Dapagliflozin improves insulin resistance and glucose intolerance in a novel transgenic rat with chronic glucose overproduction and glucose toxicity

Christos N. Joannides, Salvatore P. Mangiafico, Matthew F. Waters, Benjamin J. Lamont and Sofianos Andrikopoulos*

University of Melbourne, Department of Medicine (AH), Austin Hospital, Heidelberg, Victoria 3084, Australia

Short Title: Dapagliflozin and insulin resistance

Word Count: Abstract: 319
MainText: 4147

Number of Figures: 5

Number of Tables: 1

Number of References: 45

***Corresponding author:**
A/Prof Sofianos Andrikopoulos
University of Melbourne
Department of Medicine (Austin Health)
Level 7 Lance Townsend Building
Austin Hospital
145 Studley Road
Heidelberg, Victoria 3084
Australia
Phone: +61 3 9496 2403
Fax: +61 3 9457 5485
Email: sof@unimelb.edu.au

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/dom.12923

Abstract

Aims: Insulin resistance and impaired insulin secretion are cardinal defects that contribute to hyperglycemia in type 2 diabetes. Recently, a new class of drugs called sodium-glucose linked transporter 2 (SGLT2) inhibitors has been introduced that reduce blood glucose by inhibiting glucose reabsorption in the kidney and is not dependent on glucose metabolism or insulin action. The purpose of the present study was to determine whether the excretion of glucose would improve insulin resistance, impaired insulin secretion or both. **Materials and Methods:** Appropriate methods were used to assess insulin sensitivity (euglycemic/hyperinsulinemic clamp) and insulin secretion (hyperglycemic clamp) in insulin resistant and hyperglycemic PEPCK transgenic rats following treatment with the SGLT2 inhibitor dapagliflozin. **Results:** In 14-week old animals with hyperglycemia, insulin resistance and glucose intolerance, 6 weeks dapagliflozin treatment resulted in lower weight gain, plasma glucose and insulin levels, improved glucose tolerance associated with enhanced insulin sensitivity (Rate of glucose disappearance: 51.6 ± 2.3 vs 110.6 ± 3.9 μmol/min/kg, $P<0.005$) and glucose uptake in muscle (0.9 ± 0.1 vs 1.7 ± 0.3 μmol/min/100 g, $P<0.05$) and fat (0.23 ± 0.04 vs 0.55 ± 0.10 μmol/min/100 g, $P<0.05$). Additionally, adipose tissue GLUT4 protein levels were increased (0.78 ± 0.05 vs 1.20 ± 0.09 arbitrary units, $P<0.05$), adipocyte number was higher (221.4 ± 17.7 vs 302.3 ± 21.7 per mm^2 fat area, $P<0.05$) and size reduced (4631.8 ± 351.5 vs 3397.6 ± 229.4 μm^2, $P<0.05$). However, there was no improvement in insulin secretion. To determine whether earlier intervention is necessary, 5 week-old PEPCK transgenic rats were treated with dapagliflozin for 9 weeks and insulin secretion assessed. Dapagliflozin resulted in improved plasma glucose and insulin levels, lower weight gain but again insulin secretion was not improved. **Conclusions:** In this transgenic model of low-grade chronic hyperglycemia, SGLT2 inhibitor treatment resulted in reduced blood glucose and insulin levels and enhanced glucose tolerance associated with improved muscle and fat insulin resistance but not improved insulin secretory function.

Introduction

Type 2 diabetes is characterized by hyperglycemia caused by insulin resistance and islet β-cell dysfunction (1). Management of the disease is therefore targeted at improving insulin resistance and insulin secretion (2). In the last 25 years there has been a significant increase in the number of therapeutic classes for the treatment of type 2 diabetes (3). This means that treatment algorithms can now offer individualization of care for the person with diabetes in the pursuit of better glycemic control (4).

A new class of agents used in type 2 diabetes is the sodium-glucose linked transporter 2 (SGLT2) inhibitor. SGLT2 inhibitors reduce plasma glucose levels by inhibiting the re-absorption of glucose in the S1 segment of the proximal tubule in the kidney, resulting in glucose being excreted in the urine (5-7). Thus, unlike other drug classes (e.g. metformin, sulfonylureas, thiazolidinedione, incretins), SGLT2 inhibitors act independently of insulin or glucose metabolism thus offering a different mode of action that is not tissue specific (8). More importantly, by removing the excess glucose from the circulation, SGLT2 inhibitors have the potential of improving glycemic control by preventing glucose toxicity.

Glucose toxicity, as result of persistently high plasma glucose levels, is a mechanism that can impair islet β-cell function as well as cause insulin resistance in liver and muscle/fat. We have generated a rat model overexpressing the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK) in liver and kidney, which results in hyperglycemia, glucose intolerance, muscle and fat insulin resistance as well as a defective insulin secretion (9-11). Therefore the PEPCK transgenic rat is an excellent model of glucose toxicity-induced impairments in insulin action and secretion. In addition, we have previously shown that high glucose exposure of susceptible islets can result in reduced glucose-mediated insulin secretion and this was improved with anti-oxidant treatment (12, 13).

Given the deleterious effects of glucose toxicity, it makes sense that SGLT2 inhibitor agents that reduce glycemia by causing the excess glucose to be excreted in the urine may also have beneficial effects on insulin resistance and impaired insulin secretion. Clinical studies have suggested that short-term SGLT2 inhibitor treatment of patients with type 2 diabetes can indeed improve both insulin resistance and insulin secretion (14-16). Preclinical studies have also suggested that use of SGLT2 inhibitors can improve β-cell function, insulin sensitivity and glucose tolerance (17-20). However, these studies have not used suitable models of glucose toxicity to conclusively shown that the use of SGLT2 inhibitors improve both insulin

resistance and insulin secretion defects, and whether the effect on the islet β-cell is indirect by enhancing insulin sensitivity in diabetes.

