



Canagliflozin Prevents Hyperglycemia-Associated Muscle Extracellular Matrix Accumulation and Improves the Adaptive Response to Aerobic Exercise

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Chronic hyperglycemia is associated with low response to aerobic exercise training in rodent models and humans, including reduced aerobic exercise capacity and impaired oxidative remodeling in skeletal muscle. Here, we investigated whether glucose lowering with the sodium–glucose cotransporter 2 inhibitor (SGLT2i), canagliflozin (Cana; 30 mg/kg/day), could restore exercise training response in a model of hyperglycemia (low-dose streptozotocin [STZ]). Cana effectively prevented increased blood glucose in STZ-treated mice. After 6 weeks of voluntary wheel running, Cana-treated mice displayed improvements in aerobic exercise capacity, higher capillary density in striated muscle, and a more oxidative fiber-type in skeletal muscle. In contrast, these responses were blunted or absent in STZ-treated mice. Recent work implicates glucose-induced accumulation of skeletal muscle extracellular matrix (ECM) and hyperactivation of c-Jun N-terminal kinase (JNK)/SMAD2 mechanical signaling as potential mechanisms underlying poor exercise response. In line with this, muscle ECM accretion was prevented by Cana in STZ-treated mice. JNK/SMAD2 signaling with acute exercise was twofold higher in STZ compared with control but was normalized by Cana. In human participants, ECM accumulation was associated with increased JNK signaling, low VO_{2peak} , and impaired metabolic health (oral glucose tolerance test–derived insulin sensitivity). These data demonstrate that hyperglycemia-associated impairments in exercise adaptation can be ameliorated by cotherapy with SGLT2i.

Improved VO_{2peak} is a key health benefit of aerobic exercise that is strongly associated with reduced morbidity and mortality (1–5). However, VO_{2peak} levels are lower in

people with type 1 and type 2 diabetes compared with control participants without metabolic disease (6–10). In people with or without diabetes, measures of chronic hyperglycemia (e.g., HbA_{1c}) and impaired glucose tolerance are consistently associated with lower VO_{2peak} , even when exercise levels are matched (9,10). These clinical data suggest that hyperglycemia may impart a resistance toward improvements in VO_{2peak} with aerobic training—a phenotype known as low response to training (LRT) (11–14). Consistent with this, our previous work demonstrates that induction of hyperglycemia in mice prior to aerobic training can prevent improvements in VO_{2peak} in response to voluntary wheel running (15). Importantly, VO_{2peak} was blunted to a similar extent whether hyperglycemia was induced by decreasing insulin secretion with streptozotocin (STZ) treatment, or by Western diet-feeding (15). Thus, data from animals and humans demonstrate that hyperglycemia, irrespective of etiology, may lead to LRT, resulting in low VO_{2peak} .

The mechanisms that lead to hyperglycemia-associated decreases in VO_{2peak} are incompletely understood. However, low VO_{2peak} in people with diabetes has been linked with altered function of skeletal muscle, including low muscle blood flow and oxidative capacity (16–20). Our data from rodent models of hyperglycemia demonstrate that blunted improvements in VO_{2peak} are associated with impaired remodeling of skeletal muscle toward an aerobic phenotype, leading to lower capillary density and fewer oxidative fibers (15). Moreover, we identified glucose-induced accumulation of the skeletal muscle extracellular matrix (ECM), leading to hyperactivation of c-Jun N-terminal kinase (JNK)/SMAD2 mechanical signaling after acute exercise as potential mechanisms underlying low

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exercise response (15). Thus, existing evidence suggests that impaired remodeling of skeletal muscle may play a key role in low VO_{2peak} associated with hyperglycemia.

Sodium–glucose cotransporter 2 inhibitors (SGLT2i), such as canagliflozin (Cana), are effective in reducing blood glucose levels in patients and rodent models with impaired glucose tolerance, type 1 diabetes, and type 2 diabetes (21–26). Given strong clinical links between hyperglycemia and low VO_{2peak} , it follows that pharmacological therapies designed to reduce blood glucose may enhance the response to training in individuals with hyperglycemia. Whether the glucose-lowering effects of SGLT2i can enhance improvements in VO_{2peak} with training in humans with hyperglycemia is unknown, as only results from euglycemic participants have been reported (27). Preclinical studies of SGLT2i and exercise training are also limited. Data from obese rodent models demonstrate weight loss and improved submaximal exercise capacity when Cana is combined with exercise training (28,29), suggesting potential benefits of combination therapy. However, the use of obese models can make it difficult to dissect the effects of glucose-lowering from significant weight loss with SGLT2i.

Our investigation aimed to test whether blood glucose lowering with Cana in a lean model of hyperglycemia can restore muscle remodeling and improvements in maximal aerobic capacity with training. We also aimed to elucidate the molecular mechanisms that contribute to hyperglycemia-associated reductions in VO_{2peak} in humans and animal models and whether these are ameliorated by SGLT2i in a preclinical model. Here, we demonstrate that skeletal muscle ECM accumulation is associated with aberrant exercise-induced signal transduction in humans and mice with hyperglycemia. We also present data from STZ-treated mice demonstrating that Cana can prevent ECM accumulation and aberrant signal transduction and improve VO_{2peak} with aerobic training. These data support the premise that hyperglycemia is a negative regulator of aerobic training adaptations and identify SGLT2 inhibition as a potential therapy to ameliorate acute and chronic exercise response in populations with hyperglycemia.

