Glucose regulates secretion of exogenously expressed insulin from HepG2 cells *in vitro* and in a mouse model of diabetes mellitus *in vivo*

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Abstract

Glucose-controlled insulin secretion is a key component of its regulation. Here, we examined whether liver cell secretion of insulin derived from an engineered construct can be regulated by glucose. Adenovirus constructs were designed to express proinsulin or mature insulin containing the conditional binding domain (CBD). This motif binds GRP78 (HSPA5), an endoplasmic reticulum (ER) protein that enables the chimeric hormone to enter into and stay within the ER until glucose regulates its release from the organelle. Infected HepG2 cells expressed proinsulin mRNA and the protein containing the CBD. Immunocytochemistry studies suggested that GRP78 and proinsulin appeared together in the ER of the cell. The amount of hormone released from infected cells varied directly with the ambient concentration of glucose in the media. Glucose-regulated release of the hormone from infected cells was rapid and sustained. Removal of glucose from the cells decreased release of the hormone. In streptozotocin-induced diabetic mice, when infected with adenovirus expressing mature insulin, glucose levels declined. Our data show that glucose regulates release of exogenously expressed insulin from the ER of liver cells. This approach may be useful in devising new ways to treat diabetes mellitus.

Key Words

- Diabetes
- Non-beta cell insulin secretion
- Glucose-dependent

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Introduction

Diabetes mellitus is a terrible disease that is becoming a worldwide epidemic. The worldwide prevalence of this disease will likely reach 4.4% by 2030 (Wild *et al.* 2004). A primary defect in diabetes mellitus is an absent or insufficient activity of insulin, leading to type 1 or 2 diabetes respectively. Exogenously injected insulin lowers blood glucose and thus it remains a cornerstone for treating the disease (Rodbard *et al.* 2009, American Diabetes Association

2011). But problems arise when the dose of insulin injected fails to match caloric intake resulting in unwanted hyper- or hypoglycemia leading to life-threatening consequences. Whereas chronic hyperglycemia underlies the long-term complications of the disease, hypoglycemia gives rise to acute events with more dire outcomes (Cryer *et al.* 2003, Turchin *et al.* 2009). Thus, treatment guidelines for diabetes mellitus include targets that define both upper and lower

limits of glucose (Bhattacharyya *et al.* 2009, Rodbard *et al.* 2009, American Diabetes Association 2011).

Current therapies for diabetes cannot eliminate undesirable swings in glucose. Thus, interest to restore or mimic the body's ability to sense insulin action and in turn regulate levels of the hormone to avoid hyper- or hypoglycemia remains high. This issue has been addressed using islet and non-islet cells (Efrat 1998, Yoon & Jun 2002, Olson & Thule 2008). In non-islet cell studies, proof of concept studies, in animals, using gene transfer to produce insulin involves a glucose-sensitive promoter that controls insulin gene transcription and thus in turn the synthesis of the hormone but not its secretion (Thule *et al.* 2000, Chen *et al.* 2001, Alam & Sollinger 2002, Hsu *et al.* 2008). In the absence of glucose-regulated insulin release, transcriptional control by itself might produce a lag phase in the secretory response of insulin to meals, which may lead to initial hyperglycemia followed later by hypoglycemia.

Glucose control of pancreatic insulin secretion is a key step in regulating activity of the hormone. The use of glucose to manipulate secretion of engineered insulin from a non-pancreatic cell remains a challenge. Information derived from such a system would help us understand more the role of rapidly released insulin in regulating plasma glucose *in vivo*. The recent use of hyaluronidase mixed with rapid acting analog insulin (lispro) in clinical trials to enable faster absorption of insulin supports the need for such knowledge (Hompesch *et al.* 2011, 2012). Therefore, to mimic glucose control of insulin release *in vivo*, especially the first phase of pancreatic insulin secretion, we expressed and stored a chimeric hormone in liver cells followed by the use of glucose to regulate secretion of insulin *in vitro* and *in vivo*.