The purpose of the present study was to use the PEPCK transgenic rat as an appropriate model of glucose toxicity-induced impairments in insulin action and secretion to determine whether SGLT2 inhibition can improve insulin resistance or β-cell dysfunction, respectively. Our data suggest that the SGLT2 inhibitor dapagliflozin selectively improves insulin sensitivity and not β-cell function. Furthermore, dapagliflozin-treated PEPCK transgenic rat displayed an increase in adipose tissue glucose uptake and GLUT4 protein levels as well as reduced adipocyte size and increased adipocyte number.

RESEARCH DESIGN AND METHODS

Animals

PEPCK transgenic rats were generated on the PVG/c background as previously described (11). The control used in this study was the age-matched non-transgenic PVG/c strain. Studies were performed in male rats housed in a temperature- and humidity-controlled environment with 12 h light/dark cycle, and water and standard laboratory chow *ad libitum* (w/w: 74% carbohydrate, 20% protein and 3% fat - Ridley AgriProducts Pakenham, VIC, Australia). All studies were performed with n=4-6 animals per group. The SGLT2 inhibitor, dapagliflozin, was administered in drinking water at a final concentration of 60 mg/L from 5 weeks of age for 9 weeks (intervention study), and from 14 weeks of age for 6 weeks (treatment study). The drug solution was freshly prepared and changed at least three times per week. The vehicle treated group was provided with drinking water not containing the drug. All animal care and procedures were conducted according to the protocols and guidelines approved by the Austin Health Animal Ethics Committee. Body weight and food intake were assessed weekly (10).

Hyperglycemic clamp

The hyperglycemic clamp was conducted as previously described (21). Following a 6 hour fast (0600hrs–1200hrs), a bolus of glucose (375 mg/kg body weight) was injected intravenously to initially raise blood glucose levels. Blood glucose concentrations were monitored every 5 min using a hand-held glucose meter and maintained between 15-17 mmol/L by infusion of a glucose solution. Plasma samples were collected every 20 min for insulin determination.

Basal turnover and hyperinsulinemic-euglycemic clamp

Glucose turnover was assessed in 6-hour fasted rats as previously described (9, 10). An initial 2 min priming dose of radio-labelled glucose tracer [6-^{3}H]-glucose at a rate of 100 µBq/min followed by a constant infusion of tracer at a rate of 5.5 µBq/min in 0.9% saline was administered during basal and hyperinsulinemic-euglycemic clamp experiments to measure whole-body glucose turnover. During the clamp experiments, following an initial priming dose, insulin was infused at a constant rate at either 6 mU/kg/min in control and PEPCK transgenic rats treated with dapagliflozin or 4 mU/kg/min in PEPCK transgenic rats to produce similar plasma insulin concentrations in the three groups. Blood glucose was monitored every 5 min for adjustment of the glucose infusion rate. Blood samples were

collected during steady state conditions at 90, 100 and 110 min. Under steady state conditions, the rate of glucose appearance equals the rate of glucose disappearance. The rate of glucose appearance (R_a) was calculated by dividing the infusion rate of [6-^{3}H]-glucose (dpm/min) by the plasma [6-^{3}H]-glucose specific activity. The rate of endogenous glucose production (EGP) was determined as the difference between total R_a and the rate of exogenously infused glucose.

Measurement of glucose uptake into peripheral tissues

Following collection of the last sample in the clamp, a bolus of [1-^{14}C]-2-deoxy-glucose (370 μBq) was infused and blood collected at 2, 5, 10, 15, 30 and 45 min. Immediately following the last blood sample at 45 min, rats were sacrificed by sodium pentobarbitone overdose and tissues including white quadriceps, red gastrocnemius and epididymal adipose tissue were removed and stored at -80 °C for subsequent analysis. The rate of glucose uptake (Rg') into individual tissues was calculated as previously described (9, 10). Adipose tissue weights were removed and weighed. Subcutaneous adipose tissue was dissected by peeling back the skin from the leg revealing the dorsal adipose tissue. Infrarenal (perinephric) adipose tissue was dissected from below the kidney and epididymal adipose tissue was dissected along the epididymis and testis.

Intraperitoneal glucose tolerance test

Intraperitoneal glucose tolerance tests were carried out after an overnight fast (22). Following basal glucose, insulin and ² -hydroxybutyrate measurements (0 min), a bolus of glucose (1 g/kg body weight) was injected and blood taken at 15, 30, 45, 60 and 120 min.

Analysis of glucose, insulin and ² -hydroxybutyrate concentrations

The presence of glucose in the urine was determined by using Diastix glucose test strips (Bayer, Leverkusen, Germany). Blood glucose concentrations were measured on an Accu-Check Performa glucose meter. Plasma glucose concentrations were determined using a GM7 Analox glucose analyser (Helena Laboratories, Mount Waverley, Victoria, Australia). Plasma insulin concentrations were determined by a commercially available radioimmunoassay kit specific for rodent insulin (Merck Millipore, Temecula, CA, USA). Blood ² -hydroxybutyrate concentrations were measured on a Medisense Optium glucose meter.

Islet isolation

Islets were isolated by intraductal collagenase IX (Sigma-Aldrich, Castle Hill NSW Australia) perfusion, histopaque-1077 gradient (Sigma-Aldrich) purification and hand picking (13, 23).

Immunohistochemistry

Pancreata were isolated, fixed with 10% buffered formalin and embedded in paraffin (24). Histological sections of 5 µm were stained for insulin using a guinea pig anti-porcine insulin antibody diluted 1:100 and counterstained with an anti-guinea pig conjugated horseradish peroxidase secondary antibody and treated with 3,3'-diamonobenzidine (DAB) for visualisation. Whole slide sections were line scanned at 40X magnification using an Aperio ScanScope XT (Aperio Technologies, Vista, CA, USA). Digital images were analysed using the Aperio image software, ImageScope version 12.0.

Morphological analysis of adipose tissue

Epididymal WAT was fixed in 10% buffered formalin and embedded in paraffin. Histological sections of 4 µm were stained with hematoxylin and eosin as previously described (24).

