liver tissues from euthanized mice in control vector (E) and insulin vector (F and G) groups were fixed in 4% paraformaldehyde for 4 h. Cryosections were subjected to anti-insulin immunohistochemistry, followed by examination under immunofluorescent microscopy at ×40 magnification (E and F) and ×100 magnification (G). Bar, 50 μm *p < 0.05 and **p < 0.001 vs. ctrl.
production derived from the elongation factor-1 alpha (EF-1a) promoter, whose transcriptional activity is not subject to glucose regulation. In the present studies, we used the 4xGRE multimer to render hepatic insulin production responsive to glucose. Our studies provided proof-of-concept for reconstituting a glucose-responsive system for regulating insulin production in the liver for improving glycemic control without fasting hypoglycemia in type 1 diabetes. Further research is needed to refine the glucose-regulated system for coupling hepatic insulin secretion with changes in blood glucose levels and to address whether hepatic insulin production is capable of providing long-term physiological glycemic control in spontaneous diabetic NOD mice.

ACKNOWLEDGMENT

This project was supported by American Diabetes Association (ADA 1-06-CD-05). We thank Sandra Slusher for technical assistance and Dr. Howard Towle for providing plasmids encoding the GRE DNA from the L-PK and ACC promoters.

CONFLICT OF INTEREST

None of the authors has a conflict of interest to declare in this manuscript.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at doi:10.1016/j.molmet.2014.10.005.

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