Materials and methods

Adenovirus constructs

The conditional binding domain (CBD) is a heptapeptide (FYQLAKT) that binds glucose-regulated protein 78 (GRP78 (HSPA5)) (Shiu *et al.* 1977, Fourie *et al.* 1994, Gething 1999), an endoplasmic reticulum (ER) protein in eukaryotes. Oligonucleotides encoding CBD when placed behind the signal sequence of human preproinsulin cDNA will express a fusion hormone (Dorner *et al.* 1990). Mutations in preproinsulin cDNA were created using Gene Tailor (Invitrogen). Adenoviruses were constructed using the AdEasy system (Stratagene, La Jolla, CA, USA) as per instructions and amplified in HEK293 cells, purified by double CsCl density-gradient centrifugation, and dialyzed

(10 mmol/l Tris-Cl, pH 8.0; 2 mmol/l MgCl₂; and 4% sucrose (w/v)). Optical density at 260 nm provided particle number and expressed as optical particle units per milliliter (OPU/ml).

Cell culture

HEK293 (ATCC, Manassas, VA, USA; CRL-1573) cells were grown in DMEM, 10% FBS (v/v), and 1% penicillin-streptomycin (v/v) (Invitrogen). HepG2 (ATCC HB-8065) cells grown in MEM α with 10% FBS and 1% penicillin-streptomycin were infected with adenovirus at 70–80% confluency.

Quantitative real-time and semiquantitative PCR

RNA isolation and RT were performed using standard methodology with RNeasy Mini Kit (Qiagen), random hexamers, and M-MLV reverse transcriptase (Invitrogen). Real-time PCR primers and probes were designed using Primer3 Software (Applied Biosystems): proinsulin sense 5'-GGGGAACGAGGCTTCTTCTA-3'; probe FAM-5'-GAG-GCAGAGGACCTGCAG-3'-MGB; antisense 5'-CACAA-TGCCACGCTTCTG-3'. 20 $\times$ 18S rRNA FAM/MGB probe (Applied Biosystems) was used as the endogenous control. Amplification was performed in MicroAmp Fast Optical 96-well reaction plates (Applied Biosystems) using the 7900HT Fast Real-Time PCR System (Applied Biosystems), and results were analyzed with SDS 2.3 and RQ Manager Software Applied Biosystems). Semiquantitative reverse transcriptase PCR were performed using an MJ Research PTC-100 thermal cycler with denaturation at 94 °C, annealing at 60 °C, and extension at 72 °C, for a total of 30 cycles. PCR products were analyzed on 1.5% agarose gel. Primers for proinsulin sense: 5'-CCGGGATCCATTTGT-GAACCAACACCTGTGC-3'; antisense: 5'-GCGGCGGCCGCCTAGTTGCAGTAGTTCTCCAG-3'. Primers for GAPDH were used as previously reported (Zerbini *et al.* 2003).

Immunoblotting

HepG2 cells or mouse liver lysates were extracted in buffer (50 mmol/l Tris-HCl, pH 7.4, 150 mmol/l NaCl, 1% NP-40 (v/v), 1 mmol/l of Na₃VO₄, phenylmethylsulfonyl fluoride, NaF, and 10 μ g/ml aprotinin and leupeptin) and then separated on 13% SDS-polyacrylamide gel and transferred to PVDF membrane for western blot analysis using anti-insulin, anti-GRP78, anti-GRP94 antibodies (Santa Cruz Biotechnology), or anti- β -tubulin (Sigma) and peroxidase-conjugated secondary antibodies before visualization with the

ECL detection system (Amersham Pharmacia). Signal intensity of the bands was normalized to the corresponding first or β -tubulin band on the same membrane.