Gene expression studies

Total RNA was extracted from liver and islets (at least 100) using the TRIzol method (Invitrogen, Mount Waverley, VIC, Australia). Total RNA (1 µg for liver and 4 µg for islets) was reverse transcribed using the Promega Reverse Transcription System with random primers (Promega Madison WI USA) and any contaminating DNA removed by treatment with DNaseI (RNase-free; Ambion, Mount Waverley, VIC, Australia). Real-Time PCR was conducted in a ViiA 7 Real-Time PCR thermocycler system and analysed using the ABI ViiA 7 version 1.2 software. The relative mRNA transcript level of *Acc1* (Rn00573474_m1), *Acc2* (Rn00588290_m1), *Cpt1a* (Rn00580702_m1), *G6pc* (Rn00689876_m1), *Pck1* (Rn01529014_m1), *Glut2* (Rn00563565_m1), *Gck* (Rn00561265_m1), *Sur1* (Rn01476317_m1) and *Kir6.2* (Rn01764077_s1) were measured using pre-developed rat TaqMan Gene Expression Assays (Applied Biosystems, Scoresby, VIC, Australia) as previously described (23), normalised to the mRNA transcript level of *18S* and reported as fold change. The comparative Ct (" Ct) method was used for relative quantification.

Western blotting

Total protein lysates of red gastrocnemius and epididymal fat were separated by SDS-PAGE as previously described (25), transferred to PVDF membranes, and blocked in 5% milk in TBS-T before incubation with primary antibodies overnight at 4 °C. The primary antibodies for GLUT4 (ab48547, Abcam, Melbourne, VIC, Australia) and HSP90 used as the control (AC88, Abcam, Melbourne, VIC, Australia) were diluted 1:2,500 in 5% milk. Densitometry was quantified using Fuji film Multi Gauge software.

Statistical analysis

All data are presented as mean ± SEM. Statistical significance between two groups was assessed using a two-tailed, unpaired Student's t-test with equal variances. In three groups, statistical significance was assessed using a one-way ANOVA with Tukey's multiple comparison *post hoc* test. Incremental area under the curve was calculated using the trapezoid rule. A $P<0.05$ was considered significant.

RESULTS

Creating a glucose sink reduces adiposity and improves glucose metabolism in obese and glucose intolerant PEPCK transgenic rats

In order to determine whether removing glucose from the body using dapagliflozin can improve glucose homeostasis, 14 week-old PEPCK transgenic rats that are obese, hyperglycemic, hyperinsulinemic and glucose intolerant were treated with dapagliflozin for 6 weeks. There were no differences in the starting body weights between PEPCK transgenic rat groups treated with vehicle or dapagliflozin (327.7 ± 7.8 g *vs*. 354.7 ± 12.7 g, n=4-6, P=0.34). However, both PEPCK transgenic rat groups were heavier than control rats (244.5 ± 6.5 g, P<0.0001). Administration of dapagliflozin over the 6 week treatment period reduced body weight gain (Fig 1A) and adiposity levels (Fig 1C) in the PEPCK transgenic rat when compared with the vehicle treated group. Interestingly, dapagliflozin treatment of PEPCK transgenic rats resulted in higher food intake (Fig 1B). Treatment with dapagliflozin also resulted in a significant increase in wet kidney weight compared with vehicle treated and lean control rats respectively (Fig 1D). Fluid intake was significantly increased in PEPCK transgenic rats treated with dapagliflozin compared with vehicle (62.9 ± 2.8 vs 28.9 ± 1.3 ml/day n=4 P<0.001).

Fasted blood glucose and plasma insulin concentrations were lower in the PEPCK transgenic rats treated with dapagliflozin compared with vehicle treated (Fig 1E&F). As expected the PEPCK transgenic vehicle treated group displayed glucose intolerance and this was improved by dapagliflozin treatment (Fig 1G). In addition, blood ketone bodies (as assessed by β-hydroxybutyrate) were reduced in the PEPCK transgenic vehicle treated group compared with control and this was increased with dapagliflozin treatment of the transgenic rats (Fig 1H). Expression of hepatic genes in the gluconeogenic and fatty acid oxidation pathways showed no difference between PEPCK transgenic dapagliflozin treated and vehicle groups (Fig 1I).

Dapagliflozin treatment improved peripheral insulin resistance in PEPCK transgenic rats

To determine whether insulin resistance was improved in PEPCK transgenic rats following dapagliflozin treatment, euglycemic-hyperinsulinemic clamps were performed (Table 1 and Fig 2). There were no differences in plasma glucose and insulin levels during the

hyperinsulinemic clamp (Table 1). Glucose infusion rate and rate of glucose disappearance (R_d) were reduced in PEPCK transgenic vehicle treated rats and this was normalized with dapagliflozin treatment (Fig 2A&B). Endogenous glucose production (EGP) was higher in the PEPCK transgenic vehicle treated group and this was not altered with dapagliflozin treatment (Fig 2C). Muscle and fat tissue specific glucose uptake was reduced in the PEPCK transgenic vehicle treated group and this was improved with dapagliflozin treatment (Fig 2D). Muscle GLUT4 levels were not different among the 3 groups of rats (Fig 2E). However, adipose tissue GLUT4 levels were decreased in PEPCK transgenic vehicle treated rats compared with control and this was increased with dapagliflozin treatment (Fig 2F). Further investigation of the adipose tissue showed that adipocyte size was larger (Fig 2G&H) and adipocyte number (Fig 2I) was lower in the PEPCK transgenic vehicle treated group compared with control and both these parameters were improved with dapagliflozin treatment.

