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Dapagliflozin restores insulin and growth hormone secretion in obese mice

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Abstract

The well-documented hormonal disturbance in a general obese population is characterised by an increase in insulin secretion and a decrease in growth hormone (GH) secretion. Such hormonal disturbance promotes an increase in fat mass, which deteriorates obesity and accelerates the development of insulin resistance and type 2 diabetes. While the pathological consequence is alarming, the pharmaceutical approach attempting to correct such hormonal disturbance remains limited. By applying an emerging anti-diabetic drug, the sodium-glucose cotransporter 2 inhibitor, dapagliflozin (1 mg/kg/day for 10 weeks), to a hyperphagic obese mouse model, we observed a significant improvement in insulin and GH secretion as early as 4 weeks after the initiation of the treatment. Restoration of pathological disturbance of insulin and GH secretion reduced fat accumulation and preserved lean body mass in the obese animal model. Such phenotypic improvement followed with concurrent improvements in glucose and lipid metabolism, insulin sensitivity, as well as the expression of metabolic genes that were regulated by insulin and GH. In conclusion, 10 weeks of treatment with dapagliflozin effectively reduces hyperinsulinemia and restores pulsatile GH secretion in the hyperphagic obese mice with considerable improvement in lipid and glucose metabolism. Promising outcomes from this study may provide insights into drug intervention to correct hormonal disturbance in obesity to delay the diabetes progression.

Key Words

- ▶ SGLT2 inhibitor
- ▶ growth hormone
- ▶ hyperinsulinemia
- ▶ obesity

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Introduction

Hormonal disturbance, in either secretion amount or function, often occurs in parallel with impairment of glucose/lipid/protein metabolism in obesity. Two pivotal hormones, insulin and growth hormone (GH), which synergistically promote protein anabolism whereas exhibit

antagonistic effect on lipid and glucose metabolism, play pivotal roles in the development of obesity (Berryman *et al.* 2013, Templeman *et al.* 2017). Insulin is secreted from pancreatic beta cells to promote glucose uptake and utilisation as well as lipogenesis (Templeman *et al.* 2017).

Excessive insulin secretion (hyperinsulinemia), as often seen in overeating and obesity, leads to desensitisation of insulin receptor and its downstream signalling (Shanik *et al.* 2008), adipose tissue inflammation (Pedersen *et al.* 2015), ectopic lipid accumulation in liver and muscle (Morita *et al.* 2017), and the development of insulin resistance (Samuel & Shulman 2016). Opposite to insulin, GH exhibits a catabolic effect on lipid metabolism by favouring lipolysis and lipid oxidation (Chia 2014). Secreted from somatotrophs within the anterior pituitary gland, GH displays a pulsatile secretion pattern, with low plasma concentration at baseline and large peaks in rhythmic intervals in all mammals (Tannenbaum *et al.* 1976, Winer *et al.* 1990). This specific secretion pattern is crucial for its effect on lipid metabolism as only pulsatile GH augments the rate of lipolysis in obese humans (Surya *et al.* 2009). Dynamic analysis revealed a reduction of both GH secretion rate and burst frequency in obese individuals in proportion to the severity of obesity (Veldhuis *et al.* 1991). Such impairment of GH secretion further impairs fat utilisation.

Therefore, the hormonal disturbance (high insulin and low GH) in obese individuals concurrently drives fuel metabolism towards fat accumulation, which aggravates morbid obesity and may further accelerate insulin resistance and the development of type 2 diabetes (T2D). Restoration of physiological levels of insulin and GH in obesity is of particular importance to maintain healthy body composition and insulin sensitivity, as well as lipid and glucose metabolism.

Sodium-glucose cotransporter 2 inhibitor (SGLT2i) is a new class of drug for overt T2D patients in the clinic, which lowers blood glucose levels by promoting urinary glucose excretion. However, SGLT2i has still not been used for obese patients without T2D in the clinic. Part of the reason is that the general prerequisite for prescription of SGLT2i is hyperglycaemia, whereas the blood glucose level is only slightly elevated in obese individuals due to the compensation of insulin hypersecretion. However, hyperinsulinaemia in obesity leads to on-going hormonal and metabolic disturbances (Miller & Chapman 2001). Although SGLT2i has been well documented in the improvement of insulin sensitivity in overt T2D patients (Merovci *et al.* 2016) and obese animals (Nakano *et al.* 2015, Benetti *et al.* 2016, Obata *et al.* 2016, Ji *et al.* 2017, Xu *et al.* 2017), its effect on obesity-related hormonal disturbances, in particular, on pulsatile GH secretion remains undefined. Addressing such a knowledge gap is essential to elucidate some of the untangled observations in previous studies. For example, preserved lean mass