RESEARCH DESIGN AND METHODS

Animal Experiments

Mice

Mouse experiments were approved by the Joslin Diabetes Center Institutional Animal Care and Use Committee. Male 8-week-old CD-1 mice (strain IGS, stock no. 022) were purchased from Charles River Laboratories. Mice were group housed in a pathogen-free facility and maintained on a 12-h normal light/dark cycle with ad libitum access to diet. All mice consumed a low-fat diet (Research Diets, D14020502) consisting of 17% kcal from protein, 73% carbohydrate, and 10% fat. To induce hyperglycemia, STZ (Tocris Bioscience, cat. no. 1621,) was dissolved in citrate buffer (114 mmol/L) and administered in 40 mg/kg intraperitoneal injections on 2 consecutive days after a

4-h fast. Control (Con) mice were injected with citrate buffer vehicle. Two weeks after injection, a subset of STZ-treated mice was randomized to receive the SGLT2i Cana in their diet. For Cana treatment, the D14020502 diet was supplemented with lyophilized Cana (240 mg/kg diet; Advanced ChemBlocks, cat. no. 10300). Mice consumed ~4–5 g of food per day, resulting in a Cana dose of ~30 mg/kg/day. This approach was previously reported to successfully reduce blood glucose concentrations in hyperglycemic mice (22).

Voluntary Wheel Running

Following 8 weeks of Con, STZ, and Cana treatment, mice were assigned to remain sedentary, or undergo exercise training by voluntary wheel running (Fig. 2A for timeline). Sedentary mice were group housed (two to three mice) in a cage without a running wheel. Mice assigned to exercise training were single housed in a cage with a running wheel. Exercise-trained mice were excluded if they ran <100 km over the duration of the study. Voluntary running behavior was recorded in 10-min intervals using a Hall Effect Sensor (0297-0501), Wheel Counter 8 Channel Interface (0297-0050), and a Quad CI-Bus to interface with CI Multi Device Software (v.1.5.5) from Columbus Instruments. Sample tracings of temporal running behavior shown in Fig. 2D were collected over a 24-h diurnal period during the 4th week of exercise training. Mice were allowed to run uninterrupted for the first 6 weeks of the exercise training period. During the final 2 weeks of training, wheels were removed intermittently for 24 h to accommodate washout periods before metabolic testing (glucose tolerance testing [GTT] and DEXA) and terminal dissections.

Blood Glucose, Body Weight, Body Composition, and Food Intake

Blood glucose, body weight, and food intake were measured weekly for each mouse (Fig. 1). Random blood glucose was measured from individual mice via tail vein at ~3:30 P.M. to minimize circadian fluctuations. An Infinity (US Diagnostics) handheld glucose-monitoring system was used for all blood glucose measurements. Body composition was measured near the end of the study period in anesthetized mice using a Lunar PIXImus2 mouse densitometer (DEXA).

GTT

To assess whole-body glucose tolerance, mice were administered an intraperitoneal bolus of glucose (2 g/kg) after a 5-h fast. Blood glucose was sampled from the tail vein and recorded using an Infinity (US Diagnostics) glucose-monitoring system 0, 15, 30, 45, 60, and 120 min after injection. GTTs were performed 8 weeks after initiation of Con, STZ, or Cana treatment and repeated near the end of the exercise training period. Area under the curve (AUC) was calculated using GraphPad Prism 8 software (GraphPad Software), with calculations using $Y = 0$ to account for differences in fasting glucose.

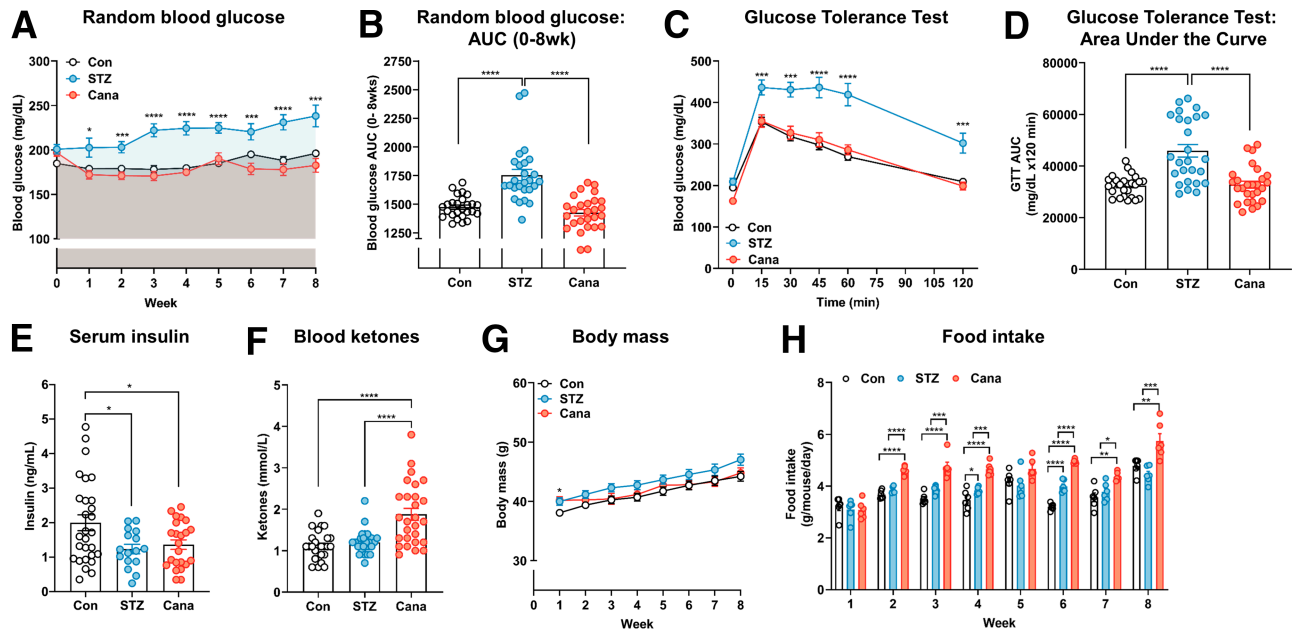


Figure 1—Metabolic phenotypes in Con, STZ, and Cana-treated mice. Blood glucose was sampled once weekly (A), and the AUC was calculated for each mouse as an index of chronic glycemia (B). After 8 weeks of Con, STZ, or Cana treatment, glucose tolerance tests were performed (C), and the AUC was calculated for each mouse (D). After a 12-h overnight fast circulating insulin (E) and ketones (F) were measured in the same mice. Over the initial 8 weeks of treatment, body mass (G) and food intake (H) were measured weekly. $n = 16$ – 30 per group in A–G; $n = 6$ cages per group ($n = 4$ – 5 mice/cage) for food intake in H. Points and columns with error bars represent mean \pm SEM. Groups were compared using one-way ANOVA with Holm-Šidák post hoc testing. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Circulating Insulin and Ketones

