The combination of exercise training and sodium-glucose cotransporter-2 inhibition improves glucose tolerance and exercise capacity in a rodent model of type 2 diabetes

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ABSTRACT

Purpose: Exercise is recommended in addition to pharmacotherapies for the management of type 2 diabetes, but metformin and exercise training may have non-additive or even inhibitory effects on exercise-induced improvements in glycemic control and exercise capacity. The objectives of this report were to determine if co-treatment with a sodium-glucose cotransporter-2 inhibitor and exercise could (1) further improve glycemic control when compared to either monotherapy and (2) not worsen exercise capacity when compared to exercise alone.
Methods: A rodent model of type 2 diabetes (30 mg/kg streptozotocin and high-fat feeding in male Sprague-Dawley rats) was used to assess 12 weeks of co-treatment with a sodium-glucose cotransporter 2 inhibitor (SGLT2i) and exercise (EX; treadmill running) on glycemic control and exercise capacity. Animals were randomized to the following conditions (n = 7–10/group): vehicle (0.5% methyl cellulose) sedentary (VEH SED), VEH EX, canagliflozin (3 mg kg^{-1} d^{-1}) SED (SGLT2i SED), or SGLT2i EX.
Results: Both EX and SGLT2i independently improved indices of glycemic control. The combination of SGLT2i and EX further improved glucose tolerance (glucose area under the curve 1109 ± 51 vs 1427 ± 82 mmol/L 120 min^{-1} for SGLT2i EX vs. SGLT2i SED, respectively; p < 0.05) and insulin responses (insulin area under the curve 24,524 ± 4126 vs. 41,208 ± 2714 pmol L^{-1} 120 min^{-1} for SGLT2i EX vs. VEH EX, respectively; p < 0.05) during an oral glucose tolerance test. Only the combination of SGLT2i EX lowered body weight compared to VEH SED (p < 0.01). SGLT2i caused several metabolic adaptations including increased ketone production and a greater reliance on fat as a source of energy during normal cage activity. Interestingly, animals that were given the SGLT2i and underwent exercise training (SGLT2i EX) had better submaximal exercise capacity than EX alone, as indicated by distance run prior to fatigue (882 ± 183 vs.433 ± 33 m for SGLT2i EX and VEH EX, respectively; p < 0.01), and this was accompanied by a greater reliance on fat as an energy source during exercise (p < 0.01).
Conclusions: If these findings with the combination of SGLT2i and exercise translate to humans, they will have important clinical health implications.

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1. Introduction

Exercise training improves glycemia and insulin sensitivity in people with type 2 diabetes [1,2], but these individuals may derive less benefit from and have more difficulty tolerating exercise than healthy individuals [3,4]. Pharmacotherapies that improve glycemic control when combined with exercise could help improve exercise tolerance and produce additive benefits on health outcomes, including glycemic control. Metformin, the first line pharmacotherapy, lowers circulating glucose concentrations, at least in part, by improving hepatic glycemic control [5]. Similar to regular exercise, metformin also upregulates ATP producing pathways including glycolysis, fatty acid oxidation, and

mitochondrial biogenesis [6]. Therefore, it may be hypothesized that combining metformin treatment with exercise training may have additivie benefits on glucose regulation. Despite the positive adaptations with metformin treatment, recent preclinical and human studies demonstrate that co-treatment with exercise training and metformin, the first line pharmacotherapy for type 2 diabetes, may not provide additional benefits over either treatment on circulating lipids [7,8] or metabolic syndrome status [7]. Metformin may even blunt exercise-induced improvements in glycemic control, mitochondrial quality, and aerobic capacity [8–15]. Thus, it is important to understand if alternative pharmacotherapies for type 2 diabetes are permissive or additive to the positive effects of exercise training.

Sodium-glucose cotransporter-2 inhibitors (SGLT2i) are a class of type 2 diabetes medications in which the primary mechanism of action is through increased urinary glucose excretion [16]. SGLT2i treatment in humans decreases body weight [17,18], causes a greater reliance on fat as an energy source [19,20], and improves diastolic function, blood pressure, and cardiovascular outcomes [18,21–23]. Yet, it is unknown if co-treatment with SGLT2i and exercise has more favorable metabolic benefits than metformin with exercise. The objectives of this report were to determine if the combination of SGLT2i and exercise could (1) further improve glycemic control when compared to either monotherapy and (2) not worsen exercise capacity compared to exercise alone. We hypothesized that co-treatment with SGLT2i and exercise would further improve glycemic control but not worsen exercise capacity.

2. Methods

Procedures performed were in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Pfizer Institutional Animal Care and Use Committee. Pfizer animal care facilities are accredited by AAALAC International. Four-week-old male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) were fed a high-fat diet (45% kcal fat; D12451i, Research Diets Inc., New Brunswick, NJ). One week later, rats were injected intraperitoneally with a low-dose of streptozotocin (STZ; 30 mg/kg, Sigma Aldrich, St. Louis, MO) in sodium citrate buffer (50 mM, pH 4.5) after a 5 h fast. This model exhibits insulin resistance, hyperglycemia, and hypertriglyceridemia [24–26]. While high-fat diet alone causes insulin resistance in normal rats it does not result in overt type 2 diabetes, due to pancreatic beta cell reserves. Low-doses of STZ in combination with high-fat diet cause insulin resistance and type 2 diabetes by partially reducing the number of beta-cells, causing more robust hyperglycemia while maintaining some insulin response [24,27,28]. Pharmacotherapies for type 2 diabetes and exercise can independently reduce low-dose STZ-induced hyperglycemia [24,29,30]. A healthy control group (VEH CHOW) was fed a low-fat standard rodent chow (Pico Lab Rodent Diet 5053, Lab Diet, St. Louis, MO, USA) and received a vehicle rather than STZ. At 16 weeks of age, animals within the same cage were randomized using an internet-based program to the following conditions (n = 7–10 animals/group): vehicle-treated and sedentary (VEH SED), vehicle-treated and exercise trained (VEH EX), canagliflozin-treated and sedentary (SGLT2i SED), or canagliflozin-treated and exercise trained (SGLT2i EX) for 12 weeks. Because EX or SGLT2i administration can lower body weight, a group of STZ-injected animals were provided adjusted amounts of high-fat diet to maintain body weights similar to SGLT2i EX (~15% kcal reduction vs. VEH SED; weight matched). No differences in body weight were observed between any of the STZ and high-fat fed groups after randomization. The SGLT2i SED group had significantly higher fed glucose concentrations than all other STZ-injected groups prior to the intervention (data not shown; p < 0.05). Rats were housed two animals/cage in a reverse 12 h dark (0800–2000), 12 h light cycle (22 °C). Weight matched animals were individually housed to control food intake. Body weight and food intake were measured weekly. Following 12 weeks of treatment, animals were fasted 14 h and euthanized with carbon dioxide and exsanguination. Euthanasia occurred ~18 h after the last bout of exercise and ~2 h after the last dose of drug. Weight matched animals were given ~1/3 of their daily food allocation 2 h prior to fasting. No adverse events occurred during the duration of this study.

2.1. SGLT2i administration

Canagliflozin (3 mg kg^{-1}d^{-1} in 0.5% methylcellulose) or VEH (0.5% methylcellulose) were administered once daily by oral gavage between 0600 and 0900.

2.2. Peak aerobic capacity

All rats underwent five treadmill acclimatization sessions (5–10 min, 10–12 m/min, 10% incline). Maximal oxygen consumption (VO$_{2\ peak}$) and maximal running velocity (V$_{max}$) were determined using a graded exercise test and an Oxymax indirect calorimetry system (Columbus Instruments, Columbus, OH), using a protocol similar to those used by others [31–33]. After a three-minute warm-up (10 m/min, 10% incline), treadmill speed was increased to 12 m/min for two minutes, followed by 15 m/min for an additional two minutes. Thereafter, the speed was increased 5 m/min, every two minutes until volitional fatigue (spending three consecutive seconds on the electrical grid). VO$_{2peak}$ was the highest observed oxygen consumption. VO$_{2peak}$ tests were repeated at seven weeks of training in the EX groups, and all groups after the 12-week intervention. All sedentary animals underwent a second treadmill acclimatization prior to testing.

2.3. Exercise training

Treadmill running duration and speed were increased over the first three weeks until the animals ran for 60 min/d, 5d/wk., 10% incline at ~50–55% of V$_{max}$ from the VO$_{2peak}$ test. Running speed was increased following seven weeks of training.