Immunoprecipitation

Cell lysate containing 700 μ g total protein was diluted to a final volume of 300 μ l with lysis buffer. To this, 20 μ l protein A/G agarose beads (Santa Cruz Biotechnology) were added and the mixture was incubated for 1 h at 4 °C with gentle rocking. Next, the beads were pelleted and the supernatant saved to which was added 2 μ g anti-GRP78 or anti-insulin antibody or normal IgG (Santa Cruz Biotechnology) and 20 μ l agarose beads followed by incubation at 4 °C with gentle rocking overnight. The mixture was separated by centrifugation and the pellet was washed with lysis buffer before release of bound material by boiling and then SDS-polyacrylamide gel separation.

Immunofluorescence

HepG2 cells grown on coverslips or frozen mouse liver sections were fixed with 4% paraformaldehyde or methanol, permeabilized with 0.5% Triton X-100 (v/v), and incubated with anti-insulin, anti-GRP78, or anti-GRP94 antibodies (Santa Cruz Biotechnology) before exposure to Alexa Fluor-conjugated secondary antibody (Molecular Probes, Burlington, ON, Canada) and viewed using an inverted microscope controlled by NIS-Elements Imaging Software (Nikon, Melville, NY, USA).

Streptozotocin-diabetic mice and virus injection

Female Balb/CJ mice (3–6 weeks, Jackson Lab, Bar Harbor, ME, USA) were fed standard chow plus water *ad libitum* and diabetes was induced by daily injections of streptozotocin (STZ, 50 mg/kg) for 5 days. Tail vein glucose was monitored using the FreeStyle Mini meter (Abbott). Diabetes was present if glucose ≥ 11.1 mmol/l for 3 consecutive days before injecting animals ($n \geq 6$ /group) with AdSsCbdInsF3 (0.7×10^9 – 1.0×10^9 pfu) or control (AdEGFP or diluent) via tail vein (Shayakhmetov *et al.* 2004). All procedures were approved and complied with guidelines at University of Calgary.

Intraperitoneal glucose tolerance test

Mice fasted ≥ 6 h were injected i.p. with 2 g/kg glucose (Davalli *et al.* 1995) and glucose monitored for 15 or 30 min up to 120 min.

Serum assays

Serum was separated from clotted blood collected by cardiac puncture and then stored at < -70 °C. Human insulin was assayed in serum aliquots (25 μ l) using the ultrasensitive human insulin ELISA (Mercodia, Uppsala, Sweden).

Statistical analysis

Analyses were performed using Student's *t*-test or one-way ANOVA and results expressed as mean values $\pm$ s.d. (*in vitro* studies) or $\pm$ s.e.m. (*in vivo* studies). Results were considered statistically significant at $P < 0.05$.

Results

CBD motif containing constructs and expression in HepG2 cells

In order for glucose to regulate secretion of exogenously expressed (pro)insulin from HepG2 cells, we exploited features of the CBD. This heptapeptide binds GRP78, an ER protein with ATPase activity (Fourie *et al.* 1994). When glucose is abundant, so is ATP leading to formation of GRP78 monomers, which do not bind CBD. But when ATP is low, GRP78 becomes a dimer and it binds to CBD-proinsulin to keep this fusion protein in the ER (Dorner *et al.* 1990, Toledo *et al.* 1993). Thus, we inserted nucleotides encoding CBD between the signal peptide and proinsulin of the human preproinsulin cDNA (Fig. 1A, AdSsCbdProins) to yield chimeric CBD-proinsulin. CBD-containing cDNA was placed into adenovirus vectors to yield AdSsCbdProins. The existence of CBD in the resulting mRNA was confirmed by RT-PCR assay using CBD sequence-specific primers (data not shown).

Next, adenovirus (10^8 – 10^{10} OPU/ml) derived from the above construct was used to infect HepG2 cells for a fixed period of 24 h. Proinsulin mRNA expression determined by RT-PCR (Fig. 1B) showed that proinsulin was expressed successfully using 10^8 OPU/ml of virus. Although mRNA abundance appeared higher in cells infected with more (10^9 or 10^{10}) virus, cells appeared less healthy in the latter. Additionally, differing infection times (Fig. 1C) revealed proinsulin mRNA expression at 24 h. Longer infection times of 48–72 h appeared to yield slightly higher levels of the mRNA. In subsequent studies, cells were infected with 10^9 OPU/ml of virus for 24 h.