For insulin, whole blood was collected in the morning, following a 12-h fast, from the tail vein by capillary tube (Sarstedt, cat. no. 16440100). Serum was isolated by centrifugation at $10,000g$ for 20 min, and insulin concentration was measured using a commercially available kit (Millipore, cat. no. EZRMI-13K). Ketones were measured following a 12-h fast from blood collected from the tail vein using a commercially available handheld meter (Precision Xtra; Abbott, cat. no. 9881465).

Exercise Capacity Testing

Sedentary and exercise-trained mice were acclimated to a modular treadmill for 2 consecutive days before undergoing aerobic exercise capacity testing to exhaustion using the graded mouse maximal exercise test (GXTm) protocol (30). Mice were motivated to run with a shock grid operating at 0.56 mA. Exhaustion was defined as failure to return to the treadmill from the rest platform after three consecutive attempts within the last minute of running. Exercise capacity was expressed as time to exhaustion (TTE). Pretraining exercise capacity was measured after 8 weeks of Con, STZ, or Cana treatment and repeated after 6 weeks of voluntary wheel running in exercise-trained mice. Age-matched sedentary Con mice were tested alongside exercise-trained mice to control for the effects of age, repeated testing, and experimental conditions on exercise capacity. Access to running wheels was restricted for ~ 24 h before testing.

VO_{2peak} Testing

VO_{2peak} testing was performed in STZ- and Cana-treated mice. Mice underwent exercise capacity testing using the GTXm protocol, as described above. A comprehensive laboratory animal monitoring system (CLAMS) was used to collect VO_2 and VCO_2 in 30-s intervals, and mice ran to exhaustion. All mice reached a plateau in VO_2 at or before the last gas collection interval. VO_{2peak} values were normalized to lean mass determined by DEXA to avoid potentially confounding effects of reduced fat mass due to Cana treatment in sedentary mice.

Acute Exercise Experiments

A separate cohort of CD-1 mice was treated with Con, STZ, or Cana protocols for 16 weeks in the absence of exercise training. To mimic the glycemic phenotype observed in the exercise training study, only STZ mice with >200 mg/dL random glucose were included in acute exercise experiments. All mice were treadmill acclimated for 2 consecutive days (Panlab, Harvard Apparatus). On the morning of experiments, mice were fasted for 2 h before completing an acute exercise bout of 30-min moderate-intensity running at a 5° incline and speed of 11 m/min. A shock grid was activated on contact (0.1 mA intensity) during the acute exercise, but motivational stimulus was seldom required at this moderate running speed. Following completion of the exercise bout, mice were immediately anesthetized with isoflurane. Gastrocnemius muscle was quickly harvested, snap frozen in liquid N_2 , and stored at