2.4. Substrate utilization during exercise and submaximal running capacity

Substrate utilization and submaximal running endurance were assessed in the exercise-trained animals. These tests were completed only in the exercise-trained animals, due to low- compliance to treadmill exercise in the chronically sedentary animals despite re-acclimatization to the treadmill. Animals ran at 12 m/min and the speed was increased over five minutes until their daily exercise running speed was achieved. This speed was maintained for 15 min to calculate mean respiratory exchange ratio (RER) and VO$_2$ and estimate exercise energy expenditure/session. The speed was increased to 75% of V$_{max}$ and maintained until volitional fatigue (three consecutive seconds on the electrical grid). Total distance run (daily running velocity + 75% V$_{max}$) was recorded.

2.5. 24 h calorimetry and physical activity

RER, energy expenditure (EE), and ambulatory activity were measured for 24 h during the ninth week with an Oxymax indirect calorimetry system and CLAMS monitoring system (Columbus Instruments). Means were calculated for dark and light cycles. Animals were acclimatized to the cages for 18–24 h prior to data collection. VEH or SGLT2i were administered in the last hour of the light cycle and data acquired from the beginning of the dark cycle for 24 h. Animals in the exercise groups completed a bout of exercise ~18 h prior to data collection. Weight matched animals were fed during the last hour of the light cycle prior to data collection. Weekly energy expenditure was extrapolated based on 24 h energy expenditure and exercise energy expenditure (five sessions/wk. of EX).

2.6. Body composition

Body composition was assessed pre-and post- intervention using an EchoMRI 4in1-1100 analyzer (EchoMRI, Houston, TX).

2.7. Oral glucose tolerance test (OGTT)

During the twelfth week of treatment, animals underwent an OGTT. Following a 14 h fast, a 0 min glucometer reading (AlphaTrak 2, Zoetis Inc., Parsippany, NJ) and plasma samples were collected from the tail. Glucose was administered (2 g/kg via oral gavage) and glucometer readings and plasma samples were collected at 30, 60, and 120 min post- glucose administration. VEH/SGLT2i was given 24 h and the last bout of exercise occurred ~46 h prior to testing.

2.8. Plasma analyses

Fasting glucose, total cholesterol, triglyceride, NEFA, lactate, and β hydroxybutyrate were assessed using the Advia Chemistry XPT Clinical analyzer (Siemens Corporation, Washington, D.C.). Insulin (Alpco, Salem, NH), proinsulin (Mercodia, Uppsala, Sweden), glucagon (Mercodia), and lactate (BioVision Inc., Milpitas, CA) were assessed using commercially available assays.

2.9. Urine collection and analyses

Animals were dosed with VEH or SGLT2i and immediately placed into metabolic cages to collect urine samples and 24 h volume. EX animals completed a bout of exercise ~18 h before beginning urine collection. Urine glucose (Wako Diagnostics, Mountain View, CA) and β-hydroxybutyrate concentrations (Cayman Chemicals, Ann Arbor, MI) were determined using commercially available assays.

2.10. Tissue collection and glycogen content

Livers and the gastrocnemius-plantaris muscle complexes were quickly excised and placed in liquid nitrogen. Tissue glycogen content was assessed as previously described [34].

2.11. Western blot analyses

Western blot analyses were conducted as previously described [8]. To control for protein loading and transfer, membranes were stained with 0.1% amido-black (Sigma Aldrich) and total protein staining quantified [35]. Western blot analysis was used to determine the protein content of OXCT1 (Thermo Fisher, Waltham, MA), AMPK (Cell Signaling Technology, Danvers, MA), AMPK phosphor-specific Thr 172 (Cell Signaling Technology), AKT (Cell Signaling Technology), AKT phospho-specific Ser 473 (Cell Signaling Technology), glycogen synthase (Cell Signaling Technology), glycogen synthase phospho-specific Ser 641 (Cell Signaling Technology), CPT-1m (Santa Cruz Biotechnology, Dalla, TX), and LCAD (Abcam) in the gastrocnemius-plantaris complex. AMPK, AMPK phosphor-specific Ser Thr 172, AKT, AKT phospho-specific Ser 473, glucose 6 phosphatase (Abcam), phosphoenolpyruvate carboxykinase (PCK) 1 (Cell Signaling Technology), CREB (Cell Signaling Technology), CREB phosphor-specific Ser 133 (Cell Signaling Technology), HMGCS1 (Abcam), and HMGCS2 (Abcam) were assessed in the liver. Primary antibodies were diluted 1:1000 and secondary antibodies 1:5000. Protein bands were identified using the manufacturers' estimated molecular weight.

2.12. Statistical Analyses

Main effects of exercise or SGLT2i treatment and significant interactions were determined using a two-way ANOVA. Fisher LSD post-hoc comparisons were used to identify differences between treatment groups when significant main effects or interactions were present (GraphPad Prism 7, San Diego, CA). A two-way ANCOVA (body weight as a covariate) with Fisher LSD post-hoc comparisons was used to determine differences between groups for energy expenditure (IBM SPSS v.25, Armonk, NY). VEH CHOW and weight matched animals are included as points of reference in the figures and Table 1 but were not included in the statistical analyses. Unpaired t-tests were used to determine differences in mean VO_2, mean RER, and distance traveled between the VEH EX and SGLT2i EX during submaximal exercise. Values are reported as mean $\pm$ SEM, with significance at $p < 0.05$. Individual data points were determined as outliers and excluded if they were $\pm$ 2SD from the mean.

3. Results

3.1. Body weight, body composition, and food intake

SGLT2i administration resulted in smaller gains in body weight ($p < 0.01$ vs. VEH) and fat mass ($p < 0.01$ vs. VEH), despite increased food intake ($p < 0.0001$; Table 1). EX had lower body weight ($p < 0.05$), less weight gain ($p < 0.0001$), lower percent body fat ($p < 0.01$), less fat gain ($p < 0.0001$), and higher percent lean mass ($p < 0.05$; Table 1) than SED. While SGLT2i SED had similar body weight to VEH SED, SGLT2i SED gained less weight ($p < 0.01$) and less fat mass ($p < 0.001$) during the intervention. EX was more effective than SGLT2i at preventing the gain of adiposity, as VEH EX and SGLT2i EX gained less fat mass than SGLT2i SED ($p < 0.05$ and $p < 0.01$ vs SGLT2i SED, respectively). Interestingly, SGLT2i SED and SGLT2i EX had ~15–20% greater relative food intake than VEH SED ($p < 0.001$ and $p < 0.0001$, respectively), yet SGLT2i EX had ~15% lower body weight.

3.2. Aerobic capacity

EX had higher VO2peak than SED ($p < 0.05$; Table 1), with ~6–8% increases in relative maximal oxygen consumption in the VEH EX and SGLT2i EX groups, respectively. However, only SGLT2i EX had a higher VO_{2peak} than VEH SED ($p < 0.05$).

3.3. Plasma lipid profile

Total cholesterol and triglycerides were not different amongst treatment groups (Table 1). SGLT2i treatment modestly increased circulating NEFA ($p < 0.05$), but only SGLT2i SED was higher than VEH SED ($p < 0.01$).

3.4. Urine volume and glucose excretion

Twenty-four-hour urine volume ($p < 0.0001$), urine glucose concentrations ($p < 0.0001$), and glucose excretion ($p < 0.0001$) were significantly higher in SGLT2i SED and SGLT2i EX compared to all other groups (Table 1), with no differences between the SGLT2i-treated groups.

3.5. Measures of glycemia

Low dose STZ and high-fat diet caused VEH SED to have elevated fasting glucose. SGLT2i lowered fasting glucose ($p < 0.0001$), proinsulin ($p < 0.0001$), and insulin concentrations ($p < 0.0001$; Fig. 1a–c). SGLT2i SED and SGLT2i EX had similar indices of glycemic regulation during fasting conditions. EX lowered proinsulin concentrations ($p < 0.01$ VEH SED vs VEH EX). Interestingly, EX only had a lowering effect on proinsulin in VEH treated animals (interaction, $0 < 0.01$). Hepatic pAMPK (Thr 172)/AMPK was increased with SGLT2i ($p < 0.01$ vs VEH; Fig. 1d), while there were no effects of either SGLT2i or EX in the gastrocnemius-plantaris complex (Fig. 1f). Conversely, no differences were observed between groups for pAKT (Ser 473)/AKT in the liver

Table 1
The effects of co-therapy of SGLT2i and EX on animal characteristics, aerobic capacity, and urinary glucose excretion.

