SGLT2 knockout prevents hyperglycemia and is associated with reduced pancreatic β-cell death in genetically obese mice

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ABSTRACT
Inhibition of the sodium-glucose co-transporter type 2 (SGLT2) has received growing acceptance as a novel, safe and effective means to improve glycemic control in patients with type 2 diabetes. Inhibition of SGLT2 lowers the renal glucose threshold and reduces plasma glucose by promoting glucose excretion in urine. Both animal studies and clinical trials in man suggest that SGLT2 inhibition has the potential to improve pancreatic β-cell function by reducing glucose toxicity. However, there is limited data exploring how reducing gluotoxicity via SGLT2 inhibition affects rates of β-cell proliferation and death throughout life in the context of insulin resistance and type 2 diabetes. SGLT2−/− mice were backcrossed to the db/db strain to produce littermate control db/db-SGLT2+/+ and experimental db/db-SGLT2−/− mice. Mice were euthanized at 5, 12 and 20 weeks of age to collect plasma for glucose, insulin, lipid and cytokine measures, and pancreata for histological analysis including determination of β-cell mass and rates of proliferation and death. SGLT2 deletion in db/db mice reduced plasma glucose as early as 5 weeks of age and continued throughout life without changes in plasma lipids or cytokines. Reduced plasma glucose levels occurred in parallel with an increase in the relative β-cell volume and reduced frequency of β-cell death, and no apparent change in rates of β-cell proliferation. These data add to a growing body of evidence demonstrating that improved glycemic control achieved through SGLT2 inhibition can preserve β-cell function and endogenous insulin secretion by reducing glucose toxicity and rates of β-cell death.

Introduction
Inhibition of the sodium-glucose co-transporter type 2 (SGLT2) has received growing acceptance as a novel, safe and effective means to improve glycemic control in patients with type 2 diabetes. SGLT2 is responsible for the majority of glucose reabsorption in the proximal tubule of the kidney such that inhibition of SGLT2 lowers the renal glucose threshold and promotes glucose excretion in the urine, lowering plasma glucose in an insulin-independent fashion. Currently, three different SGLT2 inhibitors are approved for use in humans in the U.S. and Europe,1–3 and a great number of short- and long-term randomized, placebo-controlled clinical trials evaluating SGLT2 inhibitors as monotherapy or combination therapy have demonstrated significant reductions in hemoglobin A1C and body weight in patients with type 2 diabetes.4 Additionally, SGLT2 inhibition reduced the risk of death and slowed the progression of kidney disease in type-2 diabetes mellitus (T2DM) patients at high risk for adverse cardiovascular and renal events,5,6 demonstrating unforeseen benefits for this new class of drug.

An additional unforeseen benefit of SGLT2 inhibition that has received less attention is the potential preservation of pancreatic β-cell mass and sustained ability to produce insulin in patients with T2DM. Type 2 diabetes is often preceded by insulin resistance and hyperinsulinemia, allowing patients to maintain euglycemia at the expense of increased insulin secretion. This adaptive period
cannot be sustained indefinitely and chronic glucose toxicity is associated with loss of β-cell mass due to cell death in both man and rodents. The loss of endogenous insulin secretion marks a critical event in the pathogenesis of T2DM and comes with considerable impact on the clinical management and life of patients as it requires the initiation of insulin therapy in order to control hyperglycemia. Not surprisingly, improved glycemic control either through diet, exercise or pharmacological intervention is associated with sustained pancreatic β-cell function.

Unfortunately, life-style interventions and the existing pharmacopeia of glucose lowering agents are ineffective for the maintenance of long-term glycemic control in the majority of patients. New agents with improved durability are therefore necessary to improve glycemic control and β-cell function in patients with T2DM, and early data from clinical studies of SGLT2 inhibitors suggests this new class of drug may fill this needed gap.