following SGLT2i treatment (Obata *et al.* 2016, Morino *et al.* 2018) cannot be explained by a reduction of insulin levels but may be rationalised by the protein-preserving effect of GH. Furthermore, a close evaluation of insulin and GH in obesity may further provide mechanistic insights into potential anti-obesity targets in the modulation of metabolism and body composition. Therefore, the main aim of this study is to determine the effects of dapagliflozin, one type of SGLT2i, on two dominant metabolic-regulating hormones (insulin and GH) with the corresponding glucose/lipid/protein metabolism in hyperphagic obese melanocortin 4 receptor-knockout (MC4RKO) mice.

Materials and methods

Animal

Both MC4RKO and WT mice on C57BL6/J background were obtained from heterozygous breeding pairs and genotyped using established MC4R primer pairs (Tan *et al.* 2016). Mice were housed under a 12-h light/12-h dark cycle in $22 \pm 2^\circ\text{C}$ and $35 \pm 4\%$ humidity environment at the animal facility of the Institute for Bioengineering and Nanotechnology, the University of Queensland. Mice had free access to chow diet (Specialty Feeds, Glen Forrest, WA, Australia) and water. All experiments and procedures were approved by the Animal Ethics Committee of the University of Queensland.

Experiment design

To study the chronic effect, 10-week-old MC4RKO mice (both genders) were treated with dapagliflozin (AstraZeneca, Göteborg, Sweden) dissolved in drinking water (1 mg/kg/day) for 10 weeks. Age-matched WT and MC4RKO mice with normal drinking water were used as controls. Body weight was measured weekly. Serial blood collection for GH measurement, glucose tolerance test and insulin tolerance test and indirect calorimeter recording were performed at 4, 6, 8 weeks following the initiation of the treatment, respectively. Twenty-four-hour urine samples were individually collected via a metabolic cage. At the end of the treatment, mice were killed following an intraperitoneal injection of sodium pentobarbital (32.5 mg/mL) under fed state. Terminal blood was collected by cardiac puncture and plasma was separated by centrifugation. Tissues were either snap-frozen for gene expression measurement or fixed in 4% paraformaldehyde

for immunohistochemistry staining. To study insulin sensitivity, mice receiving 2-week treatment were fasted for 6 h and intraperitoneally injected with insulin (2 U/kg, Insulin solution human, Sigma). Five minutes later, mice were killed, and tissues were collected for insulin-stimulated Akt phosphorylation measurement.

Indirect calorimetric assays

Mice were individually housed in the recording chambers of TSE PhenoMaster (TSE Systems, Germany) with free access to food and water for 3 days. Food intake, water intake and locomotor activity were measured in the whole recording period; respiratory exchange ratio (RER) and oxygen consumption were measured hourly. Body fat mass and lean mass were determined by a NMR device (Bruker, USA).

Glucose tolerance test (GTT) and insulin tolerance test (ITT)

Mice were fasted for 14 h and 4 h before GTT and ITT, respectively. During the experiments, blood glucose levels were measured before (time point 0) oral gavage of glucose (2 g/kg) for GTT or intraperitoneal injection of human insulin (0.3 U/kg, Insulin solution human, Sigma) for ITT. The blood glucose levels were measured by a Glucose Ketone metre (Nova Stat Strip Xpress Glucose Hospital Meter, Nova Biomedical, UK) at specific time points after oral gavage or injection.

Serial blood collection for GH measurement

Blood samples were collected for pulsatile GH secretion profile measurement according to the established method (Steyn *et al.* 2011). Briefly, all mice were trained for 4 weeks before the experiment. The procedure began at 7:30 h and ended at 13:30 h. During the procedure, 2 µL of whole blood was collected from the tail tip of each mouse in a 10-min interval. For each sample, 2 µL of whole blood was added into 58 µL 0.01 M PBS supplemented with 0.05% Tween 20. Samples were mixed by vortex, snap-frozen by dry ice and stored at -80°C for measurement of GH by an in-house GH ELISA (Steyn *et al.* 2011). Due to the influence of the oestrous cycle on the female mice, GH measurement was performed only in male mice.

Hormones and metabolites measurement

Glucagon and resistin levels in the terminal plasma were measured by the Mouse Metabolic Hormone Magnetic

Bead Panel (Millipore). Plasma insulin level was measured by Rat/Mouse Insulin ELISA Kit (Millipore). Plasma FFA and ketone body levels were measured by NEFA C kit (Wako) and Glucose Ketone Meter, respectively. The triglyceride content in liver and muscle was measured as described previously (Mosa *et al.* 2017b). The glucose concentration of the urine sample was determined by Glucose Colorimetric Assay Kit (Cayman Chemical). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated by the equation: $\text{HOMA-IR} = \text{fasting blood glucose (mmol/L)} \times \text{fasting plasma insulin (mU/L)} / 22.5$.