	VEH CHOW	VEH SED	VEH EX	SGLT2i SED	SGLT2i EX	Weight-matched	Pair-wise comparisons
Body weight (g)	588 ± 15	733 ± 26	692 ± 21	716 ± 25*	627 ± 25*	640 ± 10	VEH SED vs SGLT2i EX, p < 0.01 SGLT2i SED vs SGLT2i EX, p < 0.05
Change in body weight (g)	80.1 ± 6.8	135.0 ± 6.0	55.6 ± 9.0†††	86.7 ± 15.9**	31.4 ± 13.5**, †††	16.0 ± 11.0	VEH SED vs VEH EX, p < 0.0001 VEH SED vs SGLT2i SED, p < 0.01 VEH SED vs SGLT2i EX, p < 0.0001 SGLT2i SED vs SGLT2i EX, p < 0.01
Mean relative food intake (kcal/kgBW/wk)	1390 ± 27	1079 ± 25	1073 ± 35	1250 ± 23****	1269 ± 26****	941 ± 22	VEH SED vs SGLT2i SED, p < 0.001 VEH SED vs SGLT2i EX, p < 0.0001 VEH EX vs SGLT2i SED, p < 0.001 VEH EX vs SGLT2i EX, p < 0.0001
% body fat	17.6 ± 0.9	25.3 ± 1.2	20.8 ± 1.5††	26.3 ± 1.6	20.0 ± 1.9††	16.8 ± 1.2	VEH SED vs SGLT2i EX, p < 0.05 VEH SED vs SGLT2i EX, p < 0.05 VEH EX vs SGLT2i SED, p < 0.05 SGLT2i SED vs SGLT2i EX, p < 0.01
Δ fat mass (g)	33.4 ± 4.1	70.9 ± 7.5	6.5 ± 5.9†††	33.9 ± 6.2**	−5.4 ± 10.1**, †††	−25.7 ± 7.3	VEH SED vs VEH EX, p < 0.0001 VEH SED vs SGLT2i SED, p < 0.001 VEH SED vs SGLT2i EX, p < 0.0001 VEH EX vs SGLT2i SED, p < 0.05 SGLT2i SED vs SGLT2i EX, p < 0.01
% lean mass	68.6 ± 1.0	60 ± 0.6	63.7 ± 1.0*	60.4 ± 1.4	63.7 ± 2.2*	67.3 ± 1.2	
Δ lean mass (g)	14.7 ± 3.0	24.2 ± 4.8	23.0 ± 6.5	10.7 ± 7.7**	−3.5 ± 7.5**	−9.8 ± 4.9	VEH SED vs SGLT2i EX, p < 0.01 VEH EX vs SGLT2i EX, p < 0.05
VO$_2$ peak (ml/kg/h)	3535 ± 64	3077 ± 177	3446 ± 100*	3303 ± 152	3588 ± 144*	3499 ± 86	VEH SED vs SGLT2i EX, p < 0.05
Plasma triglyceride (mmol/L)	0.57 ± 0.06	0.57 ± 0.05	0.68 ± 0.07	0.59 ± 0.05	0.54 ± 0.06	1.05 ± 0.19	
Plasma total cholesterol (mmol/L)	73.1 ± 3.9	79.3 ± 6.7	65.1 ± 4.3	83.9 ± 7.6	72.6 ± 10.1	78.6 ± 5.1	
NEFA (mmol/L)	0.52 ± 0.03	0.47 ± 0.04	0.51 ± 0.03	0.61 ± 0.03*	0.55 ± 0.04*	0.48 ± 0.04	VEH SED vs SGLT2i SED, p < 0.01
24 h urine volume (ml)	17.1 ± 2.3	15.7 ± 1.6	13.9 ± 1.9	51.7 ± 4.6****	46.6 ± 4.1****	23.9 ± 4.8	VEH SED vs SGLT2i SED, p < 0.0001 VEH SED vs SGLT2i EX, p < 0.0001 VEH EX vs SGLT2i SED, p < 0.0001 VEH EX vs SGLT2i EX, p < 0.0001
Urine glucose (mmol/L)	1.5 ± 0.2	1.8 ± 0.5	2.9 ± 1.0	629.0 ± 64.9****	597.0 ± 33.1****	1.4 ± 0.6	VEH SED vs SGLT2i SED, p < 0.001 VEH SED vs SGLT2i EX, p < 0.001 VEH EX vs SGLT2i SED, p < 0.001 VEH EX vs SGLT2i EX, p < 0.0001
Urine glucose (g/24 h)	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	5.06 ± 0.24****	4.84 ± 0.43****	0.00 ± 0.00	VEH SED vs SGLT2i SED, p < 0.001 VEH SED vs SGLT2i EX, p < 0.0001 VEH EX vs SGLT2i SED, p < 0.0001 VEH EX vs SGLT2i EX, p < 0.0001

Values are mean ± SEM. N = 7–10/group for most measures. N = 4–7/group for VO2 peak measures and N = 5–10/group for urine glucose measures.

   * p < 0.05 main effect of SGLT2i treatment.
  ** p < 0.01 main effect of SGLT2i treatment.
**** p < 0.001 main effect of SGLT2i treatment.
  †† p < 0.01 main effect of exercise.
 ††† p < 0.001 main effect of exercise.

(Fig. 1e), but pAKT/AKT in the gastrocnemius-plantaris complex was lower with SGLT2i treatment (p = 0.01), with no differences between SGLT2i SED and SGLT2i EX (Fig. 1g).

Low dose STZ and high-fat diet caused VEH SED to become glucose intolerant while maintaining some insulin response. SGLT2i and EX independently improved glucose AUC compared to VEH SED (p < 0.05 and p < 0.001, respectively; Fig. 2a–b) and SGLT2i lowered insulin AUC (p < 0.001; Fig. 2c–d). SGLT2i EX further reduced glucose AUC compared to SGLT2i SED (p < 0.05) and insulin AUC compared to VEH EX (p < 0.05). When taken together, SGLT2i EX may be more efficacious than SGLT2i or EX alone during a glucose challenge.

### 3.6. Measures associated with endogenous glucose production

Plasma glucagon did not differ amongst groups (Fig. 3a) but SGLT2i and EX independently lowered insulin: glucagon (p < 0.0001 and p < 0.05, respectively; Fig. 3b). The combination of SGLT2i EX resulted in lower insulin: glucagon than VEH EX (p < 0.01), but was similar to SGLT2i SED. Lowering of insulin: glucagon could promote glycogenolysis and gluconeogenesis. Although there were no differences in phospho-cAMP response element-binding protein (CREB)/CREB (Fig. 3c) protein content in the liver, SGLT2i and EX treatments independently affected hepatic content of key proteins

in gluconeogenesis. EX had higher hepatic phosphoenolpyruvate carboxykinase 1 (PCK1, p < 0.01; Fig. 3d), while SGLT2i treatment had higher hepatic glucose 6 phosphatase (G-6-Pase, p < 0.001; Fig. 3e).

3.7. Substrate utilization during cage conditions

EX increased energy expenditure in both the dark (p < 0.01) and light cycles (p < 0.05), while SGLT2i increased energy expenditure during the light cycle (p < 0.01; Fig. 4a–b). The combination of SGLT2i EX resulted in greater energy expenditure during the light cycle than EX alone (p < 0.05 vs VEH EX). Weekly energy expenditure (non-exercise +exercise energy expenditure) was increased with EX and SGLT2i independently (p < 0.001 and p < 0.05, respectively; Fig. 4c). Importantly, SGLT2i treatment induced an ~6% increase in energy expenditure (within respective exercise condition) and exercise training induced an ~10% increase in energy expenditure (within the respective drug treatment group), but the combination of SGLT2i EX seemed to have additive effects on energy expenditure, as SGLT2i EX had ~25% higher weekly energy expenditure than VEH SED.