Dapagliflozin prevents increased weight gain and adiposity and improved glucose metabolism

The above data suggest that treating older PEPCK transgenic rats with established defects in glucose metabolism improved insulin resistance but not the impairment in insulin secretion. To determine whether early intervention would lead to improved β-cell function, 5-week-old PEPCK transgenic rats were treated with vehicle or dapagliflozin for 9 weeks. There were no differences in the starting body weights between PEPCK transgenic rat groups treated with vehicle or dapagliflozin (109.4 ± 3.9 g *vs*. 121.0 ± 4.8 g, n=4-6). However, the starting body weight was lower in the control rat group compared with both PEPCK groups (93.4 ± 3.8; P<005 vs PEPCK+vehicle, P<0.01 vs PEPCK+dapagliflozin). Body weight gain and adiposity was higher in the PEPCK transgenic vehicle rats compared with control and this was normalized with dapagliflozin treatment (Fig 3A&C). Average food intake was higher in the PEPCK transgenic vehicle treated rats compared with control and this was further increased in the dapagliflozin treated group (Fig 3B). Kidney wet weight was also higher in the PEPCK transgenic dapagliflozin treated group compared with vehicle treatment (Fig 3D). At 14 weeks of age fasting blood glucose and insulin levels were higher in the PEPCK transgenic vehicle treated group compared with control and these were reduced with dapagliflozin treatment (Fig 3E&F). Glucose tolerance was impaired in the PEPCK transgenic vehicle treated group compared with control and this was normalized with

dapagliflozin treatment (Fig 3G). Interestingly, as with the older rats, blood ketone levels were reduced in PEPCK transgenic vehicle treated rats compared with control and this was increased with dapagliflozin treatment (Fig 3H).

Dapagliflozin does not improve β-cell dysfunction in glucose intolerant PEPCK transgenic rats

To assess β-cell function following dapagliflozin treatment we performed hyperglycemic clamps and the data are shown in Fig 4. The glucose infusion rate was lower in the PEPCK transgenic vehicle treated group indicative of insulin resistance (Fig 4A). Blood glucose levels were similarly clamped at around 16 mM in all 3 groups (Fig 4B). Plasma insulin levels were lower in the PEPCK transgenic vehicle compared to control, indicative of β-cell dysfunction (Fig 4C). Importantly, dapagliflozin treatment did not improve insulin secretion in the PEPCK transgenic rat (Fig 4C). Islet size tended to be higher (Fig 4D&E) and β-cell mass tended to be lower (Fig 4G) in both PEPCK transgenic groups compared with control. Interestingly the number of islets per pancreas area was lower in the PEPCK transgenic groups compared with control (Fig 4F).

Early treatment of dapagliflozin does not improve β-cell function in PEPCK transgenic rats

Hyperglycemic clamps were performed in 14 week old rats following 9 weeks of treatment to determine whether early intervention with dapagliflozin can prevent the development of β-cell dysfunction (Fig 5). The glucose infusion rate to maintain blood glucose levels at 16 mM was lower in the PEPCK transgenic vehicle treated rats indicative of insulin resistance (Fig 5A&B). Insulin secretion was reduced in the PEPCK transgenic vehicle treated group compared with control (Fig 5C). Of importance, PEPCK transgenic rats treated with dapagliflozin from 5 weeks of age did not show improved insulin secretory function compared with control rats and indeed their response tended slightly lower than the PEPCK vehicle treated group (Fig 5C).

Islet size was larger in the PEPCK vehicle treated compared with control and treatment with dapagliflozin reduced this parameter (Fig 5D&E). Islet number and β-cell mass was lower in both PEPCK groups compared with control rats (Fig 5F&G).

DISCUSSION

In this study we sought to determine whether treatment with the SGLT2 inhibitor dapagliflozin could improve insulin resistance or insulin secretion (or both) in a transgenic rat model overexpressing the gluconeogenic enzyme phosphoenolpyruvate carboxykinase in the liver and kidney (11). As a result PEPCK transgenic rats are characterised by primary chronic low-grade hyperglycemia which in turn is associated with muscle and fat insulin resistance and impaired insulin secretion (9, 10). Our results clearly show that when initiated following established hyperglycemia, hyperinsulinemia, insulin resistance and impaired insulin secretion, dapagliflozin caused reduced plasma glucose and insulin levels as a result of improved insulin resistance, increased muscle and fat glucose uptake and GLUT4 protein levels as well as reduced adipocyte size and increased adipocyte number, but not increased insulin secretion. Indeed using the hyperglycemic clamp, we found that insulin secretion was not improved whether dapagliflozin was administered in adult PEPCK transgenic rats with established dysglycemia or in young animals when defects in insulin release are not evident (10).

Studies in humans have shown that SGLT2 inhibitor treatment of patients with type 2 diabetes improved both insulin resistance (14, 16) and insulin secretion as assessed by a meal tolerance test (14). This is despite increases in plasma glucagon and endogenous glucose production. In fact, Ferrannini and colleagues showed that there was increased β-cell glucose sensitivity - that is, for any given plasma glucose level, the patients treated with the SGLT2 inhibitor empagliflozin secreted more insulin compared with baseline (14). In support of this, a study in patients with type 2 diabetes with inadequate glycemic control using dapagliflozin for 12 weeks compared with placebo on usual care showed a significant improvement in glucose disposal during a euglycemic-hyperinsulinemic clamp and a non-significant improvement in acute insulin secretion following an intravenous glucose tolerance test (26). These studies support the use of SGLT2 inhibitors in the management of hyperglycemia in type 2 diabetes by predominantly improving insulin resistance and possibly insulin secretion. However studies in appropriate animal models are required to further explore the mechanism of SGLT2 inhibitor improvements in glucose metabolism.

Previous studies in preclinical models of diabetes have shown that SGLT2 inhibition can lead to improved glycemia, glucose tolerance, insulin resistance and improved β-cell function and mass. Specifically treatment of C57BL/Ks-*db/db* mice with BI-38335 resulted in improved glucose tolerance, insulin resistance and glucose-mediated insulin secretion in isolated islets

(27). Similarly, high fat-fed and streptozotocin-induced diabetic mice treated with dapagliflozin or impragliflozin showed improved glucose tolerance, insulin resistance and islet insulin secretion, with a significant increase in pancreatic insulin content (18, 20). The effect of the SGLT2 inhibitor on glycemic control was further enhanced when combined with other anti-diabetic medications (18, 20, 27). Whether the improved insulin secretory function is due to a reduction in hyperglycemia or improved insulin resistance is not clear.