Pancreas immunohistochemistry

According to the established methods (Mosa *et al.* 2017a), sections of paraffin-embedded fixed pancreas (7 µm) were incubated with a mixture of primary antibodies, including mouse anti-insulin (Merck) and rabbit anti-glucagon (Thermo Fisher Scientific) overnight, and then incubated with Alexa Fluor® 488-conjugated and 555-conjugated secondary antibodies (Life Technology). Cell nucleus was stained by Hoechst 33342 (Thermo Fisher). Stained slides were scanned by Nikon Upright Stereology and Slide Scanning Microscope (Nikon), and morphometric measurement was performed using Imaris Image Analysis Software. The mass of alpha or beta cells was calculated by multiplying the percentage of glucagon or insulin-positive area in each section and the weight of the pancreas. In one animal, three non-consecutive pancreas sections and 20–40 islets were measured. All parameters of the microscope and settings were kept constant throughout the whole experiment.

Gene expression measurement

The total RNA from the liver, muscle, inguinal and epididymal adipose tissues was extracted and purified by PureLink RNA Mini Kit (Thermo Fisher Scientific). 1 µg RNA was synthesised into cDNA by iScript™ RT Supermix for RT-qPCR (Bio-Rad). For quantitative PCR (qPCR), cDNA was amplified using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) and quantified by QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific). Primer pairs were shown in Supplementary data (Supplementary Table 1, see section on [supplementary materials](#) given at the end of this article). The fold change of target gene expression compared to the housekeeping gene (beta-actin) was achieved by the $2^{-\Delta\Delta\text{CT}}$ method.

Western blot

Mouse livers were homogenised in buffer containing Protease Inhibitor Cocktail (5892953001, Roche) and phosphatase inhibitor (1 mM Na₃VO₄, 30 mM NaF, 10 mM Na₄P₂O₇), heat-denatured, separated in 8% SDS-PAGE, and incubated with the following primary antibodies: anti-pSTAT5 (Tyr694, #9351, Cell Signalling Technology), anti-STAT5 (#94205, Cell Signalling Technology), anti-pAkt (Ser473, #4060, Cell Signalling Technology), anti-Akt (#4685, Cell Signalling Technology), and anti- α -tubulin (ab4074, Abcam). Immunoblots were detected by Odyssey LI-COR CLx Imaging System and quantified using NIH ImageJ software.

Statistics

Statistical analysis was performed using GraphPad Prism 7 software (excluding deconvolution analysis and analysis of covariance (ANCOVA) for energy expenditure). All results were presented as the mean $\pm$ S.E.M. One-way ANOVA and Student's *t*-test were used for comparisons among groups. A *P* value less than 0.05 was considered significant. The quantitative parameters of GH secretion and clearance associated with observed GH concentration profiles were determined by deconvolution analysis following established parameters (Steyn *et al.* 2011). Energy expenditure was analysed by ANCOVA following an established method (Speakman *et al.* 2013) in the MC4RKO mice with or without drug intervention. We excluded wild-type mice in ANCOVA analysis as the genotypic effect was not the observation target. Exclusion criteria also applied to wild-type mice due to the significant body weight and lean mass difference compared to MC4RKO mice as detailed in discussion.

Results

Dapagliflozin promotes urinary glucose excretion and improves glucose tolerance in MC4RKO mice

Reminiscent of glucose homeostasis in human obesity, MC4RKO mice had slightly increased fasting (4.59 ± 0.24 vs 3.89 ± 0.15 mmol/L) and random blood glucose (8.00 ± 0.64 vs 6.72 ± 0.21 mmol/L) (Fig. 1A), as well as slightly impaired glucose tolerance (Fig. 1C and D), compared to WT mice. Dapagliflozin treatment significantly increased daily urinary glucose excretion (Fig. 1B) and water intake

Figure 1

Dapagliflozin promotes urinary glucose excretion and improves glucose tolerance in MC4RKO mice (A), blood glucose level in fasting and random conditions. (B) Daily urinary glucose excretion. (C and D) Glucose tolerance test (GTT) and the area under the curve. Data were presented as means $\pm$ S.E.M., *n* = 6–8 each group for daily glucose excretion, *n* = 12–15 for blood glucose and GTT. #*P* < 0.05, ##*P* < 0.01 vs WT group; **P* < 0.05, ***P* < 0.01, *****P* < 0.0001 vs MC4RKO group.