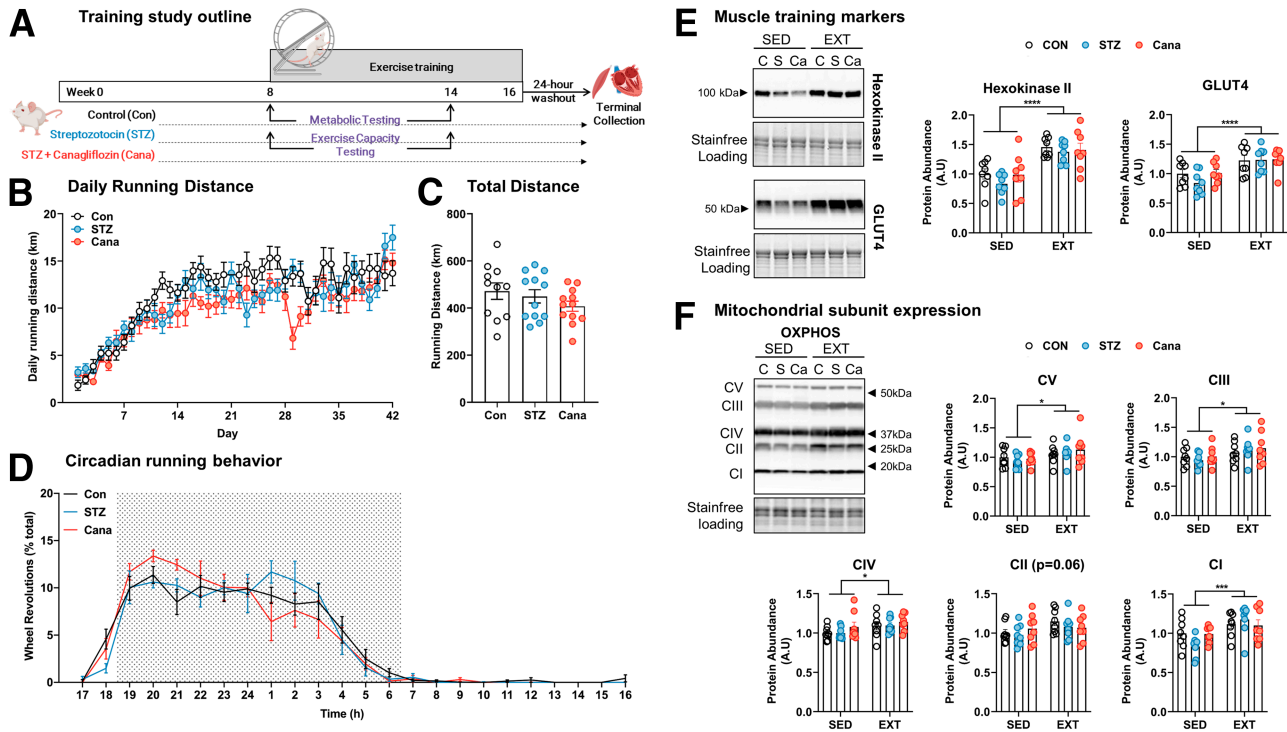


Figure 2—Hyperglycemia and Cana do not alter running behavior or protein markers of training in muscle. **A**: The exercise training paradigm and time points of key measurements are indicated in the schematic diagram. Voluntary running distance was measured daily over 6 weeks (**B**), and the cumulative distance was recorded from mice with access to wheels (**C**). **D**: Wheel running behavior was assessed within a 24-h window, and hourly revolutions were recorded. At the end of the wheel running period, key training markers hexokinase (HKII) and GLUT4 (**E**) and OXPHOS (mitochondrial complexes CI–CV) (**F**) were measured by Western blotting in gastrocnemius muscle from sedentary (SED) and exercise-trained (EXT) groups. Exercise-trained mice selected for Western blotting analysis ($n = 8$) were matched for total running distance. $n = 11$ – 15 per group in **B**–**D** and $n = 8$ per group in **E** and **F**. Points and columns with error bars represent mean \pm SEM. Group differences were assessed by one-way ANOVA in **B**–**D** or two-way ANOVA in **E** and **F**. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$. Panel **A** was created with Biorender.com.

–80°C for subsequent analysis. For signaling experiments, tissues were collected in the same manner from sedentary Con mice that did not complete an acute exercise bout.

Steady-State Lactate Testing

A separate cohort of Con, STZ, and Cana mice performed an acute bout of treadmill running at 11 m/min at a 5° incline for 30 min. Blood was collected pre- and postexercise via tail snip using a handheld analyzer to measure blood lactate (Lactate Plus, Nova Biomedical).

Western Blotting

Gastrocnemius muscle samples were pulverized in liquid N₂, lysed in modified radioimmunoprecipitation assay buffer (50 mmol/L Tris-HCl [pH 7.5], 1 mmol/L EDTA, 10% [v/v] glycerol, 1% [v/v] Triton-X, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/L dithiothreitol, Pierce protease/phosphatase inhibitor cocktail), and homogenized with a TissueLyser (Qiagen). Loading samples containing equal protein concentrations were heated to 95°C for 5 min in Laemmli buffer, except for GLUT4 and oxidative phosphorylation (OXPHOS) blots, as these antibodies recommend unboiled samples. Criterion TGX 4–15% gradient

gels (Bio-Rad) were used to separate proteins. Stain-free technology (Bio-Rad) was used to determine equal loading, and gels were transferred to nitrocellulose membranes. Membranes were blocked with Tris-buffered saline (TBS) with Tween + 5% nonfat dry milk for 1 h at room temperature or overnight at 4°C for phosphorylated (p)-JNK. Membranes were incubated with primary antibodies overnight at 4°C or 1 h at room temperature for p-JNK, followed by incubation with appropriate horseradish peroxidase-conjugated secondary antibodies and visualization with a ChemiDoc Touch imaging system (Bio-Rad). The following antibodies were used for the detection of total or phosphorylated protein levels: hexokinase II (CST 2867), GLUT4 (Abcam 654), OXPHOS (Abcam 110413), p-JNK (CST 4668), and p-SMAD2-L (CST 3104). All primary antibodies were used at 1:1,000 dilution in TBS with Tween + 5% BSA, except for p-SMAD2-L, which was diluted at 1:500.

Mouse Muscle Histology

Gastrocnemius muscles were frozen with liquid N₂-cooled isopentane during terminal dissections. Muscles were sectioned in a cryostat at a thickness of 6 μ m at –20°C. For ECM quantification, Oregon Green wheat germ agglutinin

(WGA; 1:1,000; Thermo Fisher, W6748) in 1% BSA in TBS + 1% normal goat serum (NGS) was applied to muscle cross sections for 1 h at room temperature. After imaging, fractional ECM area was analyzed via Fiji software. Capillary density was measured with fluorescein-conjugated *Griffonia simplicifolia* lectin (1:100; Vector Laboratories, cat. FL-1101-2) in PBS for 1 h. To quantify muscle fiber type, slides were incubated with primary antibodies against myosin heavy chain I (1:25; Hybridoma Bank) and myosin heavy chain IIa (1:25; Hybridoma Bank, SC-71) in TBS + 1% BSA + 1% NGS overnight at 4°C. After rinsing, mouse IgG1 fluorescent conjugate (A21124; red) and mouse IgM (A21042; green) secondary antibodies were added at 1:1,000 in 1% BSA in TBS + 1% NGS for 1 h. Oregon Green WGA (1:1,000; Thermo Fisher, W6748) was added with secondary antibodies for visualization of unstained fibers. The percentage of oxidative fibers was determined using counts of ~1,500 fibers from images of the red gastrocnemius, which contains the highest density of oxidative fibers.

Human Participants

Characteristics of human participants were originally published (15). Additional muscle ECM analysis was performed using samples from these participants, as described below. A sufficient sample was available to perform ECM analysis on 21 of 23 study participants. VO_{2peak} , muscle JNK activation with acute exercise, and the oral glucose tolerance test (OGTT)-derived insulin sensitivity (siOGTT) index was calculated as previously described (15) and used for new correlation analysis with ECM in the present investigation. Written informed consent was obtained for all participants enrolled. The Joslin Diabetes Center Committee on Human Studies approved the study and its procedures.