Differences in energy expenditure occurred independently of spontaneous cage activity, as EX actually had lower spontaneous activity during both the light and dark cycles (p < 0.05; Fig. 4d–e). Only VEH
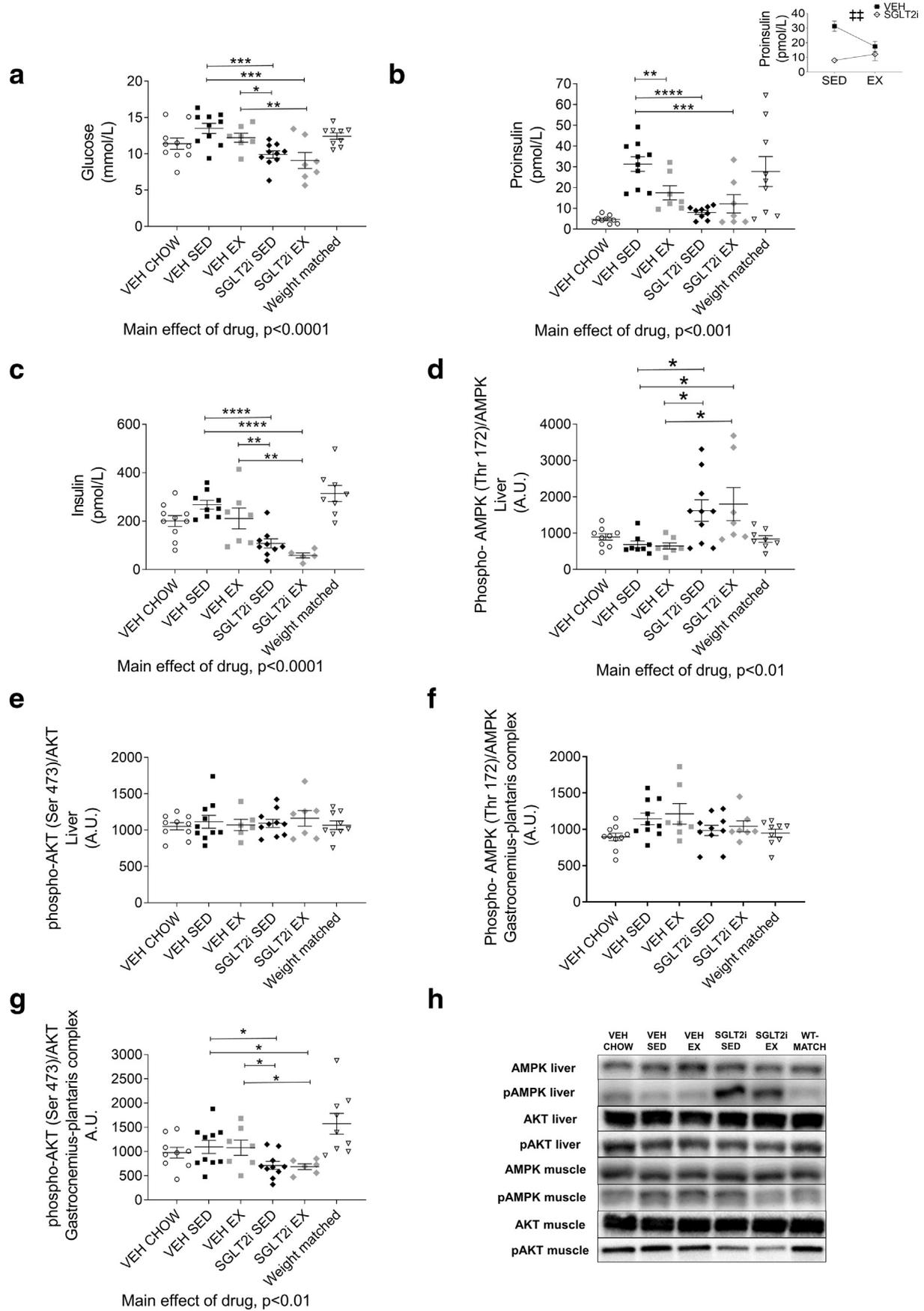
Fig. 1. SGLT2i treatment improves indices of glycemic control under fasting conditions. Fasting glucose (a), fasting proinsulin (b), fasting insulin (c), phospho- AMPK (Thr 172)/total AMPK in liver (d), phospho- AKT (ser473)/total AKT in liver (e), phospho- AMPK (Thr 172)/total AMPK in gastrocnemius-plantaris complex (g), and phospho- AKT (ser473)/total AKT in gastrocnemius-plantaris (h), and representative Western blot images (f). n = 5–10 animals/group. Values are mean ± SEM. ^{H}p < 0.01 significant interaction. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 significant difference between groups.
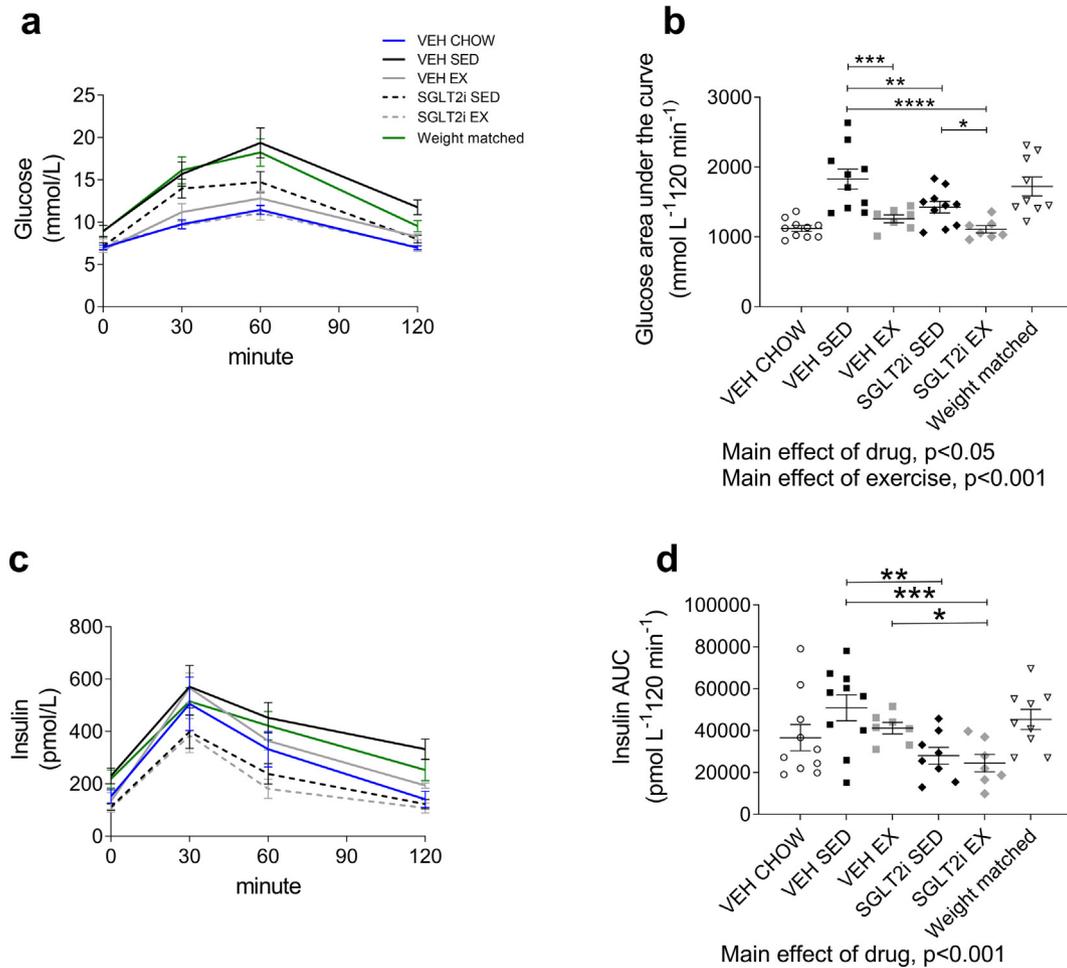
Fig. 2. SGLT2i EX further improves responses to a glucose challenge than either EX or SGLT2i alone. Glucose response curves (a), glucose area under the curve (b), insulin response curves (c), and insulin area under the curve (d). n = 7–10 animals/ group. Values are mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 significant difference between groups.

EX were less active than VEH SED (p < 0.01 light and p < 0.05 dark). EX increased RER during the light cycle (p < 0.05; Fig. 4f–g). SGLT2i caused a greater reliance on fat for energy, as indicated by lowered RER in both the light (p < 0.05) and dark cycles (p < 0.01); however, SGLT2i EX had a higher RER than SGLT2i SED during the light cycle (p < 0.05).

3.8. Tissue glycogen content

Glycogen content, glycogen synthase (GS) protein expression, and phospho-GS/GS in the gastrocnemius-plantaris did not differ with treatment (Fig. 5a–d). Liver glycogen content was lowered with SGLT2i treatment (p < 0.0001; Fig. 5e), but did not differ between SGLT2i SED and SGLT2i EX. This is consistent with lower insulin: glucagon. Although SGLT2i increased total hepatic GS (p < 0.0001), the phosphorylation status of GS was lower with SGLT2i treatment (p < 0.001), with no differences between SGLT2i SED and SGLT2i EX (Fig. 5f–h).

3.9. Measures associated with nutrient metabolism and ketones

SGLT2i administration resulted in lower lactate concentrations (p < 0.01; Fig. 6a) and higher β-hydroxybutyrate (p < 0.0001; Fig. 6b) than VEH treatment, with no differences between SGLT2i SED and SGLT2i EX. No differences were observed amongst any of the treatment groups in hepatic 3 hydroxy 3 methylglutaryl-CoA synthase (HMGCS) 1 or HMGCS2, proteins associated with ketone body production (Fig. 6c–d). SGLT2i administration resulted in higher 24 h urinary excretion of β hydroxybutyrate (p < 0.01; Fig. 6e).

SGLT2i administration resulted in higher 3 oxoacid CoA-transferase 1 (OXCT1), a protein associated with ketolysis, in the gastrocnemius-plantaris compared to VEH (p < 0.05; Fig. 6f). On the other hand, EX lowered OXCT1 content compared to SED (p < 0.001), resulting in significantly lower OXCT1 in SGLT2i EX compared to SGLT2i SED (p < 0.01).