(Supplementary Fig. 1A), normalised random blood glucose level (Fig. 1A) and improved glucose tolerance (Fig. 1C and D) in MC4RKO mice.

Dapagliflozin corrects the disturbed insulin and GH levels in MC4RKO mice

Generally, fasting insulin reflects basal insulin secretion, while fed insulin reflects beta cell function. Therefore, we measured plasma insulin level in both fasting and fed conditions. Pulsatile GH secretion changes significantly upon physiological challenges such as fasting (Ho *et al.* 1988, Steyn *et al.* 2012). To better clarify the effect of SGLT2i without physiological disturbance, we measured the GH secretion profile under fed conditions. MC4RKO mice had a 4-fold increase in fasting and a 20-fold increase in fed insulin levels than those in WT mice. Dapagliflozin treatment reduced approximately 70% of both fasting and fed insulin levels in MC4RKO mice (Fig. 2A and B). The pulsatile and basal GH secretion was suppressed in MC4RKO mice (Fig. 2C, D, E, F and G), reflecting an impairment of GH secretion similar to the diet-induced obese mice that we previously reported (Huang *et al.* 2012). Following 4 weeks of dapagliflozin treatment, the amount of pulsatile GH secretion (Fig. 2E and F) in MC4RKO mice was increased by around three-fold of untreated levels with an increasing trend in basal GH secretion (Fig. 2G). The number of secretory

Figure 2

Dapagliflozin reduces hyperinsulinemia and restores pulsatile GH secretion in MC4RKO mice (A), fasting insulin level. (B) Fed insulin level. (C) Representative examples of GH level (left) and the onset of GH secretion pulse (right) in each group during 6-h measurement period. (D, E, F, G and H) Deconvolution analysis revealed the changes of total GH secretion (D), pulsatile GH secretion (E), mass of GH secretion per burst (F), basal GH secretion (G), and the number of secretory burst per 6 h (H). (I) Gene expression levels of *Igf1* in liver. (J) STAT5 phosphorylation in liver determined by Western blot. Tubulin served as a loading control. Data were presented as means $\pm$ S.E.M., $n = 6-8$ each group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$; ns, non-significance.

bursts per 6 h among WT, MC4RKO and dapagliflozin treatment group remained unchanged (Fig. 2H). STAT5 phosphorylation and insulin-like growth factor 1 (IGF-1) are often used as biomarkers for the downstream signalling of the GH receptor in the liver. Fully restored IGF-1 gene expression and STAT5 phosphorylation in the liver of MC4RKO mice treated by dapagliflozin (Fig. 2I and J) confirmed the effect of restored pulsatile GH secretion. Muscle *Igf1* expression in MC4RKO was also fully restored by dapagliflozin (Supplementary Fig. 2C), although plasma IGF-1 levels remain comparable among all groups (Supplementary Fig. 3C).

Dapagliflozin prevents beta cell overgrowth and improves insulin sensitivity in MC4RKO mice

To further address the plausible explanation for reduced hyperinsulinaemia, we assessed islet morphology and whole-body insulin sensitivity. MC4RKO mice had a 4-fold increase in beta cell mass and a 3-fold increase in the ratio of beta/alpha cell mass compared to that in WT mice. Dapagliflozin treatment reduced both parameters by approximately 60 and 40%, respectively (Fig. 3A, B and D). Dapagliflozin treatment did not change alpha cell mass or plasma glucagon level (Fig. 3C and Supplementary Fig. 3B). Impaired Insulin sensitivity in MC4RKO mice was significantly improved following dapagliflozin treatment, as demonstrated by ITT (Fig. 3E and Supplementary Fig. 1E) and

HOMA-IR (Fig. 3F). At the cellular level, insulin-stimulated Akt phosphorylation in both white adipose tissue (WAT) and liver was significantly increased within 2 weeks of dapagliflozin treatment (Fig. 3G and H).