Treatment of Zucker Diabetic Fatty (ZDF) rats with SGLT2 inhibitors resulted in improved glycemia, glucose tolerance and higher plasma insulin levels associated with increased islet and β-cell mass (17, 28) as well as suppressed endogenous glucose production with no difference in rate of glucose disappearance or muscle and white adipose tissue-specific glucose uptake during a hyperinsulinemic clamp (29, 30). This is in contrast to our study in the PEPCK transgenic rats which clearly showed a significant improvement in the rate of glucose disposal and increased glucose uptake specifically in muscle and white adipose tissue associated with higher GLUT4 protein levels. It is not clear why we were able to see an improvement in muscle and adipose tissue insulin resistance in the PEPCK transgenic rat in contrast to the ZDF rat studies above. It may well be that glucose toxicity is the primary cause of insulin resistance in the PEPCK transgenic rat, which is ameliorated with SGLT2 inhibition while there may be other contributory factors in the ZDF rat including lipotoxicity (31, 32) (weight was not reduced with dapagliflozin treatment (29, 30)) and inflammation (33, 34). Finally, while endogenous glucose production was increased in PEPCK transgenic compared with control rats, this was not reduced with dapagliflozin treatment. This is contrary to the studies with the ZDF rat and SGLT2 inhibitors, which showed suppression of endogenous glucose production (28, 29) during a euglycemic-hyperinsulinemic clamp, associated with liver glucokinase activation (30). It is important to note in this context that in our model, the PEPCK transgene is driven by a promoter that does not respond to the suppressive effects of insulin (11), thus we were not expecting to see a substantial reduction in endogenous glucose production with the plasma insulin levels achieved during the hyperinsulinemic clamp (Table 1) as we have previously shown (9).

It was surprising that insulin secretory function as assessed by the hyperglycemic clamp was not improved in the treatment group and was not protected in the intervention group in our study. Indeed, islet area and β-cell mass were not different and if anything trended lower in dapagliflozin compared with vehicle treated PEPCK transgenic rats. Of interest is that PEPCK transgenic rats have fewer islets from 5 weeks of age compared with control rats and

it may hence be more difficult to show an improvement in insulin secretory function with interventions such as dapagliflozin treatment. Glucose toxicity has been shown to be an important mechanism causing β-cell dysfunction (35) and treatments that improve hyperglycemia have been suggested to be beneficial to impairments in insulin secretion in type 2 diabetes (2). Indeed, we have shown that chronic high glucose treatment of islets can impair insulin secretory function via generation of advanced glycation end products and oxidative stress (12, 13). Furthermore as discussed above, improved β-cell insulin sensitivity was shown in patients with type 2 diabetes treated with empagliflozin (14). Interestingly, a study using the hyperglycemic clamp in ZDF rats treated with dapagliflozin showed reduced plasma insulin and C-peptide levels and no effect on β-cell mass compared with the vehicle treated group (36), which support the findings of our present study. However, they did show that the disposition index, which takes into account the prevailing insulin resistance state, was improved in the ZDF rats treated with dapagliflozin (36). Moreover, in a study using C57BL/Ks-*db/db* mice that were crossed to SGLT2 whole body knockout mice, insulin secretion was improved *in vivo* but not *in vitro* using isolated islets in static or perifusion experiments and this was associated with an increase in β-cell volume (37). Therefore, while there may not have been a direct increase in insulin secretory capacity with SGLT2 inhibitor treatment, improvements in glycemia and insulin sensitivity would lessen the burden and provide an islet β-cell sparing effect.

A number of clinical and animal studies in type 2 diabetes have shown that SGLT2 inhibitor treatment leads to weight loss, which stabilises despite continued glucose/energy loss in the urine (5, 18, 38-41). In fact, a recent study in overweight patients with type 2 diabetes showed that SGLT2 inhibitor treatment resulted in a 3 kg weight loss rather than 11 kg that was predicted by calculating the urinary energy loss (42). Furthermore this study suggested an increase in energy intake as a possible mechanism for this disparity in the predicted and actual weight loss (42). Indeed studies in diet-induced obese rats treated with dapagliflozin showed a modest decrease in weight loss attenuated by an increase in energy intake despite an increase in plasma β-hydroxybutyrate levels (41). These observations corroborate the results in our study showing reduced weight gain and adiposity in the PEPCK transgenic rats treated with dapagliflozin but higher food intake and blood β-hydroxybutyrate levels. An increase in ketone levels have been associated with reduced appetite in obese individuals (43, 44) and in transgenic mice with a reduced body weight phenotype (45). The reason for increased energy intake in response to SGLT2 inhibition-induced glycosuria is not clear.

There is obviously a sensing mechanism that is activated with glycosuria that results in increased energy intake, which is worth investigating further as greater weight loss would lead to even further improvements in blood glucose and lipid levels and blood pressure in type 2 diabetes.

In conclusion, we showed that treatment of the hyperglycaemic, hyperinsulinaemic and glucose intolerant PEPCK transgenic rat with the SGLT2 inhibitor dapagliflozin resulted reduced blood glucose and insulin levels and enhanced glucose tolerance associated with improved muscle and fat insulin resistance. However, insulin secretory function was not affected in this transgenic model of chronic low-grade hyperglycemia. Understanding the mechanism by which dapagliflozin exerts its effect will inform us of the most appropriate combinations of medications to use in type 2 diabetes to result in the most effective and durable reductions in blood glucose levels.

Acknowledgements

This study was funded by an Investigator-initiated study grant from AstraZeneca to SA. We would like to thank Professor Joseph Proietto for helpful discussions and Dr Maria Stathopoulos for experimental assistance.

Author Contributions

CNJ conducted experiments, analyzed data and wrote the first draft of the manuscript, SPM conducted experiments, analyzed data and edited the manuscript, MFW and BJL conducted experiments, analyzed data and provided feedback on the manuscript and SA conceived the experimental design, applied for funding, assisted in experiments and analyzing the data and wrote the manuscript.

Declarations of Interest

This study was funded by an investigator-initiated study grant from AstraZeneca to SA.

REFERENCES

1. Kahn SE, Hull RL, Utzschneider KM. Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature. 2006;444(7121):840-6.

2. Aston-Mourney K, Proietto J, Andrikopoulos S. Investigational agents that protect pancreatic islet beta-cells from failure. Expert Opin Investig Drugs. 2005;14(10):1241-50.

3. Kahn SE, Cooper ME, Del Prato S. Pathophysiology and treatment of type 2 diabetes: perspectives on the past, present, and future. Lancet. 2014;383(9922):1068-83.