Human Muscle Histology

Skeletal muscle biopsy samples were frozen in N_2 -cooled isopentane, and 6- μ m sections were cut using a cryostat. ECM was visualized by staining with WGA for 1 h. Exposure time was held equally across samples, and average pixel intensity per image was measured using Fiji. Two images per participant were measured, and the average value was used for correlation analysis.

Statistics and Calculations

Column and point graphs with error bars are presented as mean \pm SEM. One-way ANOVA was used to compare effects between Con, STZ, and Cana groups. For all comparisons between sedentary and exercise-trained groups, a two-way ANOVA was used to determine main effects of 1) glycemia or 2) training. Both ANOVA approaches were followed by Holm-Šidák post hoc testing. Nonparametric Spearman coefficients were calculated where correlations are indicated. In all analyses, significance was accepted as $P < 0.05$. Statistics and calculations were completed in GraphPad Prism 8 software (GraphPad Software).

Data and Resource Availability

The data sets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

RESULTS

Cana Normalizes Blood Glucose in STZ-Treated Mice

Our previous work demonstrated that hyperglycemic mice fail to improve VO_{2peak} after 6 weeks of voluntary wheel running (15). To test whether glucose lowering with the SGLT2 inhibitor, Cana, can restore the response to training, hyperglycemia was induced in CD-1 mice using STZ, and half of the mice were treated with Cana. Con mice did not receive STZ or Cana treatment. STZ treatment significantly increased blood glucose compared with Con; however, Cana prevented increased blood glucose in STZ-treated mice (Fig. 1A). As a result, the AUC for glucose over the first 8 weeks of the study period was significantly higher in STZ compared with Con and Cana (Fig. 1B).

Metabolic Impact of Cana Treatment

Consistent with a Cana-induced improvement in glycemia, a glucose tolerance test administered 8 weeks following treatment initiation revealed that Cana prevented severe glucose intolerance in STZ-treated mice (Fig. 1C and D). Improved glycemia in Cana-treated mice occurred despite a similar ~40% reduction in circulating insulin in both STZ and Cana, compared with Con (Fig. 1E). In contrast, blood ketones were increased only in the Cana group (Fig. 1F). In obese models, glucose excretion with Cana treatment can also result in significant weight loss (28,29). We chose to study a lean model of hyperglycemia to mitigate weight loss and found that body weights were similar among groups over the initial 8-week treatment period (Fig. 1G). Body weight was maintained in Cana likely due to increased food intake, which was elevated above the level of Con and STZ (Fig. 1H).

Running Behavior Is Maintained With Hyperglycemia and Cana

To determine the effect of Cana-induced glucose lowering on training response, mice were assigned to undergo exercise training (voluntary wheel running) or remain sedentary for a further 6 weeks (Fig. 2A). Daily running distance was similar among groups and was not affected by hyperglycemia or Cana treatment (Fig. 2B). Total distance run over the study period was ~450 km for all groups (Fig. 2C). Circadian running behavior was quantified in a subset of mice and demonstrated no substantial differences in running patterns among groups (Fig. 2D). Common skeletal muscle training markers, including GLUT4, hexokinase II, and mitochondrial (OXPHOS) subunits were increased similarly by exercise training in all groups (Fig. 2E and F). These results demonstrate equal training stimulus was achieved in all trained mice and indicate that any