Dapagliflozin promotes negative energy balance, reduces fat mass and increases lipid oxidation in MC4RKO mice

To determine the phenotypic changes following the dapagliflozin treatment, body composition and indirect calorimeter were measured in these mice. With a deficit in satiety signalling (Huszar *et al.* 1997), MC4RKO mice had significantly increased food intake compared to WT mice, and this was not affected by the dapagliflozin treatment (Fig. 4A). Compare to WT mice, the body weight gain of MC4RKO mice was significantly greater and was attenuated by the dapagliflozin treatment (Fig. 4B and C). NMR measurement showed that MC4RKO mice had an approximately 4-fold increase in fat mass and a 30% increase in lean mass compared to WT mice (Fig. 4D and E). Dapagliflozin treatment reduced around 50% of fat mass in MC4RKO mice without changing lean mass (Fig. 4D and E). The decreased weight of different adipose depots (Supplementary Fig. 1B) further confirmed the reduction of fat mass following dapagliflozin treatment. Since increased ectopic lipid accumulation is associated with insulin resistance (Boren *et al.* 2013), the triglyceride content was assessed in both liver and muscle.

Figure 3

Dapagliflozin prevents beta cell overgrowth and improves insulin sensitivity in MC4RKO mice (A), representative images of pancreas sections, stained by glucagon and insulin antibody. Scale bars: 100 μ m. (B, C and D) Quantitative analysis of beta cell mass (B), alpha cell mass (C) and the ratio of beta cell to alpha cell mass (D) were performed by Imaris Image Analysis Software. (E) Insulin tolerance test (ITT). (F) The homeostatic model assessment for insulin resistance (HOMA-IR). (G and H) Akt phosphorylation in WAT (G) and liver (H) following insulin injection in mice fasted for 6 h. Tubulin served as a loading control. Data were presented as means $\pm$ s.e.m. For Immunohistochemistry, $n = 3$ mice each group, and 20–40 islets per mouse were measured. For ITT, $n = 6$ –8 each group. ### $P < 0.001$, #### $P < 0.0001$ vs WT group; * $P < 0.05$, ** $P < 0.01$ vs MC4RKO group, ns, non-significance.

In MC4RKO mice, ectopic lipid accumulation increased in both liver and muscle, which was ameliorated by dapagliflozin treatment (Fig. 4F and G). Results from indirect calorimeter showed that MC4RKO mice had elevated respiratory exchange ratio (RER) in both light and dark phase, reflecting a dominant utilisation of carbohydrates over fats. The RER was reduced by dapagliflozin treatment (Fig. 4H and I). Together with an increase in plasma level of ketone body (Supplementary Fig. 3D), results suggested that dapagliflozin drove fuel utilisation towards fat. The decreased locomotor activity in MC4RKO mice was not changed by dapagliflozin treatment (Supplementary Fig. 1C and D). To determine whether changes in energy expenditure following dapagliflozin treatment in MC4RKO cohorts is a pure effect from the drug, we analysed the data with ANCOVA using lean mass as a covariate. There was a significant treatment effect ($F = 19.379$, $P = 0.002$) without a significant covariate effect ($F = 0.187$, $P = 0.677$) nor interaction effect ($F = 0.096$, $P = 0.766$). These results indicated that reduced energy expenditure of MC4RKO mice in the dapagliflozin treatment group was largely due to the drug effect with little impact from lean mass change (Fig. 4J and K).

Dapagliflozin increases expression of genes involved in lipid oxidation, lipolysis, WAT browning, and decreases expression of genes involved in inflammation

Obesity is often accompanied by the disturbance of gene expression involved in lipid metabolism and inflammation. To determine the effect of dapagliflozin at the molecular level, we analysed relevant genes in liver (Fig. 5A), s.c. WAT (Fig. 5B) and visceral WAT (Fig. 5C). MC4RKO mice had decreased expression of genes involved in lipid oxidation in the liver (*Ppara* and *Cpt1a*) and lipolysis in WAT (*Hsl*), and dapagliflozin treatment corrected those alterations (Fig. 5A, B and C). The lipogenic gene expression levels were increased in the liver (*Srebf1*) and decreased in visceral WAT (*Fasn* and *Ppar γ*) of MC4RKO mice, and dapagliflozin treatment partially normalised those alterations (Fig. 5A and Supplementary Fig. 2A, B). Dapagliflozin treatment increased the expression level of WAT browning markers (*Ppar γ* , *Pgc1 α* , *Cidea*, *Eva1* and *Tmem26*) in MC4RKO mice although there was no change in *Ucp1* expression level (Fig. 5B). The dissociation between the control of *Ucp1* and that of *Pgc1 α* may be attributed to an impairment of

Figure 4

Dapagliflozin promotes negative energy balance, reduces fat mass and increases lipid oxidation in MC4RKO mice (A), daily food intake. (B) Body weight gain through the experiment. (C, D and E) At the terminal of the experiment, total body weight (C), fat mass (D) and lean mass (E) were measured. (F and G) Triglyceride content in liver (F) and muscle (G). (H and J) Respiratory exchange ratio (RER) (H) and oxygen consumption (J) were measured during dark phase (18:00 h to 6:00 h next day) marked by shaded area and light phase (6:00 h to 18:00 h) in half-hour intervals. (I) The average RER in both light and dark phase. (K) Scatter plots from ANCOVA showing raw oxygen consumption values plotted against lean body mass of MC4RKO and MC4RKO + SGLT2i mice. Data were presented as means ± s.e.m., n = 6–8 each group. ####P < 0.0001 vs WT group; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 vs MC4RKO group.