4. Gunton JE, Cheung NW, Davis TM, Zoungas S, Colagiuri S. A new blood glucose management algorithm for type 2 diabetes: a position statement of the Australian Diabetes Society. The Medical journal of Australia. 2014;201(11):650-3.

5. Bailey CJ, Gross JL, Pieters A, Bastien A, List JF. Effect of dapagliflozin in patients with type 2 diabetes who have inadequate glycaemic control with metformin: a randomised, double-blind, placebo-controlled trial. Lancet. 2010;375(9733):2223-33.

6. Henry RR, Murray AV, Marmolejo MH, Hennicken D, Ptaszynska A, List JF. Dapagliflozin, metformin XR, or both: initial pharmacotherapy for type 2 diabetes, a randomised controlled trial. Int J Clin Pract. 2012;66(5):446-56.

7. Nauck MA, Del Prato S, Meier JJ, Duran-Garcia S, Rohwedder K, Elze M, et al. Dapagliflozin versus glipizide as add-on therapy in patients with type 2 diabetes who have inadequate glycemic control with metformin: a randomized, 52-week, double-blind, active-controlled noninferiority trial. Diabetes Care. 2011;34(9):2015-22.

8. Abdul-Ghani MA, Norton L, Defronzo RA. Role of sodium-glucose cotransporter 2 (SGLT 2) inhibitors in the treatment of type 2 diabetes. Endocrine reviews. 2011;32(4):515-31.

9. Lamont BJ, Andrikopoulos S, Funkat A, Favaloro J, Ye JM, Kraegen EW, et al. Peripheral insulin resistance develops in transgenic rats overexpressing phosphoenolpyruvate carboxykinase in the kidney. Diabetologia. 2003;46(10):1338-47.

10. Mangiafico SP, Lim SH, Neoh S, Massinet H, Joannides CN, Proietto J, et al. A primary defect in glucose production alone cannot induce glucose intolerance without defects in insulin secretion. J Endocrinol. 2011;210(3):335-47.

11. Rosella G, Zajac JD, Baker L, Kaczmarczyk SJ, Andrikopoulos S, Adams TE, et al. Impaired glucose tolerance and increased weight gain in transgenic rats overexpressing a non-insulin-responsive phosphoenolpyruvate carboxykinase gene. Mol Endocrinol. 1995;9(10):1396-404.

12. Kooptiwut S, Kebede M, Zraika S, Visinoni S, Aston-Mourney K, Favaloro J, et al. High glucose-induced impairment in insulin secretion is associated with reduction in islet glucokinase in a mouse model of susceptibility to islet dysfunction. J Mol Endocrinol. 2005;35(1):39-48.

13. Zraika S, Aston-Mourney K, Laybutt DR, Kebede M, Dunlop ME, Proietto J, et al. The influence of genetic background on the induction of oxidative stress and impaired insulin secretion in mouse islets. Diabetologia. 2006;49(6):1254-63.

14. Ferrannini E, Muscelli E, Frascerra S, Baldi S, Mari A, Heise T, et al. Metabolic response to sodium-glucose cotransporter 2 inhibition in type 2 diabetic patients. J Clin Invest. 2014;124(2):499-508.

15. Merovci A, Mari A, Solis C, Xiong J, Daniele G, Chavez A, et al. Dapagliflozin Lowers Plasma Glucose Concentration and Improves Beta Cell Function. J Clin Endocrinol Metab. 2015:jc20143472.

16. Merovci A, Solis-Herrera C, Daniele G, Eldor R, Fiorentino TV, Tripathy D, et al. Dapagliflozin improves muscle insulin sensitivity but enhances endogenous glucose production. J Clin Invest. 2014;124(2):509-14.

17. Hansen HH, Jelsing J, Hansen CF, Hansen G, Vrang N, Mark M, et al. The sodium glucose cotransporter type 2 inhibitor empagliflozin preserves beta-cell mass and restores glucose homeostasis in the male zucker diabetic fatty rat. J Pharmacol Exp Ther. 2014;350(3):657-64.

18. Millar PJ, Pathak V, Moffett RC, Pathak NM, Bjourson AJ, O'Kane MJ, et al. Beneficial metabolic actions of a stable GIP agonist following pre-treatment with a SGLT2 inhibitor in high fat fed diabetic mice. Mol Cell Endocrinol. 2016;420:37-45.

19. Neschen S, Scheerer M, Seelig A, Huypens P, Schultheiss J, Wu M, et al. Metformin supports the antidiabetic effect of a sodium glucose cotransporter 2 inhibitor by suppressing endogenous glucose production in diabetic mice. Diabetes. 2015;64(1):284-90.

20. Tahara A, Takasu T, Yokono M, Imamura M, Kurosaki E. Effects of the combination of SGLT2 selective inhibitor ipragliflozin and various antidiabetic drugs in type 2 diabetic mice. Archives of pharmacal research. 2016;39(2):259-70.

21. Lamontagne J, Jalbert-Arsenault E, Pepin E, Peyot ML, Ruderman NB, Nolan CJ, et al. Pioglitazone acutely reduces energy metabolism and insulin secretion in rats. Diabetes. 2013;62(6):2122-9.

22. Andrikopoulos S, Blair AR, Deluca N, Fam BC, Proietto J. Evaluating the glucose tolerance test in mice. Am J Physiol Endocrinol Metab. 2008;295(6):E1323-32.

23. Andrikopoulos S, Fam BC, Holdsworth A, Visinoni S, Ruan Z, Stathopoulos M, et al. Identification of ABCC8 as a contributory gene to impaired early-phase insulin secretion in NZO mice. J Endocrinol. 2016;228(1):61-73.

24. Kebede M, Favaloro J, Gunton JE, Laybutt DR, Shaw M, Wong N, et al. Fructose-1,6-bisphosphatase overexpression in pancreatic beta-cells results in reduced insulin secretion: a new mechanism for fat-induced impairment of beta-cell function. Diabetes. 2008;57(7):1887-95.