Figure 1

Expression of CBD-proinsulin in HepG2 cells. (A) Schematic illustration of the human preproinsulin cDNA-containing CBD inserted between signal sequence (SS) and proinsulin sequence. (B) mRNA expression of CBD-proinsulin in HepG2 cells infected with 10⁸, 10⁹, and 10¹⁰ OPU/ml AdSsCbdProins adenovirus for 24 h (RT-PCR). Control: 10¹⁰ OPU/ml AdEGFP adenovirus. (C) mRNA expression of CBD-proinsulin in HepG2 cells infected with 10⁹ OPU/ml AdSsCbdProins adenovirus for 24, 48, and 72 h (RT-PCR). Control: 10⁹ OPU/ml AdEGFP for 72 h. Both graphs (B and C) show densitometry analysis of data collected using semiquantitative RT-PCR expressed as a percentage of maximum signal (mean $\pm$ s.d., $n=3$).

* $P<0.05$ vs control group.

Glucose regulates CBD-proinsulin release from HepG2 cells

Our construct (Fig. 1A) was not designed for glucose to regulate its expression. Thus, as expected, abundance of CBD-proinsulin mRNA in HepG2 cells infected with the AdSsCbdProins was not dependent on extracellular glucose (Fig. 2A). To examine proinsulin release, HepG2 cells infected with AdSsCbdProins were exposed

to glucose from 0 to 20 mmol/l for 1 day. CBD-proinsulin in spent media was assessed using western blot analysis. Results (Fig. 2B) showed that CBD-proinsulin levels rose hand in hand with glucose concentrations such that in 0 mmol/l glucose CBD-proinsulin was low. Abundance of extracellular CBD-proinsulin was significantly higher in cells exposed to 2.5 vs 0 mmol/l glucose. CBD-proinsulin secretion into media continued to rise in the presence of 5–10 mmol/l followed by a plateau beyond 10–20 mmol/l glucose. These studies show the following: i) CBD-proinsulin levels in the media rise with the glucose concentration from 0 to 10 mmol/l, ii) thereafter, its release plateaus in the presence of glucose >10 mmol/l, and iii) abundance of CBD-proinsulin in the media increases in response to extracellular glucose.

The preceding studies do not address CBD-proinsulin release from cells in response to fluctuating glucose concentrations. Thus, infected cells were exposed to 5 mmol/l glucose followed by replacement of media containing more or no glucose (7.5 or 0 mmol/l) respectively every hour. Results (Fig. 2C) showed that levels of the chimeric hormone rose following exposure to 7.5 mmol/l glucose. By contrast, CBD-proinsulin release into the media declined but not until 2–3 h in the absence of glucose. These findings show that CBD-proinsulin release from cells was glucose regulated. Additionally, cellular release of CBD-proinsulin triggered by 7.5 mmol/l glucose appears more rapid than the decline in hormone release induced by 0 mmol/l glucose.

Kinetics of CBD-proinsulin release from HepG2 cells

The kinetics of CBD-proinsulin release was examined using infected HepG2 cells kept in 0 mmol/l glucose for 16 h before exposure to hyperglycemia (15 mmol/l). Spent media were collected at 10-min intervals $\times$ 6 followed by every hour extending to 5 h and samples were analyzed using western blot analysis (Fig. 2D). Abundance of CBD-proinsulin in media was minimal at the first 10-min point. But thereafter, abundance of the hormone rose steadily such that after 60 min of exposure to 15 mmol/l glucose, extracellular CBD-proinsulin was nearly sevenfold higher vs at the start. The CBD-proinsulin level peaked at 2–3 h of exposure to 15 mmol/l glucose and was sustained to 5 h. Glucose-regulated release of CBD-proinsulin from infected cells appears quickly within 10–20 min and reaches a plateau beyond 60 min of exposure to 15 mmol/l glucose.