Figure 5

Dapagliflozin increases expression of genes involved in lipid oxidation, lipolysis, WAT browning, and decreases expression of genes involved in inflammation. (A, B and C) Gene expression fold change was measured by qPCR, in liver (A), subcutaneous (B) and visceral (C) white adipose tissue (WAT). Data were presented as means ± s.e.m., n = 6–8 each group. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

β -adrenergic pathway in MC4R KO mice as melanocortin system modulates sympathetic outflow (Silva *et al.* 2014) and lack of β -adrenergic pathway dissociates the control of *Ucp1* from that of *Pgc1 α* in cultured β -less mouse brown adipocytes (Lehr *et al.* 2006). In addition, the increased inflammation markers (*Tnf α* and *Ccl2*) in both liver and WAT in MC4RKO mice were reduced by dapagliflozin treatment (Fig. 5A and C) with a concurrent reduction of circulation inflammation marker resistin (Supplementary Fig. 3A).

Discussion

A precise balance between insulin and GH not only regulates glucose/lipid/protein metabolism in a homeostatic state but also plays an essential role in maintaining normal body composition. This study sought to define the metabolic effects of SGLT2i in hyperphagic obese mice and focus on the dominant endocrine elements: insulin and GH. Although there are a few studies investigating the effect of SGLT2i in obese animal models (Nakano *et al.* 2015, Benetti *et al.* 2016, Obata *et al.* 2016, Ji *et al.* 2017, Xu *et al.* 2017), none of them has reported the dynamic changes of GH following the treatment. Missing this key endocrine element may underestimate the pivotal effect of GH and its interaction with insulin on glucose/lipid/protein metabolism. In this study, we showed that dapagliflozin reduced hyperinsulinaemia and restored pulsatile GH secretion in obese MC4RKO mice. The substrate metabolism switched from carbohydrates to fats following the restoration of insulin and GH secretion. These changes led to decreased fat mass, improved insulin sensitivity and glucose tolerance, as well as preserved lean mass and delayed T2D progression. Changes of insulin, GH and substrate metabolism upon SGLT2i treatment in obese MC4RKO mice are reminiscent of fasting conditions in humans (Ho *et al.* 1988), indicating a physiological benefit (Patterson *et al.* 2015) from a pharmaceutical intervention in obesity.

The mechanisms of SGLT2i in regulating insulin secretion in T2D is mainly due to the removal of excess glucose in the blood and reducing the secretion pressure on beta cells (Kaneto *et al.* 2017). In our study, the slightly elevated random blood glucose level of MC4RKO mice was normalised following dapagliflozin treatment with a concomitant reduction in beta cell mass (Fig. 3). Decreased beta cell mass led to the reduction of hyperinsulinaemia, which improves whole-body insulin sensitivity (Shanik *et al.* 2008, Ning *et al.* 2011, Pedersen *et al.* 2015), and vice versa. Unlike previous studies

on overt T2D animal models that show an increase in beta cell mass after SGLT2i treatment (Kimura *et al.* 2018), our results showed a decrease in beta cell mass in the treated mice. This contradictory results may be due to the different exposure durations of beta cells to glucotoxicity and lipotoxicity, with beta cells undergoing hyperplasia and hypertrophy (compensation) in obesity (Cerf 2013) whereas experiencing apoptosis (decompensation) in overt diabetes (Wajchenberg 2007).

Although it is well documented that dapagliflozin treatment raises plasma glucagon levels in most human and rodent study (Ferrannini *et al.* 2014, Merovci *et al.* 2014, Bonner *et al.* 2015, Timper *et al.* 2016, Millar *et al.* 2017, Saponaro *et al.* 2019), we did not observe changes in either circulating levels of glucagon or pancreatic glucagon positive cells following dapagliflozin treatment in MC4RKO mice. Such deviation may result from the loss of MC4R signalling as a recent study in MC4R-deficient mice demonstrates that MC4R is required for stimulation of glucagon release in response to hypoglycaemia or glucopaenia (Tooke *et al.* 2019). Interestingly, the expression levels of genes involved in gluconeogenesis (Fig. 5A) were upregulated following dapagliflozin treatment. Such changes may be less a matter of glucagon's effect but more likely to be a consequence of reduced hyperinsulinaemia or increased GH.