Although SGLT2i resulted in whole body substrate utilization that indicated a greater reliance on fat (lower RER), no differences were observed for protein content of CPT-1m or LCAD in skeletal muscle (data not shown).

3.10. Substrate utilization during exercise and submaximal exercise capacity

During submaximal exercise, VEH EX and SGLT2i EX had similar oxygen consumption (Fig. 7a-b); however, SGLT2i EX relied more on fat as a substrate for energy production during submaximal exercise (~60% energy from fat in SGLT2i EX vs ~50% energy from fat in VEH EX; p < 0.01; Fig. 7c–d). During the submaximal exercise bout, SGLT2i EX ran approximately twice as far as VEH EX (p < 0.05; Fig. 7e).

4. Discussion

This report is one of the first to assess the effectiveness of co-treatment with an SGLT2i, and exercise training in the management of type 2 diabetes. We found that SGLT2i EX suppressed body weight gain and lowered adiposity despite increased food consumption.
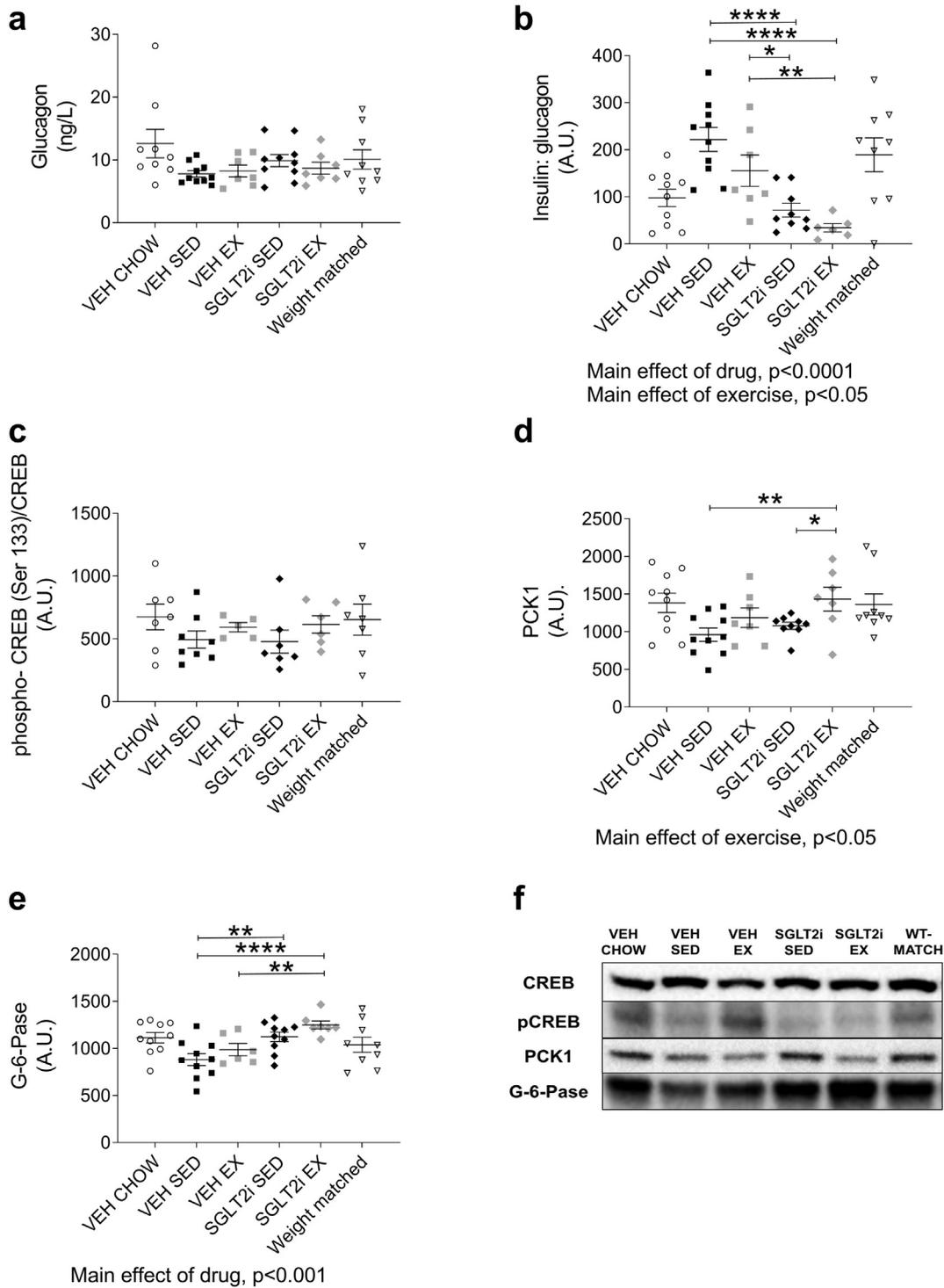
Fig. 3. SGLT2i alters indices of endogenous glucose production. Fasting glucagon (a), insulin to glucagon ratio (b), hepatic phospho-CREB (Ser133)/ total CREB (c), hepatic PCK1 (d), hepatic G-6-Pase (e), and representative Western blot images (f). n = 5–10 animals/group. Values are mean ± SEM. *p < 0.05, **p < 0.01, ****p < 0.0001 significant difference between groups.

As anticipated, EX and SGLT2i individually improved measures of glycemic control, but during an OGTT, SGLT2i EX had an additive effect compared to VEH EX or SGLT2i SED. SGLT2i increased fat utilization at rest and during exercise and increased ketone concentrations. Although VEH EX had higher energy expenditure than VEH SED over 24 h, SGLT2i EX further increased energy expenditure compared to VEH EX during the light cycle. EX increased VO_{2peak} similarly in VEH EX and SGLT2i EX, but interestingly, SGLT2i EX may actually improve submaximal exercise endurance when compared to exercise training alone. Therefore, we demonstrate that in contrast to the combination of metformin and

exercise training, SGLT2i EX may augment the effect of exercise on glycemic control and exercise capacity in a rodent model of type 2 diabetes.

In addition to pharmacotherapies, regular exercise is recommended for individuals with type 2 diabetes. However, recent evidence suggests that in some instances the combination of metformin with regular exercise may not further improve glycemia [11,12,36] and may even blunt exercise-induced improvements in glycemic control [8–13,15]. Because of this, we assessed the effects of co-treatment with SGLT2i and EX, as SGLT2i lowers circulating glucose but may also improve insulin sensitivity [37]. To our knowledge, this is the first report to assess co-therapy of
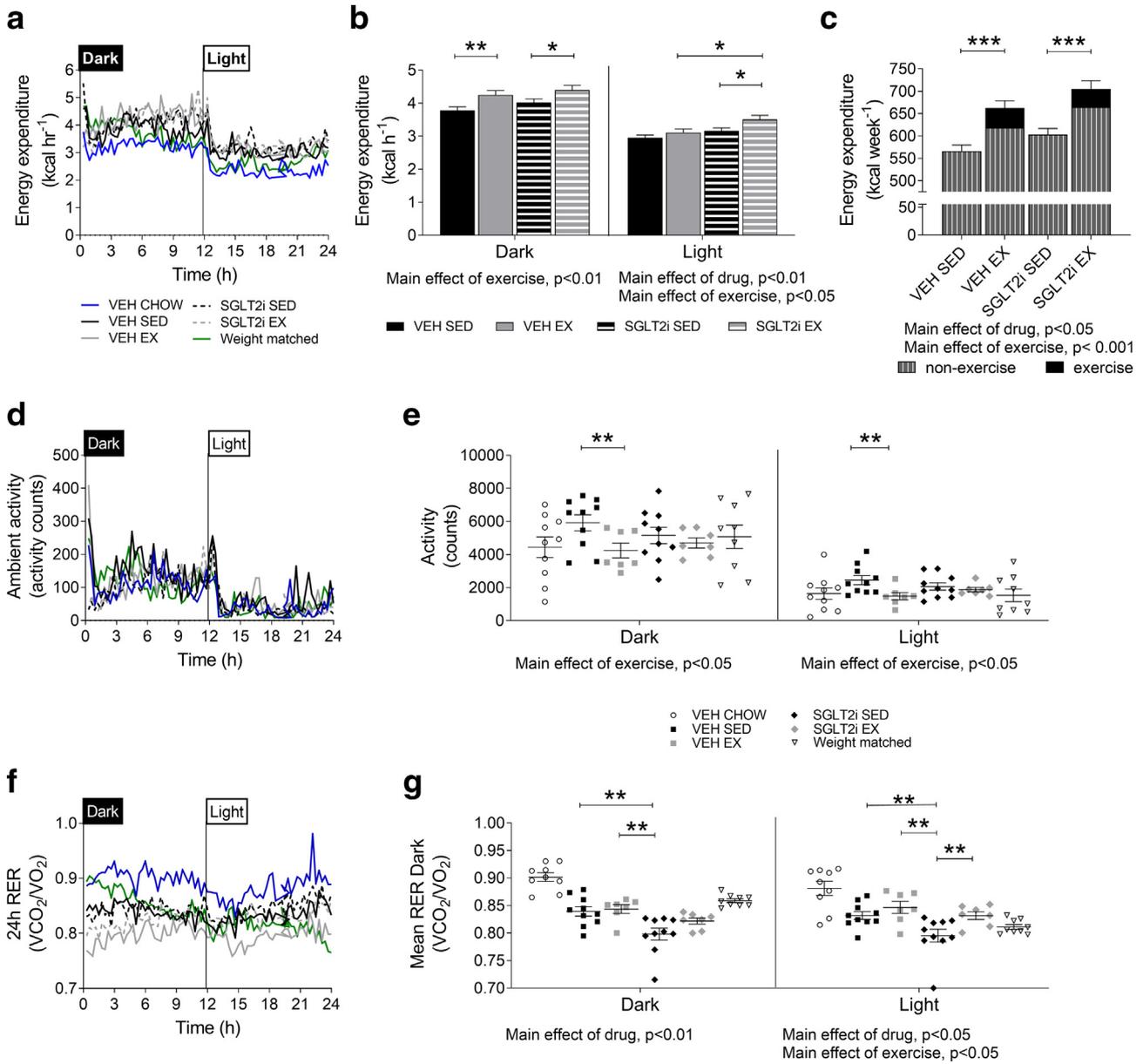
Fig. 4. SGLT2i alters substrate utilization and the combination of SGLT2i and EX increase energy expenditure throughout the day. Energy expenditure, ambient activity, and RER were assessed for 24 h using calorimetry cages and means for the dark and light cycles were calculated for energy expenditure, and RER. Total ambient activity counts were calculated for the dark and light cycles. 24 h Energy expenditure (a), mean energy expenditure in the dark and light cycles (b), weekly energy expenditure (c), 24 h ambient activity (d), mean activity in the dark and light cycles (e), 24 h RER (f), and mean RER in the dark and light cycles (g). n = 7–10 animals/group. Values are mean ± SEM. *p < 0.05 and **p < 0.01 significant difference between groups.