Figure 2

Glucose regulates release but not synthesis of CBD-proinsulin. (A) Real-time PCR results of CBD-proinsulin mRNA expression relative to 18S in HepG2 cells infected with AdSsCbdProins under varying concentrations of glucose (top panel) or varying time of exposure to 15 mmol/l glucose (bottom panel) (mean $\pm$ s.d., $n \geq 3$). (B) CBD-proinsulin secretion from HepG2 cells infected with AdSsCbdProins adenovirus detected using western blot analysis (top panel). The graph shows optical density of CBD-proinsulin signal from the western blot reflecting hormone secretion from cells treated with various concentrations of glucose (mean $\pm$ s.d., $n = 5$).

CBD-proinsulin associated with GRP78 is retained in the ER

The presence of the CBD motif within proinsulin should enable it to associate with GRP78. To test this hypothesis, antibodies against proinsulin and GRP78 were used in immunoprecipitation studies. Spent media and lysates from AdSsCbdProins-infected cells exposed to 0 or 7.5 mmol/l glucose for 24 h were studied using western blot analysis. Results (Fig. 3A) in spent media showed that CBD-proinsulin levels were higher in cells exposed to 7.5 mmol/l glucose. In lysate, the converse was seen with CBD-proinsulin (Fig. 3B, upper panel) being minimally more abundant in cells treated with 0 vs 7.5 mmol/l glucose. Additionally, GRP78 was also more abundant in 0 mmol/l glucose (Fig. 3B, middle panel).

Next, immunoprecipitation studies were performed by adding anti-insulin antibody to cell lysates and then

* $P < 0.05$, *** $P < 0.001$ vs 0 mM glucose group; NS, non-significant.

(C) CBD-proinsulin secretion from infected HepG2 cells in response to fluctuating glucose level detected using western blot analysis (top panel). Graph showing relative amounts of CBD-proinsulin secreted from cells (mean $\pm$ s.d., $n = 6$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs baseline level. (D) Kinetics of CBD-proinsulin release from infected HepG2 cells treated with 15 mmol/l glucose assessed using western blot analysis (top panel). Graph shows relative amounts of CBD-proinsulin secreted from cells (mean $\pm$ s.d., $n = 4$). * $P < 0.05$, ** $P < 0.01$ vs 10-min point.

analyzing the pull down material. Results (Fig. 3C, upper panel) showed that abundance of CBD-proinsulin was higher inside cells exposed to 0 vs 7.5 mmol/l glucose. The same pull down material also contained GRP78 (Fig. 3C, lower panel). We speculate that the lower level of CBD-proinsulin in 7.5 vs 0 mmol/l glucose in Fig. 3C, when compared to Fig. 3B, is likely due to the added immunoprecipitation step in pulling down less CBD-proinsulin before western blot analysis. The converse experiment using the anti-GRP78 antibody added to lysates pulled down material that contained GRP78 (Fig. 3D, lower panel) as well as proinsulin (Fig. 3D, upper panel). The findings using both antibodies are consistent with the idea that CBD-proinsulin and GRP78 exist as a complex inside the infected cells.

To further examine whether CBD-proinsulin and GRP78 may appear together, AdSsCbdProins-infected

Figure 3

CBD-proinsulin associated with GRP78 is retained in the ER. (A) Western blot analysis of CBD-proinsulin secreted from HepG2 cells infected with AdSsCbdProins adenovirus in response to 0 or 7.5 mmol/l glucose. (B) Lysate content of CBD-proinsulin and GRP78 in infected HepG2 cells treated with 0 or 7.5 mmol/l glucose. (C and D) Immunoprecipitation (IP) of lysate from infected HepG2 cells using anti-insulin (C) or -GRP78 (D) antibodies,

followed by western blot analysis of pull down material for proinsulin and GRP78. (E and F) Infected HepG2 cells were exposed to antibodies against proinsulin, GRP78, or GRP94 and then examined using confocal microscopy. Images were taken separately for proinsulin and GRP78 (E) or proinsulin and GRP94 (F) and finally merged together.