We previously reported that SGLT2 deletion in genetically obese db/db mice (db/db-SGLT2−/−) resulted in profound glucosuria and lowered fasting plasma glucose levels by 50%, similar to absolute levels observed in lean, non-diabetic controls. SGLT2 deletion in db/db mice was also associated with a significant improvement in whole-body insulin sensitivity, as assessed by hyperinsulinemic euglycemic clamp. The improved insulin sensitivity and reduced fasting plasma glucose in the db/db-SGLT2−/− mice was associated with a 2-fold increase in glucose-stimulated insulin secretion in vivo, as assessed by hyperglycemic clamp, demonstrating improved pancreatic β-cell function compared with db/db-SGLT2+/+ controls. The improved glucose-stimulated insulin secretion observed in vivo was not due to changes in individual β-cell function, but rather due to increased β-cell mass in the db/db-SGLT2−/− mice. These data demonstrated for the first time that long-term lowering of plasma glucose levels by promoting glucosuria in obese, hyperglycemic animals resulted in improved pancreatic β-cell function and raised several important, unanswered questions. Because we studied mice at a single time point of approximately 20 weeks of age, we could not determine at what point in life the changes in β-cell mass occurred, or whether they resulted from increased compensatory proliferation or reduced cell death, or some combination of the two. Here we describe a time course study designed to understand the progression of the diabetic phenotype in control db/db-SGLT2+/+ and experimental db/db-SGLT2−/− mice and corresponding changes in glucose homeostasis, β-cell mass and rates of cell proliferation and death.

Results

SGLT2 deletion improves hyperglycemia throughout life in db/db mice

We previously reported that SGLT2 deletion in C57BL6/J mice reduced body weight in chow-fed animals, but had no effect on body weight after four weeks of high-fat diet feeding or in genetically obese 20-week old db/db mice. Consistent with these observations, there was no effect of genotype on body weight at 5, 12 or 20 weeks when comparing db/db-SGLT2+/+ and db/db-SGLT2−/− mice (Figure 1a). As expected, age had a significant effect on body weight (P < 0.0001) independent of genotype and body weights approximately doubled from 5 to 12 weeks of age in both groups, consistent with the rapid and progressive development of obesity in the db/db strain. Db/db-SGLT2+/+ mice were hyperglycemic as early as 5 weeks of age and plasma glucose levels were greater than 250 mg/dl at each time point (Figure 1b). In contrast, plasma glucose levels remained below 200 mg/dl in db/db-SGLT2−/− mice at all ages and were 45% less at 5 weeks and 60% less at both 12 and 20 weeks compared with db/db-SGLT2+/+ mice (Figure 1b; P < 0.01 genotype; P = 0.53 age). Plasma insulin levels increased approximately 1.5-fold from 5 to 12 weeks of age in both genotypes and there was a modest, but insignificant effect of age on plasma insulin levels (Figure 1c; P = 0.09). There was no significant effect of genotype on plasma insulin levels, despite an approximate 50% reduction in plasma insulin levels in 20 week-old db/db-SGLT2−/− compared with db/db-SGLT2+/+ mice (Figure 1c; P = 0.4). This is in contrast to our previous report where plasma insulin levels were significantly reduced by 50% in 20 week-old db/db-SGLT2−/− mice and likely reflects greater variability in plasma insulin levels from ad libitum-fed (current study) versus overnight fasted.
(previous study) mice. Plasma triglyceride and fatty acid levels were highly variable within and between age groups and genotypes and there was no effect of age or genotype on either measure (Figure 1c and d; plasma triglyceride: P = 0.44 age, P = 0.95 genotype; plasma fatty acids: P = 0.79 age, P = 0.29 genotype). There was similarly no effect of age or genotype on plasma cytokine levels from a panel of seven pro- and anti-inflammatory cytokines, including, IL-10, IL-12p70, IL-6, and CXCL1 (Table 1). Plasma IFNγ, IL-1β and TNFα levels were below the lowest level of detection for both groups at all ages (Table 1).

**Improved hyperglycemia is associated with increased relative β-cell volume in db/db-SGLT2−/− mice**

To evaluate the effects a reduced glucose load may have on the pancreatic β-cell in db/db-SGLT2−/− mice, pancreata were collected from 5, 12 and 20 weeks of age.
20 week old mice for histological studies. Consistent with the reported rapid development of insulin resistance in the \textit{db/db} strain and resulting compensatory \(\beta\)-cell hyperplasia,\(^{15}\) there was a significant effect of age on relative \(\beta\)-cell volume (Figure 2a; \(P < 0.05\)). There was a 1.7- and 2-fold increase in relative \(\beta\)-cell volume from 5 to 12 weeks of age for \(db/db\)-SGLT2\(^{+/+}\) and \(db/db\)-SGLT2\(^{−/−}\) mice, respectively, followed by little to no change from 12 to 20 weeks of age (Figure 2a). Relative \(\beta\)-cell volume was on average 20%, 40% and 55% greater in \(db/db\)-SGLT2\(^{−/−}\) compared with \(db/db\)-SGLT2\(^{+/+}\) mice at 5, 12 and 20 weeks and the effect of genotype on relative \(\beta\)-cell volume trended strongly towards significance (Figure 2a; \(P = 0.06\)). There were no differences in islet number between \(db/db\)-SGLT2\(^{+/+}\) and \(db/db\)-SGLT2\(^{−/−}\) mice and similarly no significant effect of age (Figure 2b).