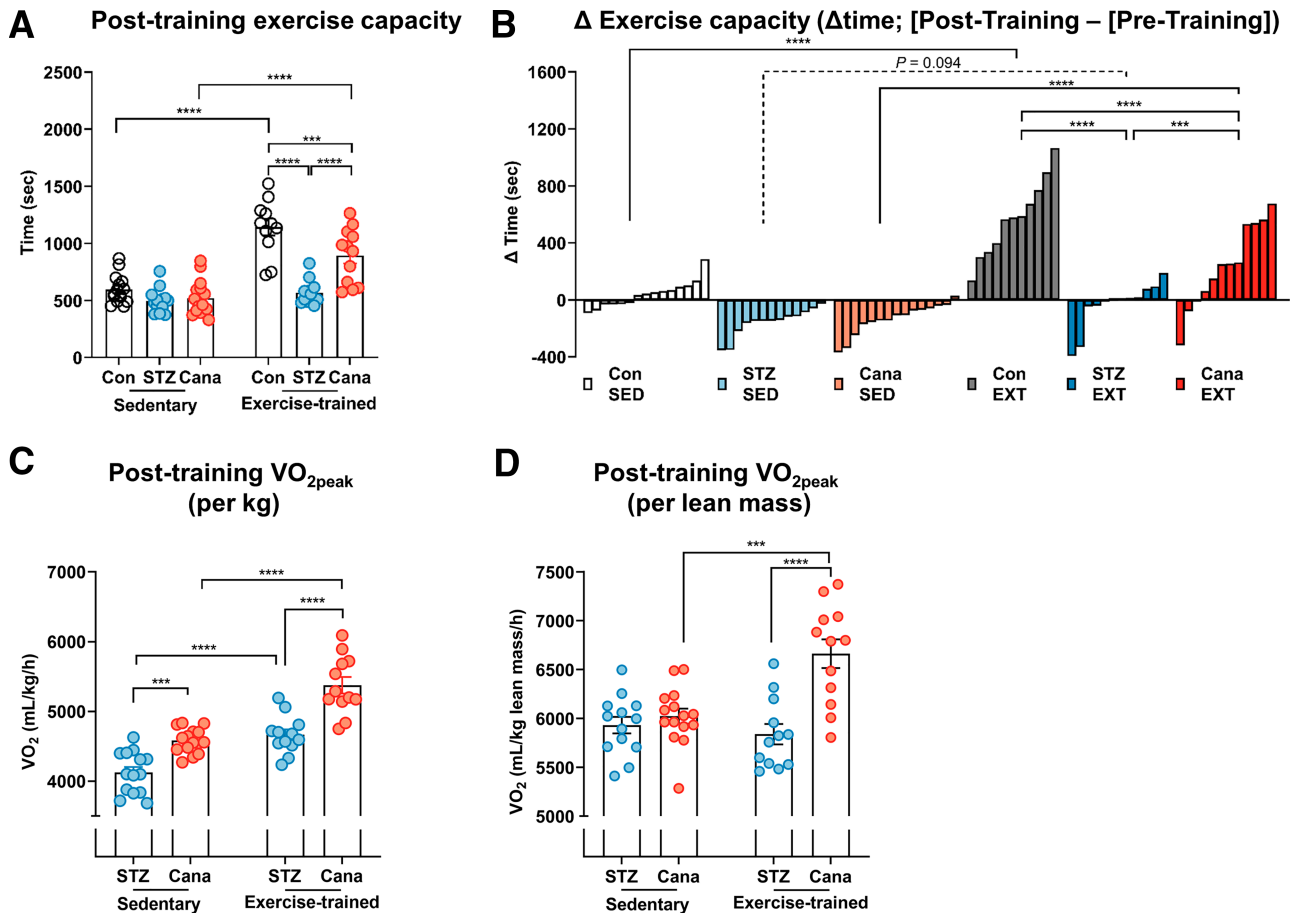


Figure 3—Improvements in exercise capacity with training are blunted in hyperglycemic mice but restored by Cana. **A:** After 6 weeks of wheel running, exercise capacity, expressed as running time to exhaustion, was similar among sedentary (SED) groups, and increased with exercise training (EXT) in Con and Cana groups. In contrast, exercise capacity did not improve in hyperglycemic STZ-treated mice. **B:** The individual change in exercise capacity (Δ exercise capacity) was calculated by subtracting baseline (pretraining) capacity from the postexercise training capacity (Δ time = posttraining – pretraining). VO_{2peak} was measured in STZ and Cana mice and expressed in per kg total body mass (**C**) and per lean mass (**D**) assessed by DEXA. $n = 11$ – 15 per group in **A–D**. Columns with error bars represent mean \pm SEM. Group differences were assessed by two-way ANOVA with Holm-Sidak post hoc testing for **A–D**. *** $P < 0.001$, **** $P < 0.0001$.

differences in training adaptation among groups were not due to alterations in running distance or patterns.

Cana Restores Improvements in Aerobic Exercise Capacity With Training

Aerobic exercise capacity was measured with an incremental treadmill running test before and after 6 weeks of voluntary wheel running in exercise-trained mice and age-matched sedentary Cons. Final exercise capacity, expressed as TTE, was not different among sedentary mice. However, exercise capacity in STZ was significantly lower than Con in exercise-trained mice and was not significantly increased by exercise training (Fig. 3A), demonstrating a phenotype of LRT. Cana restored training response in STZ-treated mice, leading to a significantly higher exercise capacity in trained mice (Fig. 3A). When data were expressed as the change in exercise capacity over time (Δ TTE = posttraining – pretraining), the mean improvement of exercise capacity in trained STZ-treated mice was significantly lower than

Con. The defective training response in STZ-treated mice was improved by Cana but remained lower than Con (Fig. 3B). When assessed per kg body weight, VO_{2peak} (mL/kg/h) was higher in sedentary and exercise-trained mice treated with Cana compared with STZ-treated mice (Fig. 3C). However, when VO_{2peak} was corrected for lean mass, higher VO_{2peak} with Cana was only observed in exercise-trained mice (Fig. 3D). These results suggest that improved VO_{2peak} in sedentary mice was due to Cana-induced reductions in fat mass rather than enhanced aerobic capacity. In contrast, improved VO_{2peak} in trained mice was independent of adiposity. Taken together, our results support the hypothesis that hyperglycemia can induce LRT and that prevention of hyperglycemia with SGLT2i can enhance training response, leading to higher aerobic capacity.

Metabolic Effects of Cana and Exercise Cotherapy

We next determined the effect of Cana treatment on other key health benefits of exercise. Random blood glucose was