HepG2 cells were exposed to media without glucose for 3 h. These cells were exposed to an antibody against proinsulin or GRP78 followed by confocal microscopy. Results (Fig. 3E) in green and red reflected staining by proinsulin or GRP78 respectively. When the two separate pictures were layered on top of each other, the merged image turned yellow suggesting co-localization of the two proteins. To gather further evidence that CBD-proinsulin was located in the ER, we stained infected cells with an antibody against GRP94, another protein that resides within the ER. An image (Fig. 3F) of the overlay stained for proinsulin (green) or GRP94 (red) showed predominant yellow, again suggesting co-localization of these two proteins. These data support the idea that both CBD-proinsulin and GRP78 appear together and likely in the ER of infected cells.

Diabetic mice infected with a construct designed to express mature insulin

While CBD-proinsulin was useful for the *in vitro* studies, in a mouse model of diabetes mellitus, mature insulin should be more efficient in lowering glucose. In order for CBD-proinsulin to be converted to mature insulin by liver cells, the chimeric hormone must be cleaved between CBD/B-chain, B-chain/C-peptide, and C-peptide/A-chain.

Previous studies showed that furin cut sites may be useful for this conversion (Nishigori *et al.* 1996). Thus, furin cut sites were inserted at strategic locations to yield AdSsCbdInsF3. The fusion hormone arising from this construct is recognized by the furin protease family found in liver and their activity on this substrate should yield mature insulin (Denault & Leduc 1996).

Balb/CJ mice made diabetic using STZ were then infected with the AdSsCbdInsF3 virus. Diabetic mice injected with either AdEGFP or diluent served as controls. Insulin expression in mouse liver was assessed using RT-PCR and western blot analysis against insulin antibody. As expected, results showed the presence of both proinsulin mRNA (Fig. 4A) and protein (Fig. 4B) in liver from diabetic mice injected with AdSsCbdInsF3 but not from control mice. Additionally, immunohistology studies showed that liver from AdSsCbdInsF3-injected mouse stained positive for insulin expression (Fig. 4C).

Next, we measured the glucose levels in these mice. In diabetic mice, 2 days following infection with AdSsCbdInsF3, the morning glucose level was lower compared with that in diabetic controls. Furthermore, the glucose levels in diabetic mice carrying AdScbdInsF3 (Fig. 4E, black triangle) continued to drop toward euglycemia from days 3 to 7 following viral infection vs diabetic control mice (black dots) in which glucose levels

Figure 4

AdSsCbdInsF3 adenovirus lowers glucose levels in diabetic mice. (A) RT-PCR results of proinsulin mRNA expression relative to GAPDH in liver tissue of Balb/CJ mice infected with AdSsCbdInsF3. (B) Western blot analysis of proinsulin expression relative to β -tubulin in liver tissue of Balb/CJ mice infected with AdSsCbdInsF3. (C) Frozen mice liver section was stained for insulin and then examined using confocal microscopy. (D) Serum human insulin levels of Balb/CJ mice infected with AdSsCbdInsF3 in comparison to

control mice (mean $\pm$ s.e.m., $n \geq 6$). ** $P < 0.01$ vs control group. (E) Morning blood glucose was measured after STZ injection and on indicated day after AdSsCbdInsF3 adenovirus injection. (F) IPGTT was performed after virus injection. White circles, normal control mice; black triangles, mice injected with AdSsCbdInsF3 adenovirus; black dots, mice injected with AdEGFP adenovirus or diluent without virus (mean $\pm$ s.e.m., $n \geq 6$).

remained high. In AdSsCbdInsF3-infected mice beyond 7 days to the end of the study, glucose levels remained much lower than those in diabetic control mice. Additionally, human insulin was assayed in mice from each group, which showed that diabetic mice infected with AdSsCbdInsF3 had significantly higher levels of human insulin in comparison to the control mice (Fig. 4D). The presence of human insulin correlated with the lower blood glucose levels in the infected animals.