\textit{Pancreatic \(\beta\)-cell death is reduced in \(db/db\)-SGLT2\(^{−/−}\) mice throughout life}

Pancreatic \(\beta\)-cell mass in rodents represents a balance of cell proliferation and death, processes that may be influenced by the glucose load to which the pancreas is exposed. To better understand whether increased proliferation or reduced cell death, or some combination of the two, accounted for the observed differences in relative \(\beta\)-cell volume in \(db/db\)-SGLT2\(^{−/−}\) mice, we determined rates of

![Figure 2](image-url)

\textit{Figure 2}. Improved hyperglycemia is associated with increased relative pancreatic \(\beta\)-cell volume in \(db/db\)-SGLT2\(^{−/−}\) mice. Pancreata from littermate \(db/db\)-SGLT2\(^{+/+}\) and \(db/db\)-SGLT2\(^{−/−}\) mice were sectioned, stained and scored to determine \(\beta\)-cell volume (a) as the ratio of islet area/pancreas area and islet number (b) corrected to pancreas area. Measurements were made with Image J software. Representative images for the data shown in A-B are shown (c). Data are the mean ± standard error of the mean for group sizes of 4–5 per genotype at 5 and 12 weeks, and 7–8 at 20 weeks. Data were analyzed by 2-way ANOVA to determine the effect of age and genotype and \(P < 0.05\) was considered significant.
proliferation and death by KI67/insulin and TUNEL/insulin co-staining. The overall frequency of KI67 positive β-cells was low in both genotypes at all ages and because of this, highly variable. We did not detect an effect of age or genotype on the frequency of KI67 positive β-cells (Figure 3a; age P = 0.85, genotype P = 0.56), suggesting similar rates of proliferation amongst groups. In contrast, there was a significant effect of genotype on rates of β-cell death (Figure 3b; P < 0.05) and the frequency of TUNEL positive β-cells was 45%, 50% and 25% less at 5, 12 and 20 weeks of age, respectively, in db/db-SGLT2−/− compared with db/db-SGLT2+/+ mice (Figure 3). There was also a modest effect of age on the frequency of β-cell death that trended towards significance and suggested declining rates with age (Figure 3b; P = 0.09).

Discussion

Our data add to a growing body of in vivo evidence demonstrating that improved glycemic control in the context of insulin resistance can preserve β-cell function and endogenous insulin secretion. This concept was previously demonstrated in partially pancreatectomized rats treated with the non-selective SGLT1/2 inhibitor phlorizin,16 as well as in a more recent study where Zucker Diabetic Fatty (ZDF) rats were treated with a selective SGLT2 inhibitor.17 In both studies, SGLT2 inhibition improved hyperglycemia and resulted in improved β-cell function assessed by hyperglycemic clamp.16,17 The improved β-cell function in the latter study was associated with significant positive changes in islet morphology, although β-cell mass was largely unaffected,17 in contrast to our previous study13 and data reported here. Notably, ZDF rats were treated with SGLT2 inhibitor for only 33 days,17 whereas the effects of SGLT2 deficiency in our study were evaluated after 5, 12 and 20 weeks, with the greatest effects on β-cell mass observed at 12 and 20 weeks (Figure 2a).13 Thus, the apparent differences with regards to the effects on β-cell mass may be due to treatment duration or potentially the partial degree of inhibition achieved during drug studies compared with effectively complete inhibition in the genetic knockout studies. This consideration also holds true when comparing our results to human studies where partial pharmacological inhibition of SGLT2 would be expected (discussed further below).