SGLT2i and exercise training in a model of type 2 diabetes. To date, there has only been one other published report looking at the interaction between SGLT2i and exercise training in insulin sensitive, overweight-obese adults [38]. In the previous report, neither EX alone or in combination with the SGLT2i, dapagliflozin, improved glucose responses during a glucose tolerance test following 12 weeks of intervention [38]. In contrast, we demonstrate, when using a rodent model of type 2 diabetes, that EX and SGLT2i independently improve indices of glycemic control, including fasting glucose, proinsulin, insulin, and responses to an OGTT. During an OGTT, SGLT2i EX better maintained glucose concentration than SGLT2i alone and lowered insulin responses compared to VEH EX. These improvements in indices of glycemic control contrast some observations with the combination of metformin and exercise training [8–13,36], and could be important for altering clinical standard of practice if the findings translate to humans with type 2 diabetes.

Although SGLT2i are known to improve glycemia, there is a metabolic paradox associated with SGLT2i, as endogenous glucose production (EGP) increases in humans during early administration [37]. Our data suggest that treatment with SGLT2i over 12 weeks may result in prolonged increases in EGP since insulin: glucagon was lower in SGLT2i SED and SGLT2i EX. Exercise may also increase the phosphorylation of CREB [39], which increases transcription of gluconeogenic

Fig. 5. SGLT2i lowers hepatic glycogen content but does not affect glycogen within the gastrocnemius-plantaris. Gastrocnemius-plantaris glycogen (a) gastrocnemius-plantaris and glycogen synthesis (GS;b), gastrocnemius-plantaris phospho- GS (Ser 641) (c), gastrocnemius-plantaris phospho-GS/total GS (d), hepatic glycogen (e) hepatic GS (f), hepatic phospho- GS (Ser 641) (g), and hepatic phospho-GS/total GS (h). n = 5–10 animals/group. Values are mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 significant difference between groups.