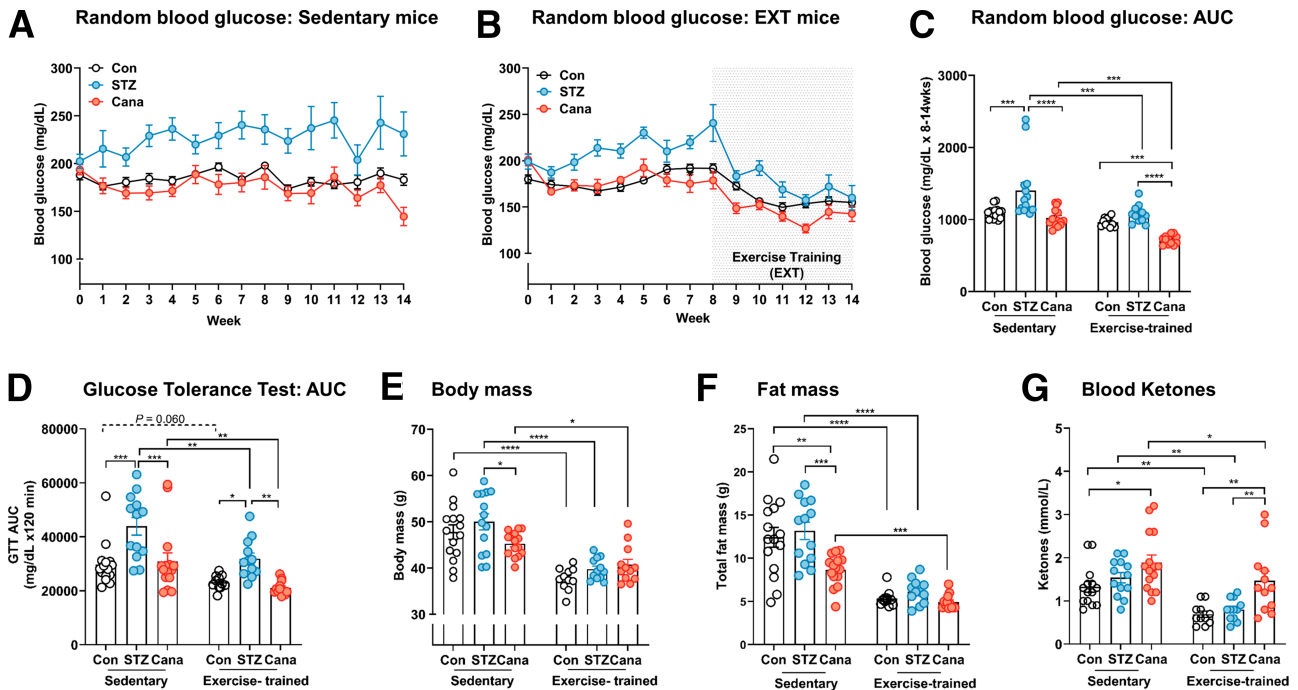


Figure 4—Exercise-training induces metabolic improvements in mice regardless of glycemic status. Random blood glucose was measured weekly in all mice for 8 weeks, after which mice were randomized to remain sedentary (A) or undergo exercise training (EXT) (B) for a further 6 weeks. Data are shown for the entire study duration in A and B, with statistical analysis shown in C. In C, the AUC for glucose was calculated per mouse to reflect the 6-week (weeks 8–14) training period. D: Glucose tolerance tests (GTT) were performed in EXT mice and age-matched sedentary controls, and the glucose AUC was calculated. Body mass was measured in sedentary mice and EXT mice (E), and body composition (fat mass) was measured by DEXA (F). G: Circulating ketones were measured in all groups after a 12-h fast. $n = 11$ – 15 per group for A–G. Points and columns with error bars represent mean \pm SEM. Group differences were assessed by one-way ANOVA in A and B or two-way ANOVA in C–G, followed by Holm-Šidák post hoc testing. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$.

improved by Cana and exercise in STZ-treated mice (Fig. 4A and B), with combination treatment resulting in the lowest AUC for glucose during the 6-week treatment period (Fig. 4C). Glucose tolerance was also improved by exercise and Cana treatments, although no additional effect of Cana was noted in exercise-trained mice (Fig. 4D). Body mass (Fig. 4E) and fat mass (Fig. 4F) were lower in all exercise-trained groups compared with sedentary. Importantly, body mass and fat mass were similar among all exercise-trained mice, indicating Cana-associated improvements in exercise capacity with training (Fig. 3) cannot be attributed to weight loss. Blood ketones were decreased in all groups by exercise but remained higher in Cana compared with Con and STZ (Fig. 4G). Overall, exercise resulted in improved metabolic health in all treatment groups, regardless of glycemic status. These results suggest that in a lean model of hyperglycemia, the primary health benefit of Cana/exercise cotherapy is preservation of improvements in aerobic exercise capacity.

Cana Prevents STZ-Induced Defects in Muscle Remodeling

Blunted improvements in exercise capacity with hyperglycemia are associated with impaired muscle remodeling toward an aerobic phenotype (15). To test whether impaired muscle remodeling can be prevented by Cana, we measured fiber

type and capillary density in sedentary and exercise-trained mice. The proportion of oxidative (type I/IIA) fibers in red gastrocnemius muscle was increased by exercise training in all groups (Fig. 5A). However, in trained mice, STZ had a significantly lower proportion of oxidative fibers than Con and Cana, indicating that oxidative fiber type switching was impaired by hyperglycemia and that this was prevented by Cana. Exercise-induced increases in capillary density were also blunted by hyperglycemia and failed to increase in STZ with training (Fig. 5B), but impaired exercise-induced angiogenesis was prevented by Cana. A similar pattern was observed in cardiac muscle, where in exercise-trained mice, Cana significantly improved capillary density compared with STZ (Fig. 5C). Muscle fiber type and capillary density were correlated with exercise capacity in trained mice (Fig. 5D–F). These data demonstrate that improvements in exercise capacity with Cana treatment are associated with improved aerobic remodeling of cardiac and skeletal muscle.

Cana Prevents Hyperglycemia-Associated Muscle ECM Accumulation and Signaling Defects

Low exercise response with hyperglycemia is associated with accumulation of ECM in skeletal muscle, and higher JNK/SMAD signaling with acute exercise (15,31). To determine whether Cana can prevent muscle ECM accumulation and restore signal transduction with acute