A growing number of short- and long-term studies evaluating SGLT2 inhibitors as monotherapy or add-on therapy in patients with T2DM have demonstrated improved β-cell function following treatment.18–22 These assessments are based primarily on indirect measures, specifically homeostasis model assessment 2 index of β-cell function,18–21 as well as functional measures such as the area under the curve ratios of C-peptide and glucose during a frequently sampled mixed meal tolerance test.20,22 While these human studies
There are no changes in β-cell function or β-cell mass, previous data from animal studies, including our own, suggests SGLT2 inhibition improves β-cell function in the short-term and has the potential to maintain β-cell morphology and mass in the long-term. Thus, although complete loss of SGLT2 function through genetic deletion would be expected to produce greater effects than partial inhibition through pharmacotherapy, both interventions appear to yield similar effects on β-cell function. Long-term follow up studies in patients prescribed SGLT2 inhibitors will be of great interest to determine if our observation of preserved pancreatic β-cell mass in rodents due to improved glycemic control translates to man. Initial results from clinical trials of up to 2 years comparing SGLT2 inhibitors to metformin, sulfonylureas or DPP4 inhibition demonstrate similar effects in terms of glucose lowering, and in some cases improved durability of the glucose lowering effect of SGLT2 compared with other classes of drugs. An additional consideration in the human studies that cannot be overlooked is the body weight reducing effect of SGLT2 therapy, which may also contribute to changes in β-cell function. We did not observe changes in body weight following SGLT2 deletion in these studies most likely due to the fact that the db/db strain is hyperphagic, which may counteract caloric loss through glucosuria. We previously observed significant reductions in body weight comparing WT to SGLT2 knockout mice on a C57BL6 background.

Pancreatic β-cell compensation in both rodents and man occurs in the setting of insulin resistance and can include increased insulin biosynthesis, expansion of β-cell mass, as well as enhanced coupling of nutrient sensing mechanisms to insulin secretion. The eventual decompensation and loss of β-cell function and mass are thought to result from glucose toxicity, as well as lipid toxicity, and determining the relative contribution of either proposed mechanism in vivo has proven difficult. However, studies in both ZDF rats and db/db mice where bezafibrate or phlorizin treatment were used to selectively reduce plasma lipids or glucose, respectively, demonstrated that changes in plasma glucose alone are sufficient to explain improvements in β-cell gene expression and function in these models. These studies are supported by in vitro studies with cultured human islets where glucose toxicity has been shown to induce cell death via pathways that are also upregulated in isolated islets from humans with type 2 diabetes and reduced β-cell mass. While incubation of isolated islets with palmitate in culture has been shown to reduce islet insulin content and mRNA levels, the effect was subsequently shown to both require and be exacerbated by high glucose. Together, available data in the literature, evidence from our previous study, where SGLT2 deletion did not affect fasting plasma fatty acids in db/db mice, and new time course data described here suggest that changes in plasma glucose resulting from SGLT2 deletion without subsequent changes in plasma lipids preserves β-cell mass by reducing glucose and not lipid toxicity. A final possibility is that improved insulin sensitivity in db/db-SGLT2−/− mice contributed to preserved islet mass by reducing the demand for insulin placed on the β-cell. We previously reported a mild, but significant improvement in whole-body insulin sensitivity by euglycemic clamp in the db/db-SGLT2−/− mice, and more recently SGLT2 inhibition in humans was also shown to produce insulin sensitizing effects. The improved insulin sensitivity in our mice may have contributed to the reduced plasma insulin levels observed at 20 weeks of age (Figure 1c), despite increased β-cell mass (Figure 2a) and persistent, albeit improved, insulin resistance. Finally, improved insulin sensitivity may also have contributed to the nonsignificant reduction in rates of β-cell proliferation at 5 weeks of age, although insulin sensitivity was not specifically assessed at this age. Future work will be necessary to discern the specific effects of insulin-independent glucose lowering through SGLT2 deletion as opposed to changes resulting from improved insulin sensitivity.

In summary, we find that SGLT2 deletion in db/db mice resulted in reduced plasma glucose as early as 5 weeks of age and continued throughout life without apparent changes in plasma lipids or cytokines. The reduced plasma glucose levels occurred in parallel with an increase in the relative β-cell volume and reduced frequency of β-cell death, suggesting that SGLT2 deletion prevented...
glucose toxicity and induction of pro-apoptotic genes that has been described in experimental models and human subjects. Relative rates of β-cell proliferation appeared relatively constant over time in the models studied, despite pronounced changes in relative β-cell volume over the same time period suggesting that relative rates of cell death, which varied by age and genotype, made a greater contribution to the differences in islet mass observed here and previously. Future studies exploring the molecular and biochemical changes within the β-cell that support sustained proliferation and protection from cell death in db/db-SGLT2−/− mice will be of interest, as will the long-term effects of treating patients with type 2 diabetes with SGLT2 inhibitors, specifically with regards to β-cell function.