The AdSsCbdInsF3 construct was created to yield mature insulin, and it should be more efficient in lowering glucose compared with the prohormone. Thus, glucose levels in diabetic mice injected with AdSsCbdProins or AdSsCbdInsF3 were compared. In mice equipped to express mature insulin, the glucose levels were lower than those producing the prohormone. Also, human insulin levels measured using ELISA assay in mice injected with AdSsCbdInsF3 were threefold higher compared with those injected with AdSsCbdProins (data not shown). Proinsulin is known to be roughly 5–10% as potent as mature insulin (Shaw & Chance 1968, Lazarus *et al.* 1970).

Next, an intraperitoneal glucose tolerance test (IPGTT) was performed in these mice following a ≥ 6 -h

fast. Blood glucose levels were measured after i.p. injection of glucose. Results (Fig. 4F) showed that mice carrying AdSsCbdInsF3 did not have hyperglycemia at the start and glucose remained lower vs diabetic control mice throughout the study. Glucose levels in the diabetic mice carrying AdSsCbdInsF3 mimicked those in non-diabetic mice and not in diabetic control mice subjected to the same IPGTT. These observations suggest that in this STZ-induced diabetic Balb/CJ mouse model, infection with AdSsCbdInsF3 lowers glucose vs that of diabetic control animals in the morning or response to IPGTT.

Discussion

Insulin therapy is a mainstay in the treatment of patients with diabetes mellitus (Rodbard *et al.* 2009, American Diabetes Association 2011). But the consequences of its actions on glucose are not perfect because it is difficult for patients to predict the exact dose of hormone to inject so that it matches the body's needs. If a mismatch occurs, it ends in hyper- or, worst, hypoglycemia. To address this issue, we want to study new ways that enable glucose to regulate insulin release from non-islet cells. This interest

led us to create a model that attempts to mimic the initial aspect of pancreatic insulin release. Our data show that the release of insulin expressed in liver cells can be regulated by glucose.

Our data show that HepG2 cells infected with the AdSsCbdProins expressed proinsulin mRNA (Fig. 1B and C) and the hormone. The amount of proinsulin released from infected cells varied directly with the ambient concentration of glucose in the media (Fig. 2B). The glucose-regulated release of the hormone from infected cells was rapid and sustained (Fig. 2D). Removal of glucose from the cells decreased release of the hormone (Fig. 2C). Immunoprecipitation (Fig. 3C and D) and immunocytochemistry (Fig. 3E and F) data suggested that GRP78 and proinsulin appeared together in the ER of the infected cell. In STZ-induced diabetic mice, when infected with adenovirus (AdSsCbdInsF3) that expressed mature insulin, glucose levels declined (Fig. 4E and F). Together, our observations *in vitro* and *in vivo* show that the constructs can direct the synthesis of insulin in liver cells but more importantly glucose regulates its activity in this animal model.

The potential importance of our data may become clearer when viewed in the light of studies by others addressing a similar issue. Previous attempts to mimic pancreatic function in regulating insulin have used non-islet cells, including hepatocytes, to express and secrete insulin (Lu *et al.* 1998, Chen *et al.* 2005, Wilson *et al.* 2005, Hsu *et al.* 2008). The general approach involved use of a glucose-responsive promoter or motif to control insulin gene transcription (Thule *et al.* 2000, Chen *et al.* 2001, Alam & Sollinger 2002, Hsu *et al.* 2008). An inherent feature of glucose-regulated insulin expression, as a control point, is that it gives rise to a lag phase during which the gene is transcribed and then translated. These time-dependent processes result in a delay that precedes the onset of action from the newly synthesized insulin. This delay may lead to an initial hyperglycemic response following exposure to glucose and then possibly hypoglycemia when insulin synthesis surpasses the needs (Lu *et al.* 1998, Olson *et al.* 2003).