Gastrocnemius-plantaris complex
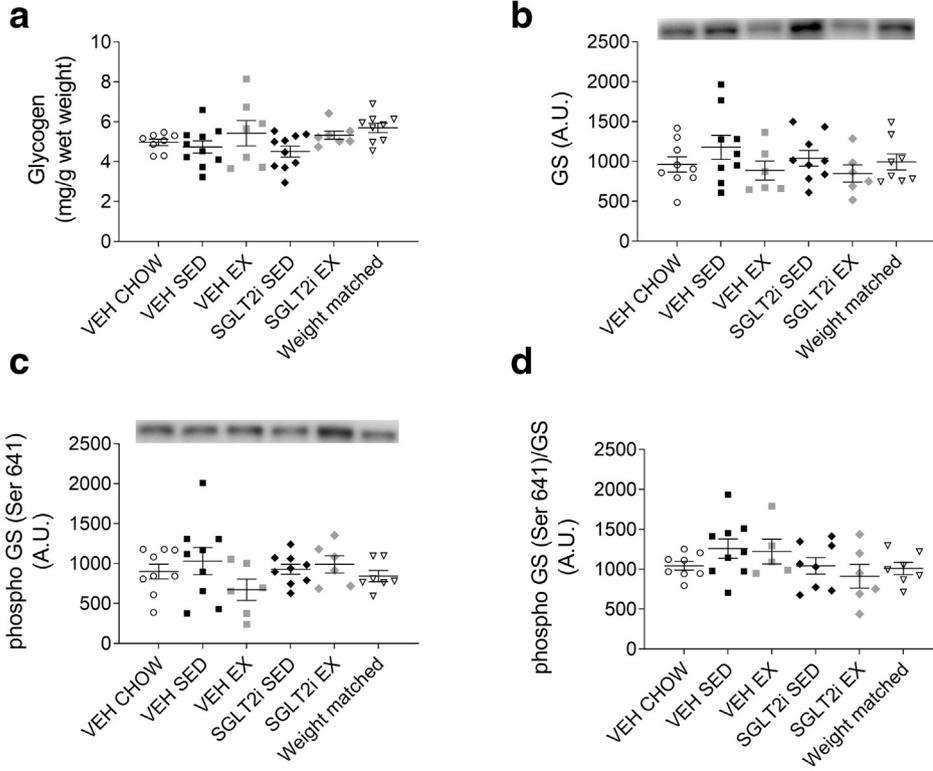
Liver
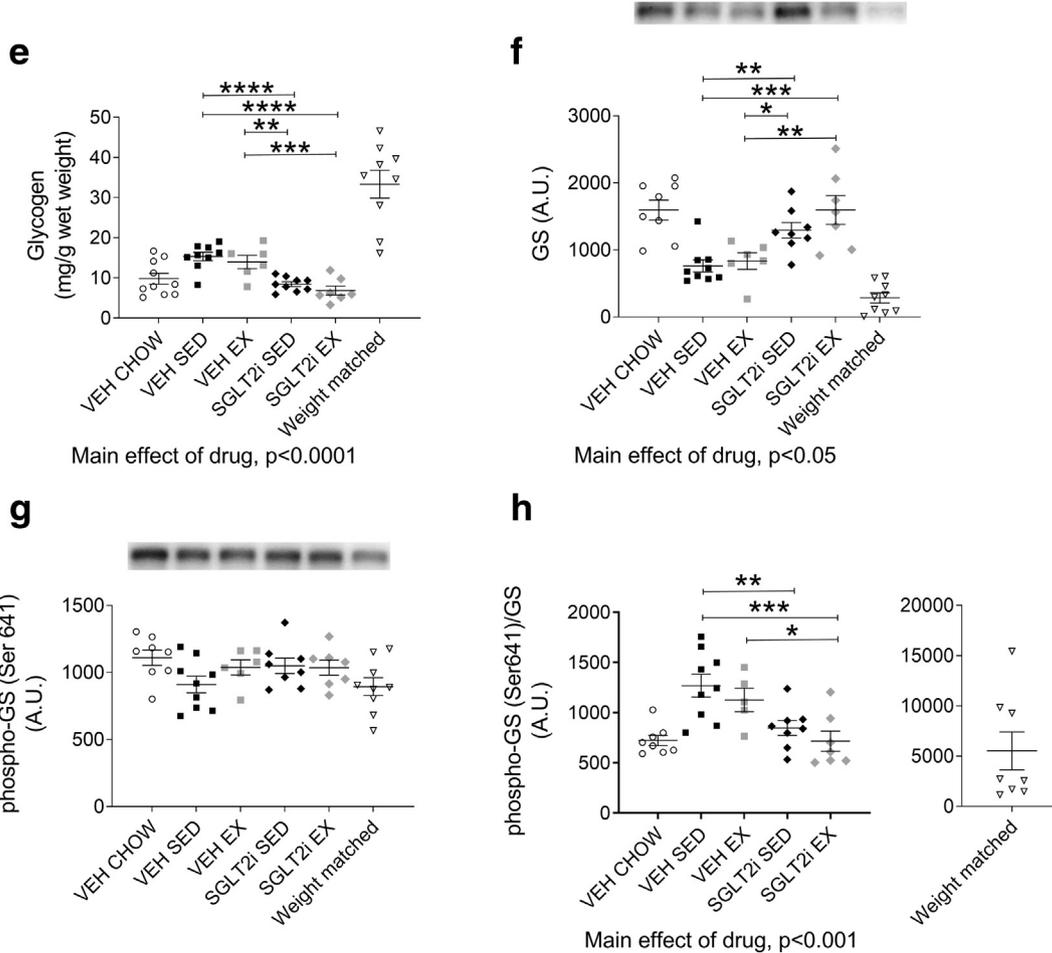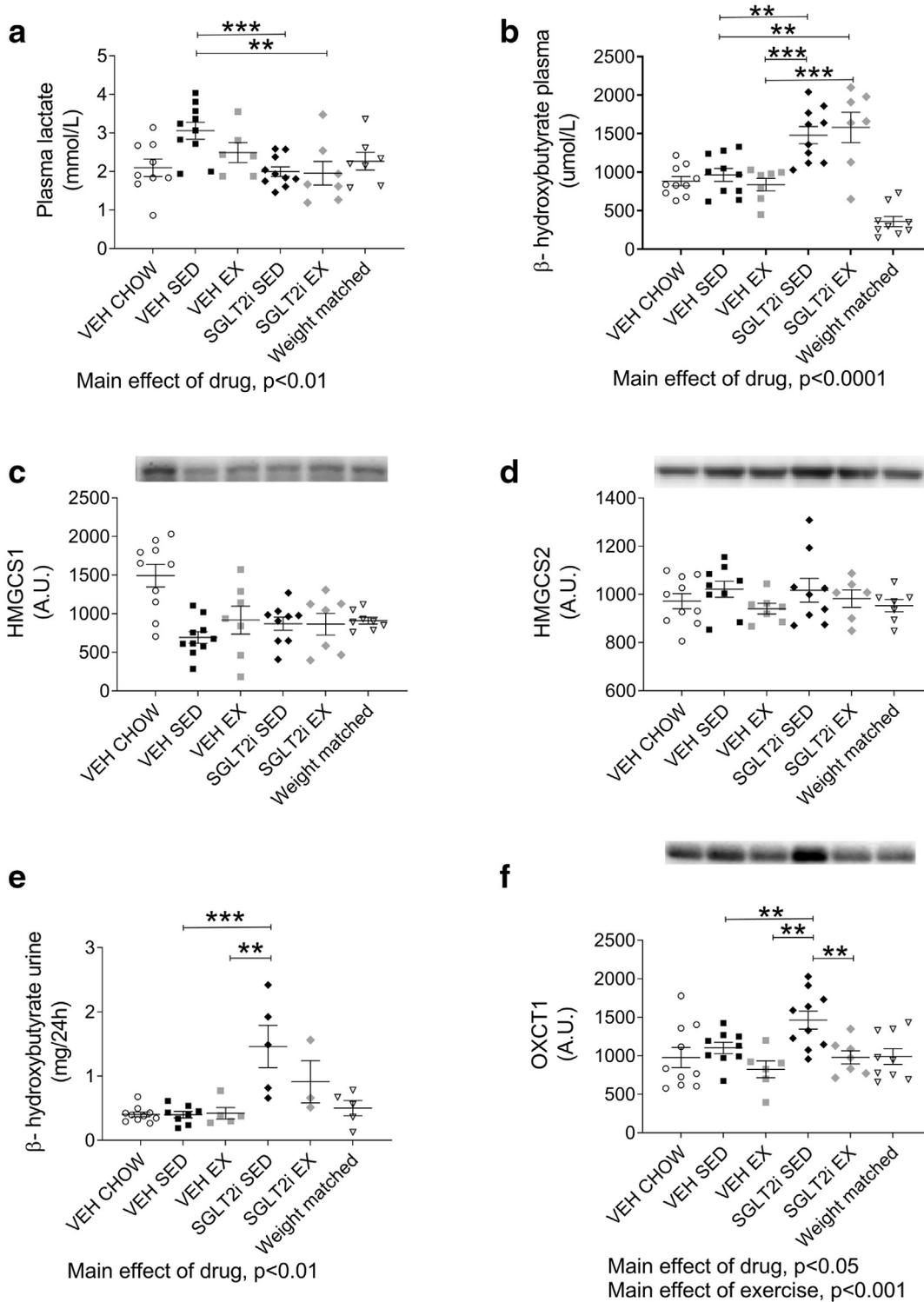
Fig. 6. SGLT2i increases circulating β hydroxybutyrate, urinary β hydroxybutyrate excretion, and the ketolysis protein OXCT1 in the gastrocnemius-plantaris. Plasma lactate (a), plasma β hydroxybutyrate (b), hepatic HMGCS1 (c), hepatic HMGCS2 (d), urine β hydroxybutyrate (e), and OXCT1 (f). n = 6–10 animals/group for most measures. n = 3–10 animals/group for urine β hydroxybutyrate. Values are mean ± SEM. **p < 0.01 and ***p < 0.001significant difference between groups.

genes [40] but there were no differences amongst groups for hepatic expression of either phospho- or total CREB in the current report. However, hepatic PCK1, the first committed step to gluconeogenesis [41], was higher in SGLT2i EX, and glucose-6-phosphatase, the final step of gluconeogenesis [42], was higher in SGLT2i SED and SGLT2i EX than VEH SED which may be compensatory to support increased EGP.

SGLT2i treatment in humans may result in modest body weight losses [17,18] and a greater reliance on fat as a source of energy

[19,20]. Our observations agree with these findings, as SGLT2i-treated rats had higher energy expenditure and lower RER at rest. Interestingly, the combination of SGLT2i EX seemed to have additive effects on energy expwnditure, resulting in a ~25% increase in energy expenditure compared to VEH SED. This increase in energy likely contributed to the observed ~15% lower body weight in SGLT2i EX despite increased food consumption. Gluconeogenesis is an energy costly process that may be induced by SGLT2i. SGLT2i-induced caloric loss through urinary glucose
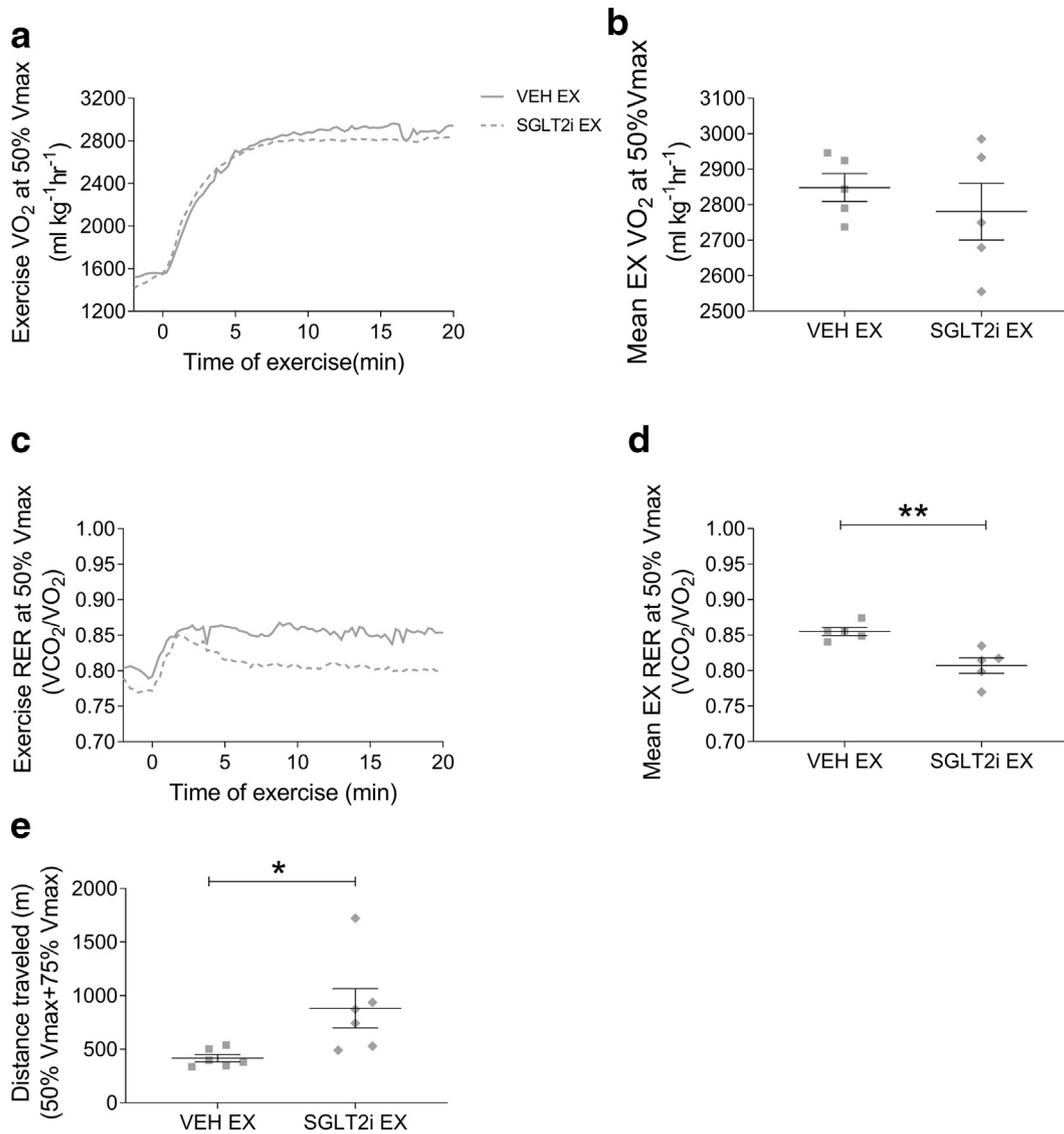
Fig. 7. The combination of SGLT2i and EX resulted in greater reliance on fat as an energy source during exercise and improved exercise capacity. Exercise VO$_2$ (a), mean exercise VO$_2$ (b), respiratory exchange ratio during exercise (RER; c), mean exercise RER (d) and distance traveled during a submaximal exercise bout (f). n = 5–7 animals/group. Values are mean ± SEM. *p < 0.05 and **p < 0.01 significant difference between groups.