25. Xirouchaki CE, Mangiafico SP, Bate K, Ruan Z, Huang AM, Tedjosiswoyo BW, et al. Impaired glucose metabolism and exercise capacity with muscle-specific glycogen synthase 1 (gys1) deletion in adult mice. Mol Metab. 2016;5(3):221-32.

26. Mudaliar S, Henry RR, Boden G, Smith S, Chalamandaris AG, Duchesne D, et al. Changes in insulin sensitivity and insulin secretion with the sodium glucose cotransporter 2 inhibitor dapagliflozin. Diabetes Technol Ther. 2014;16(3):137-44.

27. Chen L, Klein T, Leung PS. Effects of combining linagliptin treatment with BI-38335, a novel SGLT2 inhibitor, on pancreatic islet function and inflammation in db/db mice. Current molecular medicine. 2012;12(8):995-1004.

28. Thomas L, Grempler R, Eckhardt M, Himmelsbach F, Sauer A, Klein T, et al. Long-term treatment with empagliflozin, a novel, potent and selective SGLT-2 inhibitor, improves glycaemic control and features of metabolic syndrome in diabetic rats. Diabetes Obes Metab. 2012;14(1):94-6.

29. Han S, Hagan DL, Taylor JR, Xin L, Meng W, Biller SA, et al. Dapagliflozin, a selective SGLT2 inhibitor, improves glucose homeostasis in normal and diabetic rats. Diabetes. 2008;57(6):1723-9.

30. Ueta K, O'Brien TP, McCoy GA, Kim K, Healey EC, Farmer TD, et al. Glucotoxicity targets hepatic glucokinase in Zucker diabetic fatty rats, a model of type 2 diabetes associated with obesity. Am J Physiol Endocrinol Metab. 2014;306(11):E1225-38.

31. De Feyter HM, Lenaers E, Houten SM, Schrauwen P, Hesselink MK, Wanders RJ, et al. Increased intramyocellular lipid content but normal skeletal muscle mitochondrial

oxidative capacity throughout the pathogenesis of type 2 diabetes. FASEB J. 2008;22(11):3947-55.

32. Smith AC, Mullen KL, Junkin KA, Nickerson J, Chabowski A, Bonen A, et al. Metformin and exercise reduce muscle FAT/CD36 and lipid accumulation and blunt the progression of high-fat diet-induced hyperglycemia. Am J Physiol Endocrinol Metab. 2007;293(1):E172-81.

33. Ndisang JF, Jadhav A. Hemin therapy suppresses inflammation and retroperitoneal adipocyte hypertrophy to improve glucose metabolism in obese rats co-morbid with insulin-resistant type-2 diabetes. Diabetes Obes Metab. 2013;15(11):1029-39.

34. Rodriguez-Calvo R, Serrano L, Coll T, Moullan N, Sanchez RM, Merlos M, et al. Activation of peroxisome proliferator-activated receptor beta/delta inhibits lipopolysaccharide-induced cytokine production in adipocytes by lowering nuclear factor-kappaB activity via extracellular signal-related kinase 1/2. Diabetes. 2008;57(8):2149-57.

35. Robertson RP, Harmon J, Tran PO, Tanaka Y, Takahashi H. Glucose toxicity in beta-cells: type 2 diabetes, good radicals gone bad, and the glutathione connection. Diabetes. 2003;52(3):581-7.

36. Macdonald FR, Peel JE, Jones HB, Mayers RM, Westgate L, Whaley JM, et al. The novel sodium glucose transporter 2 inhibitor dapagliflozin sustains pancreatic function and preserves islet morphology in obese, diabetic rats. Diabetes Obes Metab. 2010;12(11):1004-12.

37. Jurczak MJ, Lee HY, Birkenfeld AL, Jornayvaz FR, Frederick DW, Pongratz RL, et al. SGLT2 deletion improves glucose homeostasis and preserves pancreatic beta-cell function. Diabetes. 2011;60(3):890-8.

38. Cefalu WT, Leiter LA, Yoon KH, Arias P, Niskanen L, Xie J, et al. Efficacy and safety of canagliflozin versus glimepiride in patients with type 2 diabetes inadequately

controlled with metformin (CANTATA-SU): 52 week results from a randomised, double-blind, phase 3 non-inferiority trial. Lancet. 2013;382(9896):941-50.

39. Ferrannini E, Ramos SJ, Salsali A, Tang W, List JF. Dapagliflozin monotherapy in type 2 diabetic patients with inadequate glycemic control by diet and exercise: a randomized, double-blind, placebo-controlled, phase 3 trial. Diabetes Care. 2010;33(10):2217-24.

40. Forst T, Guthrie R, Goldenberg R, Yee J, Vijapurkar U, Meininger G, et al. Efficacy and safety of canagliflozin over 52 weeks in patients with type 2 diabetes on background metformin and pioglitazone. Diabetes Obes Metab. 2014;16(5):467-77.

41. Devenny JJ, Godonis HE, Harvey SJ, Rooney S, Cullen MJ, Pelleymounter MA. Weight loss induced by chronic dapagliflozin treatment is attenuated by compensatory hyperphagia in diet-induced obese (DIO) rats. Obesity (Silver Spring). 2012;20(8):1645-52.

42. Ferrannini G, Hach T, Crowe S, Sanghvi A, Hall KD, Ferrannini E. Energy Balance After Sodium-Glucose Cotransporter 2 Inhibition. Diabetes Care. 2015;38(9):1730-5.

43. Chearskul S, Delbridge E, Shulkes A, Proietto J, Kriketos A. Effect of weight loss and ketosis on postprandial cholecystokinin and free fatty acid concentrations. Am J Clin Nutr. 2008;87(5):1238-46.

44. Sumithran P, Prendergast LA, Delbridge E, Purcell K, Shulkes A, Kriketos A, et al. Ketosis and appetite-mediating nutrients and hormones after weight loss. Eur J Clin Nutr. 2013;67(7):759-64.

45. Visinoni S, Khalid NF, Joannides CN, Shulkes A, Yim M, Whitehead J, et al. The role of liver fructose-1,6-bisphosphatase in regulating appetite and adiposity. Diabetes. 2012;61(5):1122-32.