In terms of changes in GH, the mechanisms underlying reduced GH secretion in obesity are not fully understood but may involve perturbations in GH-releasing hormone (GHRH) and somatostatin tone, as well as increased feedback inhibition by FFA and insulin (Maccario *et al.* 2000). It is known that hyperinsulinaemia inhibits GH secretion from pituitary gland via insulin receptor both *in vivo* and *in vitro*, regardless of systemic insulin resistance (Luque & Kineman 2006, Gahete *et al.* 2013). Therefore, restored pulsatile GH secretion after dapagliflozin treatment is likely to be a consequence of the reduced circulating insulin level. Detailed analysis of GH patterning further demonstrated that dapagliflozin treatment increased the amount of pulsatile GH secretion but did not change the pulse frequency (Fig. 2), indicating a restoration of the deficit in GH release without affecting the hypothalamic pulse generation by dapagliflozin. Although the direct effects of dapagliflozin on pituitary cells or GH regulatory neurons should also be considered, no *Sglt2* mRNA expression was detected in mouse pituitary gland; neither in arcuate and periventricular nuclei of the hypothalamus, where GHRH/somatostatin neuron are located (Supplementary Fig. 2D and E). Thus, it is unlikely that SGLT2 has a direct effect on pituitary cells or hypothalamic

GHRH/somatostatin neurons. The null effect of dapagliflozin on GHRH/somatostatin neurons is further supported by the unaltered GH secretion pattern following treatment.

The restoration of insulin and GH levels may play a vital role in the metabolic effects of SGLT2i in obesity. Hyperinsulinaemia causes increased lipid synthesis and inflammation in WAT, liver and muscle (Xu *et al.* 2003, Shanik *et al.* 2008, Pedersen *et al.* 2015, Samuel & Shulman 2016, Morita *et al.* 2017). Amelioration of hyperinsulinaemia reduces lipid accumulation and improves insulin sensitivity by decreasing the expression level of genes regulating lipogenesis (Ning *et al.* 2011, D'Souza A *et al.* 2016) and inflammation (Mehran *et al.* 2012, Pedersen *et al.* 2015) *in vivo*. In consensus with previous studies of SGLT2i in obese animal models (Nakano *et al.* 2015, Benetti *et al.* 2016, Ji *et al.* 2017, Xu *et al.* 2017), SGLT2i in this report reduced lipid content and inflammation in WAT, liver and muscle (Fig. 4F and G). These changes led to improved insulin sensitivity as measured by ITT, HOMA-IR and insulin-stimulated Akt phosphorylation (Fig. 3). GH, on the other hand, induces lipolysis in WAT and lipid oxidation in the liver (Chia 2014). Low GH secretion (Luque *et al.* 2011) or GH receptor knockout (Cordoba-Chacon *et al.* 2018) leads to fat accumulation in mouse models. By contrast, GH injection in obese mouse results in a reduction of fat body mass (List *et al.* 2009). These pieces of evidence support a catabolic effect of GH on fat metabolism. It should be noted that exogenous GH injection may cause insulin resistance (Berryman *et al.* 2013) due to GH-induced increase in blood glucose and FFA flux. GH injection also causes desensitisation of GH receptor signalling pathways (Ji *et al.* 2002). Besides, exogenous GH injection does not mimic physiological pulsatile GH secretion pattern, which may exert distinct metabolic effects. In line with the previous study in mice (Obata *et al.* 2016), our study showed that dapagliflozin treatment increased lipolysis in WAT and lipid oxidation in the liver of MC4RKO mice. More importantly, dapagliflozin treatment restored physiological pulsatile GH secretion without changing long-term plasma FFA level (Supplementary Fig. 3E). Therefore, both insulin sensitivity and GH receptor signalling were improved by dapagliflozin treatment in obese MC4RKO mice. No change in circulating total IGF-1 was observed in the SGLT2i treatment group (Supplementary Fig. 3C), which is consistent with obese people in general (Nam *et al.* 1997, Lewitt *et al.* 2014). However, increased *Igf1* mRNA expression was observed in muscle following the recovery of pulsatile GH (Supplementary Fig. 2C) and may exert protein preservation

effects on lean mass through autocrine/paracrine manner (Philippou *et al.* 2007). It is worth to point out that discrete components of the GH secretory pattern may differentially affect IGF-I generation. For example, pulsatile GH is more effective than continuous GH infusion in increasing IGF-I mRNA levels in skeletal muscle in rodents (Isgaard *et al.* 1988). This may explain increased levels of *Igf1* mRNA expression in muscle in our study as we show an increase in pulsatile GH secretion following dapagliflozin treatment.