Physiological release of insulin is biphasic and begins with an initial surge followed by a gentle sustained flow of the hormone (Porte & Pupo 1969). While the transcriptional approach in non-islet cells might reproduce the second phase of insulin release, it does not replicate the first phase. For example, in studies using an adenovirus carrying a glucose-regulated insulin gene, glucose levels remained high in most animals immediately after the meal (Thule & Liu 2000, Olson *et al.* 2003). Additionally,

the combined use of adeno-associated virus and adenovirus to deliver a glucose-responsive, liver-specific promoter is reported to lower glucose below 200 mg/dl in diabetic mice (Kozlowski *et al.* 2007). Despite the sustained hormone release arising from glucose-regulated insulin gene transcription, when tested in animal models of diabetes mellitus, glucose levels did not mimic physiological patterns (Thule & Liu 2000, Riu *et al.* 2002, Olson *et al.* 2003, Kozlowski *et al.* 2007).

Why transcriptional models do not mimic pancreatic control of the hormone may, in part, be due to the inability of this approach to control insulin secretion. Studies targeting this specific step have used non-pancreatic endocrine cells in which glucose regulates release of non-insulin hormones. These cells were engineered to synthesize large quantities of insulin. For example, studies of K cells in mice expressing human insulin driven by the rat GIP promoter were promising (Cheung *et al.* 2000). But unfortunately, K cells are present in crypts of the gut and thus they are very difficult to use for viral transduction due to accessibility. Additionally, intestinal epithelial cells undergo very rapid turnover, so repeated delivery of the transgenic gene into these progenitor cells is needed. Together, these features of K cells added to their inherent function of releasing non-insulin hormones affected by glucose (i.e. GIP) represent a barrier for their use in clinical applications. A central issue in using either a glucose-responsive promoter or non-pancreatic cell approaches to mimic pancreatic insulin release is the inability to replicate a bolus secretion of the hormone. The lack of this insulin surge following glucose stimulation is important in physiological control of the hormone.

Studies to address this issue have been attempted (Rivera *et al.* 2000, Tian *et al.* 2008) by controlling insulin secretion at the level of the ER using drug-induced protein disaggregation. These studies demonstrated that protein secretion could be controlled at the posttranslational step and the ER as a useful storage site. However, the use of pharmacological agents and not glucose to regulate insulin release is fraught with difficulties such as adjustment of drug dosage to address hyper- or hypoglycemia. Thus, these models still do not replicate physiological insulin release.

A potential issue of storing chimeric proinsulin in the ER is that it might induce stress leading to apoptosis. But we found no evidence of this problem on cell viability or differences in biomarkers of stress and apoptosis, GRP78, GRP94, and caspase-3 and -7 in hormone-expressing cells vs control (data not shown). Furthermore, studies summarized here and those of others showed that use of ER as

a storage site for exogenously expressed protein did not lead to cell toxicity or activation of markers denoting ER stress (Rivera *et al.* 2000, Tian *et al.* 2008).

In summary, the work of others suggests that replicating pancreatic insulin release in non-islet cells remains an elusive goal. Toward this objective, we have devised a way that enables glucose to regulate release of exogenously expressed insulin from the ER of liver cells. Both glucose regulation and ER storage of proinsulin are possible by fusing the CBD motif to the hormone (Fourie *et al.* 1994). *In vitro*, glucose controls the release of chimeric proinsulin from HepG2 cells. *In vivo*, the adenovirus that expresses mature insulin lowers glucose vs diabetic controls. While our data have many limitations, it is a step in replicating the initial phase of pancreatic insulin release.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

All authors contributed to acquisition, analysis, and interpretation of data; revised the manuscript; and approved the final version. I E W had a significant role in developing the idea for the project.

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