when combined with increased EGP could contribute to weight loss associated with medications in this class [17,18] and suppression of body weight gain in SGLT2i EX. It is important to note that SGLT2i-treated rats compensated for SGLT2i-induced energy deficits with a ~15–20% increase in food intake, which may explain why SGLT2i SED and VEH SED had similar body weights. However, body weight gain was suppressed in SGLT2i EX despite increases in food intake, which could have positive implications for individuals with type 2 diabetes.

During times of energetic stress, tissue glycogen can be used as source of energy. Here, neither pAMPK/AMPK, glycogen, nor glycogen synthase content in skeletal muscle differed amongst treatment groups. SGLT2i groups did have higher hepatic pAMPK/AMPK, lower liver glycogen content, and higher GS compared to VEH SED, which may also support increased EGP.

In addition to changes in substrate utilization, there were changes in circulating lactate and ketone bodies. SGLT2i SED and SGLT2i EX had lower lactate concentrations than VEH SED, which could reflect lower glycolytic activity or increased lactate oxidation. Additionally, circulating β-hydroxybutyrate was ~50–60% higher with SGLT2i, which corresponds with a previous report in which canagliflozin

administration increased β hydroxybutyrate 78% and total ketone bodies 73% in patients with type 2 diabetes [43]. Our observed increases in β hydroxybutyrate did not coincide with altered hepatic protein expression of HMGCS-1 or -2, key proteins in ketone body production. SGLT2i-treated animals had greater urinary excretion of β-hydroxybutyrate and higher OXCT1 protein expression in skeletal muscle, which may be compensatory responses to prevent further elevations in circulating ketones. Recently, exogenous ketone administration was shown to improve glycemic responses during a glucose tolerance test through improved insulin sensitivity [44]. Increased circulating ketone bodies and increased OXCT1 in skeletal muscle may promote ketone metabolism in skeletal muscle, but future studies are needed to better understand if SGLT2i-induced increases in endogenous ketone production have insulin-sensitizing effects.

Exercise training is recommended to those with type 2 diabetes because it can improve glycemic control. However, individuals with type 2 diabetes may derive less benefit in aerobic capacity from regular exercise and may have more difficulty tolerating exercise than their healthy counterparts [3,4]. One previous report showed that the combination of SGLT2i and exercise training had no negative impacts on

exercise-induced improvements in aerobic capacity in insulin sensitive adults [38]. Another recent report indicates that treatment with an SGLTi when combined with standard care may in fact improve cardiorespiratory fitness in individuals with type 2 diabetes [45]. We found similar improvements in VO_{2peak} between VEH EX and SGLT2i EX in our rodent model of type 2 diabetes. However, our findings build upon the previous work, as we show that SGLT2i EX ran almost twice as far before fatigue at submaximal running speeds. Submaximal exercise capacity is a better indicator of peripheral mitochondrial function and fuel use [46,47]. Individuals with type 2 diabetes tend to rely less on lipid oxidation during exercise [48], however we showed that SGLT2i EX had greater fat utilization during exercise than VEH EX (Fig. 7c–d), perhaps in part contributing to greater endurance. There is evidence in both athletes and obese subjects that either exogenous ketone administration or ketogenic diets may increase reliance on fat during exercise [49,50] and have some positive effects on endurance [49]. SGLT2i EX rats did have ketosis, which may have contributed to improved exercise tolerance. Additional studies are needed to elucidate if SGLT2i-induced alterations in ketone metabolism may improve exercise capacity in people with type 2 diabetes.

One limitation of the present report is that type 2 diabetes in this model was caused by chemical-induced damage to the pancreatic beta cells. Although this form of type 2 diabetes does not follow the natural disease progression that occurs in humans, many of the characteristics observed in this report do mimic those seen in humans (i.e. insulin response to a glucose challenge) with clinically relevant elevations in glycemia. This is often not the case with other rodent models of type 2 diabetes. Moreover, we were able to replicate many SGLT2i-induced responses seen in human populations [17–20,37] in this rodent model, including lower body weights, a greater reliance on fat as an energy source, and a greater potential for increased endogenous glucose production. A second limitation was the unanticipated unwillingness of chronically sedentary animals to complete submaximal exercise testing despite re-acclimatization to treadmill running. Due to the unwillingness of the sedentary animals to run, we were only able to assess submaximal aerobic capacity in the EX animals. Additional studies are needed to determine if improved submaximal exercise capacity can be observed in sedentary animals treated with SGLT2i.

EX and SGLT2i administration independently improved indices of glycemic control in this rodent model of type 2 diabetes. However, unlike cotreatment with metformin and exercise [11,12,36], the combination of SGLT2i and EX did not impair exercise-induced alterations in glycemic control and actually had additive effects on either glucose or insulin responses during a glucose challenge when compared to SGLT2i or EX alone. Additionally, SGLT2i EX further suppressed body weight gain, improved adiposity, and increased energy expenditure when compared to SGLT2i SED, highlighting the importance of exercise training in combination with pharmacotherapies for the improvement of disease risk factors. SGLT2i administration altered substrate metabolism towards a greater reliance on lipid metabolism at rest and during submaximal exercise, and induced ketosis, neither of which were greatly altered in SGLT2i EX compared to SGLT2i alone. Importantly, SGLT2i EX seemed to further improve exercise endurance compared to EX alone. When taken together, these findings suggest that the combination of SGLT2i and EX may have additional benefits on type 2 diabetes and exercise capacity compared to either treatment alone. If these findings translate to humans, they may change current standard of clinical practice for exercise and pharmacotherapy co-treatment for type 2 diabetes.

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Data availability

Data are available from the authors upon reasonable request.

Funding sources

Declarations of competing interest

Authors TTR, DAB, MFG and WPE are employees and shareholders of Pfizer. MAL is a former employee of Pfizer. BB has consulted for Pfizer, Inc. MAL, KLH, BFM, and BB have received research funding from Pfizer, Inc. Some of the work discussed was conducted at and funded by Pfizer Inc.

Contributions

Involved in the study concept and design (MAL, TTR, DAB, MFG, KLH, BFM, BB, WPE); acquisition of data (MAL, TTR, DAB, MFG); analysis and interpretation of data (MAL, TTR, KLH, BFM, BB, WPE); drafting of the manuscript (MAL, KLH, BFM, BB, WPE); critical revision of the manuscript for important intellectual content (MAL, TTR, DAB, MFG, KLH, BFM, BB, WPE); statistical analysis (MAL, KLH, BFM, BB, WPE), and final approval for publication (MAL, TTR, DAB, MFG, KLH, BFM, BB, WPE).

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