FIGURE LEGENDS

Figure 1:

(A) Body weight gain and (B) average food intake over the 6 week treatment period in control, PEPCK transgenic and PEPCK transgenic rats treated with dapagliflozin. (C) WAT weight, (D) wet kidney weight, overnight fasted (E) blood glucose levels, (F) plasma insulin levels and (G) ipGTT (1 g/kg i.p.) and incremental area under the glucose curve (inset) in control, PEPCK transgenic and PEPCK transgenic rats treated with dapagliflozin from 14 weeks of age. Overnight fasted (H) blood ² -hydroxybutyrate (BHB) levels and (I) relative mRNA expression of key liver genes involved in fatty acid synthesis (*Acc1*), fatty acid oxidation (*Acc2* and *Cpt1a*) and gluconeogenesis (*G6pc* and *Pck1*). Data are presented as mean ± SEM (n=4-6 *P<0.05 vs control, †P<0.05 vs PEPCK transgenic).

Figure 2:

(A) Glucose infusion rate, (B) rate of glucose disappearance, (C) endogenous glucose production during hyperinsulinemic-euglycemic clamp conditions, (D) the rate of glucose uptake (Rg') in white quadriceps, red gastrocnemius and epididymal fat, and total GLUT4 protein content with corresponding densitometry levels in (E) red gastrocnemius and (F) epididymal fat during hyperinsulinemic conditions. Representative H&E stained sections of epididymal adipose tissue (G), average adipocyte area (H) and adipocyte number per mm^2 fat area (I) in 20 week-old control, PEPCK transgenic and PEPCK transgenic rats treated with dapagliflozin for 6 weeks duration. Data are presented as mean ± SEM (n=4-6 *P<0.05 vs control, †P<0.05 vs PEPCK transgenic).

Figure 3:

(A) Body weight gain and (B) average food intake over the 9 week intervention period in control, PEPCK transgenic and PEPCK transgenic rats treated with dapagliflozin. (C) WAT weight, (D) wet kidney weight, overnight fasted (E) blood glucose, (F) plasma insulin, (G) ipGTT and incremental area under the glucose curve (inset) in control, PEPCK transgenic and PEPCK transgenic rats treated with dapagliflozin from 5 weeks of age and (H) overnight fasted blood ² -hydroxybutyrate levels. Data are presented as mean ± SEM (n=4-6 *P<0.05 vs control, †P<0.05 vs PEPCK transgenic).

Figure 4:

(A) Glucose infusion rate, (B) blood glucose levels and (C) plasma insulin levels during the course of the hyperglycemic clamp in 20 week-old control, PEPCK transgenic and PEPCK transgenic rats treated with dapagliflozin for 6 weeks duration. (D) Representative insulin stained pancreatic sections of 20 week-old control, PEPCK transgenic and PEPCK transgenic rats treated with dapagliflozin for 6 weeks duration. The progression of (E) the average islet area and (F) islet number per mm^2 pancreas area from 14 weeks to 20 weeks of age with or without dapagliflozin administration. (G) ²-cell mass in 20 week-old control, PEPCK transgenic and PEPCK transgenic rats treated with dapagliflozin for 6 weeks duration. Both average islet area and islet number per mm^2 pancreas area were calculated in 14 week-old control and PEPCK transgenic rats from the intervention study and used as a baseline. Data are presented as mean ± SEM (n=4-6 *P<0.05 vs control, †P<0.05 vs PEPCK transgenic).

Figure 5:

(A) Glucose infusion rate, (B) blood glucose levels and (C) plasma insulin levels during the course of the hyperglycemic clamp in 14 week-old control, PEPCK transgenic and PEPCK transgenic rats treated with dapagliflozin for 9 weeks duration. (D) Representative insulin stained pancreatic sections of 14 week-old control, PEPCK transgenic and PEPCK transgenic rats treated with dapagliflozin for 9 weeks duration. The progression of (E) the average islet area and (F) islet number per mm^2 pancreas area from 5 weeks to 14 weeks of age with or without dapagliflozin administration. (G) ²-cell mass in 14 week-old control, PEPCK transgenic and PEPCK transgenic rats treated with dapagliflozin for 9 weeks duration. Both average islet area and islet number per mm^2 pancreas area were calculated from a subset of 5 week-old control and PEPCK transgenic rats and used as a baseline. Data are presented as mean ± SEM (n=4-6 *P<0.05 vs control, †P<0.05 vs PEPCK transgenic).

Table 1: Plasma glucose and insulin levels under basal and hyperinsulinemic conditions in 20 week-old control, PEPCK transgenic and PEPCK transgenic rats treated with dapagliflozin for 6 weeks duration.

	Control	**PEPCK transgenic**	**PEPCK transgenic + dapagliflozin**
n	4	6	6
Basal			
Plasma glucose (mmol/L)	8.0 ± 0.2	7.5 ± 0.5	6.2 ± 0.4*
Plasma insulin (ng/mL)	3.0 ± 0.2	4.6 ± 0.9*	1.3 ± 0.2*†
Clamp			
Plasma glucose (mmol/L)	8.5 ± 0.2	8.0 ± 0.2	7.9 ± 0.2
Plasma insulin (ng/mL)	5.9 ± 0.9	4.6 ± 0.3	5.2 ± 0.3

Data are presented as mean ± SEM for the indicated number of rats (*P<0.05 vs control, †P<0.05 vs PEPCK transgenic).

Author/s:
Joannides, CN;Mangiafico, SP;Waters, MF;Lamont, BJ;Andrikopoulos, S

Title:
Dapagliflozin improves insulin resistance and glucose intolerance in a novel transgenic rat model of chronic glucose overproduction and glucose toxicity

Date:
2017-08

Citation:
Joannides, C. N., Mangiafico, S. P., Waters, M. F., Lamont, B. J. & Andrikopoulos, S. (2017). Dapagliflozin improves insulin resistance and glucose intolerance in a novel transgenic rat model of chronic glucose overproduction and glucose toxicity. DIABETES OBESITY & METABOLISM, 19 (8), pp.1135-1146. https://doi.org/10.1111/dom.12923.

Persistent Link:
http://hdl.handle.net/11343/292753