Materials and methods

Animals

Studies were performed at Yale University where all housing and experimental protocols were overseen and approved by the Yale Institutional Animal Care and Use Committee. SGLT2 (Slc5a2) knockout mice (SGLT2−/−) were generated as previously described. Briefly, a Lambda KOS phage library approach was used to generate a targeting vector where exons one through five of the Slc5a2 gene were replaced with a selection cassette. The linearized targeting vector was electroporated into 129/SvEvBrd embryonic stem cells and successfully targeted stem cell clones were injected into C57BL6/J blastocysts to generate chimeric offspring that were then backcrossed to C5BL6/J mice for ten generations. SGLT2−/− mice were then backcrossed to db/db mice on a C57BLKS (BKS) background purchased from Jackson Labs to generate double heterozygous mutants (db/WT-SGLT2±) that were subsequently crossed to produce littermate control db/db-SGLT2+/+ and experimental db/db-SGLT2−/− male mice for studies, as previously described. Db/db mice on the BKS background are characterized by rapid progression of obesity and insulin resistance, followed initially by compensatory β-cell hyperplasia and eventually β-cell failure, loss of islet mass and insulin levels, and frank hyperglycemia. Mice were housed at 22 ± 2°C on a 12-h light/dark cycle with free access to water and rodent chow diet (Harlan Teklad, TD2018S; 18% fat, 58% carbohydrate, 24% protein by calories). Mice were brought to lab in clean cages the evening prior to tissue collection and housed with ad libitum access to food and water. All mice were euthanized for tissue and plasma collection between noon and 2 pm.

Biochemical analyses

Blood was collected by cardiac puncture and plasma separated from red cells by centrifugation. Plasma glucose levels were determined by glucose oxidase method using a Beckman Glucose Analyzer II. Plasma insulin levels were measured by radioimmunoassay (Linco Research). Plasma fatty acids and triglycerides were measured using a spectrophotometric technique and commercially available kits (Wako NEFA Kit; Genzyme Triglyceride Reagent). Plasma cytokine levels were measured by electrochemiluminescence on a multiplex array using the Meso Scale Discovery platform.

Histology

Samples were prepared and stained at the Department of Pathology Histology Core at Yale University. Pancreata were fixed in 4% formalin overnight and transferred to 70% ethanol for 24 hours prior to embedding in paraffin wax for sectioning. Five µm tissue sections were cut and mounted on glass slides for paraffin removal and staining. Transferase-mediated dUTP nick-end labeling (TUNEL, Millipore, S7100) and insulin co-staining with polyclonal anti-insulin (Dako 0564) and visualization with alkaline phosphatase-based method were used to determine frequency of cell death. Cell proliferation was quantified following labeling with anti-KI67 (Biocare Medical CRM325) and anti-insulin (Dako A0564) and visualization with peroxidase and fluorescent secondary antibodies, respectively (Dako K4003; Invitrogen A11073). Quantification of histological features were performed as previously described. Briefly, the frequency of β-cell death was calculated as the number of insulin/TUNEL-positive cells corrected to relative islet area (islet area/pancreas area). Calculations were made using three sections per mouse and 10 to 15 fields per section. Frequency
of cellular proliferation was conducted in a similar fashion by counting insulin/KI67-positive cells and correcting to relative islet area. Pancreas weights were not available so relative β-cell volume was calculated as the ratio of insulin positive islet area/pancreas area. Islet number was determined by counting all islets within a field corrected to pancreas area. Three staggered sections per mouse were studied to reduce the probability of analyzing the same islet twice and approximately 30 fields per section were observed. All scoring was done blind at the microscope where magnification and light intensity could be adjusted to confirm positive staining. For 20 week samples, data from our previously published study was included (n = 3 per genotype) to increase sample size (db/db-SGLT2+/− = 8, db/db-SGLT2−/− = 7 final). All cell size measurements were made using ImageJ software.

Statistical analyses
All data reported are the mean ± standard error of the mean. The effect of age and genotype on each experimental outcome was compared using 2-way ANOVA where P < 0.05 was considered significant. All statistical analyses were made using GraphPad Prism 7.0 software.

Disclosure of potential conflicts of interest
Jean Whaley is currently an employee of Janssen Research and Development and a former employee of Bristol-Myers Squibb, where she contributed to the development of the SGLT2 inhibitor dapagliflozin. All other authors declare they have no competing interests.

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