Studies show controversial results in energy metabolism changed by SGLT2i, especially in energy expenditure. A recent review suggests that the energy loss caused by SGLT2 inhibition may trigger compensatory hyperphagia in both human and rodents in most circumstances (Rajeev *et al.* 2016). Although MC4RKO mice with dapagliflozin treatment showed no change in food intake compared to the vehicle group, they had reduced body weight. This indicates an unparalleled change between body weight loss and the anticipated reduction in food intake. Therefore, compensatory hyperphagia may exist in dapagliflozin treated MC4RKO mice. Different to food intake, various changes in energy expenditure are found in previous animal studies, with increase (Naznin *et al.* 2017, Xu *et al.* 2017), decrease (Chiba *et al.* 2016, Kern *et al.* 2016) or no change (Devenny *et al.* 2012, Yokono *et al.* 2014) reported after SGLT2i treatments. The controversial results are possibly caused by differences in experimental designs as some studies used pair-fed animals (Devenny *et al.* 2012, Xu *et al.* 2017) while others used *ad libitum* feeding (Yokono *et al.* 2014, Chiba *et al.* 2016, Kern *et al.* 2016, Naznin *et al.* 2017). Alternatively, the method used to normalise and interpret the energy expenditure data may contribute to the different outcomes. According to a recent review (Kleinert *et al.* 2018), most approaches previously used to interpret energy expenditure are based on a direct comparison of uncorrected energy expenditure per individual or with correction by body weight. None of them takes body composition into account. This may cause misinterpretation of the data, given that energy expenditure is dependent on both lean (muscle) and fat (brown adipose tissue) body mass (Kleinert *et al.* 2018). Therefore, analysis of covariance (ANCOVA), which uses body weight and body composition as covariates, is more appropriate to analyse energy expenditure (Kleinert *et al.* 2018). To use ANCOVA, the data set should meet two assumptions: (1) independence of the covariate and treatment effect and (2) homogeneity of regression slopes (Miller & Chapman 2001). By pre-analysing body weight, fat mass and lean mass as the covariate respectively,

we confirmed that lean mass was independent of the treatment (assumption 1). We also confirmed that the slopes of linear trend lines for the two groups were similar (Fig. 4K) (assumption 2). By meeting with both criterion of ANCOVA, our analysis showed that dapagliflozin treatment reduced energy expenditure in MC4RKO mice. Since food intake was not changed by dapagliflozin in our study, reduced energy expenditure was more likely due to an alternative compensatory mechanism to combat negative energy balance caused by dapagliflozin. However, with a significant reduction of body weight in the long term, the net metabolic effect of dapagliflozin remained going towards negative energy balance.

In humans, the MC4R gene mutation is the most frequent single-gene cause of obesity, presenting in 6% of severe childhood obesity patients (Yeo *et al.* 1998, Farooqi *et al.* 2003). Dapagliflozin effectively restored insulin and GH secretions, as well as lipid/glucose metabolisms in obese MC4RKO mice, providing significant clinical relevance of such drug in treating obese patients with MC4R gene mutation. Whether SGLT2i has similar effects on general obese patients await further confirmation.

Although the outcomes of this study are promising, there remain a few limitations. For example, the detailed mechanisms by which dapagliflozin regulates GH secretion were not fully explored. Further studies on primary cultured somatotrophs or hypothalamus slices are encouraged. In addition, the current study focuses on the balance of insulin and GH without distinguishing the sole effect of insulin or GH on lipid/glucose metabolism. Although the STAT5 phosphorylation and local *Igf1* expression do provide strong evidence for the direct effects of GH, further study using GH receptor blocker in MC4RKO mice or using transgenic mice with deficient GH receptor signalling may help to isolate the GH's role in glucose/lipid/protein metabolism following SGLT2i treatment.

In conclusion, this study provides evidence to support the promising effect of SGLT2i, dapagliflozin, in the restoration of pathological levels of insulin and GH in obese MC4RKO mice. This may lead to the beneficial effects in shifting substrate utilisation from glucose to lipid, reducing fat mass and improving insulin sensitivity. This study sheds lights on the potential application of SGLT2i in obese patients.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-19-0385>.

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

Z H, L H and C C designed the experiments. Z H, L H, C W, S Z, X Q and Y C conducted the experiments and collected the data. Z H, L H, J D V and Y Z performed data analysis. Z H, L H and C C wrote the manuscript. M C provided MC4RKO mice and valuable advice for the experiment design.

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