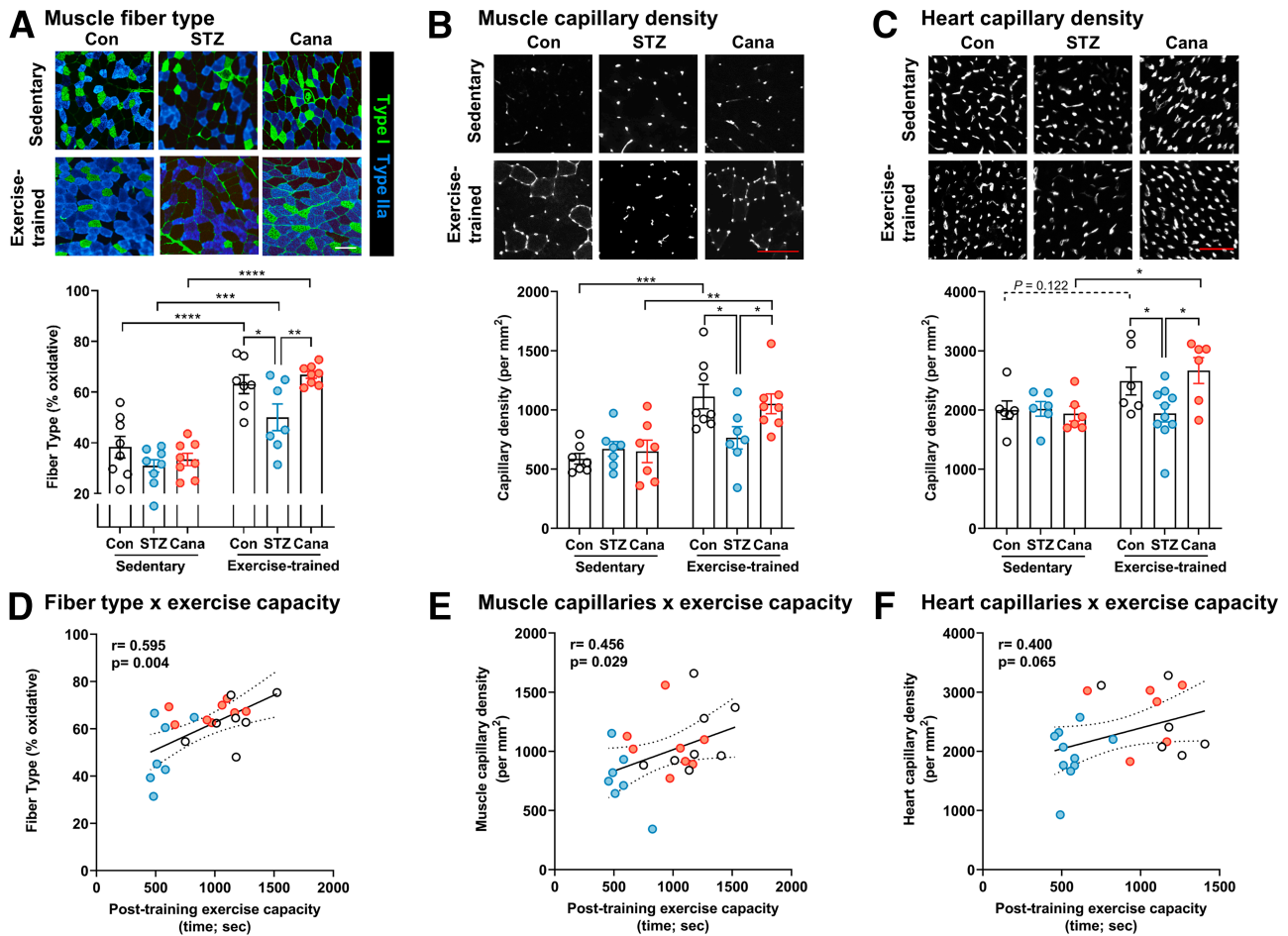


Figure 5—Aerobic remodeling of heart and skeletal muscle is restored by Cana treatment. Gastrocnemius muscle and heart (left ventricle) were collected for histological analysis of fiber type and/or capillary density in sedentary Con and exercise-trained mice as indices of training-induced muscle remodeling. **A:** Exercise-induced shifts in muscle fiber type toward an oxidative (i.e., type I and IIa) profile were blunted in STZ compared with Con, but were restored by Cana. Scale bar = 100 μm . **B:** Skeletal muscle capillary density was measured using *Griffonia* lectin staining. Increases in capillary density with exercise training were impaired in STZ but were restored by Cana. Scale bar = 100 μm . **C:** In exercise-trained mice, cardiac muscle capillary density was higher in Cana compared with STZ alone. Scale bar = 50 μm . Skeletal muscle fiber type (**D**), skeletal muscle capillary density (**E**), and heart capillary density (**F**) were each correlated with exercise capacity in exercise-trained animals. $n = 6$ –10 per group. Columns with error bars represent mean \pm SEM. Group differences were assessed by two-way ANOVA with Holm-Sidak post hoc testing. Nonparametric Spearman coefficients were calculated in **D**–**F**. Dotted lines in **D**–**F** represent the 95% confidence bands of the best-fit line. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

exercise in STZ-treated mice, we studied a separate cohort of untrained mice. Blood glucose was higher in STZ compared with Con, but normoglycemia was maintained by Cana (Fig. 6A). Skeletal muscle ECM fractional area was significantly higher in STZ compared with Con, but ECM accumulation was prevented by Cana (Fig. 6B). With acute exercise, JNK was more highly activated in STZ compared with Con (Fig. 6C and D). Importantly, this hyperactivation of JNK, which can impair aerobic remodeling with exercise, was prevented by Cana. We previously demonstrated that SMAD2—a key regulator of muscle phenotype via transforming growth factor- β /myostatin—can be phosphorylated by JNK in its Ser245/250/255 linker region (31). Consistent with this, SMAD2-linker phosphorylation was significantly correlated with JNK activation in all groups (Fig. 6E). Clinical data demonstrate that

exercise intolerance in people with diabetes is associated with higher blood lactate levels compared with control subjects without diabetes, even when working at the same exercise intensity (32). Here we demonstrate that STZ-treated mice have higher lactate levels following 30 min of moderate aerobic exercise compared with Con (Fig. 6F), but this was prevented by Cana. Taken together, our data demonstrate that treatment with Cana can prevent muscle ECM accumulation and deleterious changes to the acute response to exercise in STZ-treated mice.

Muscle ECM Accumulation Is Associated With JNK Activation and Low $\text{VO}_{2\text{peak}}$ in Humans

Rodent data demonstrate that muscle ECM accumulation and hyperactivation of JNK with acute exercise are associated with lower $\text{VO}_{2\text{peak}}$ (14,15,31). To test whether these

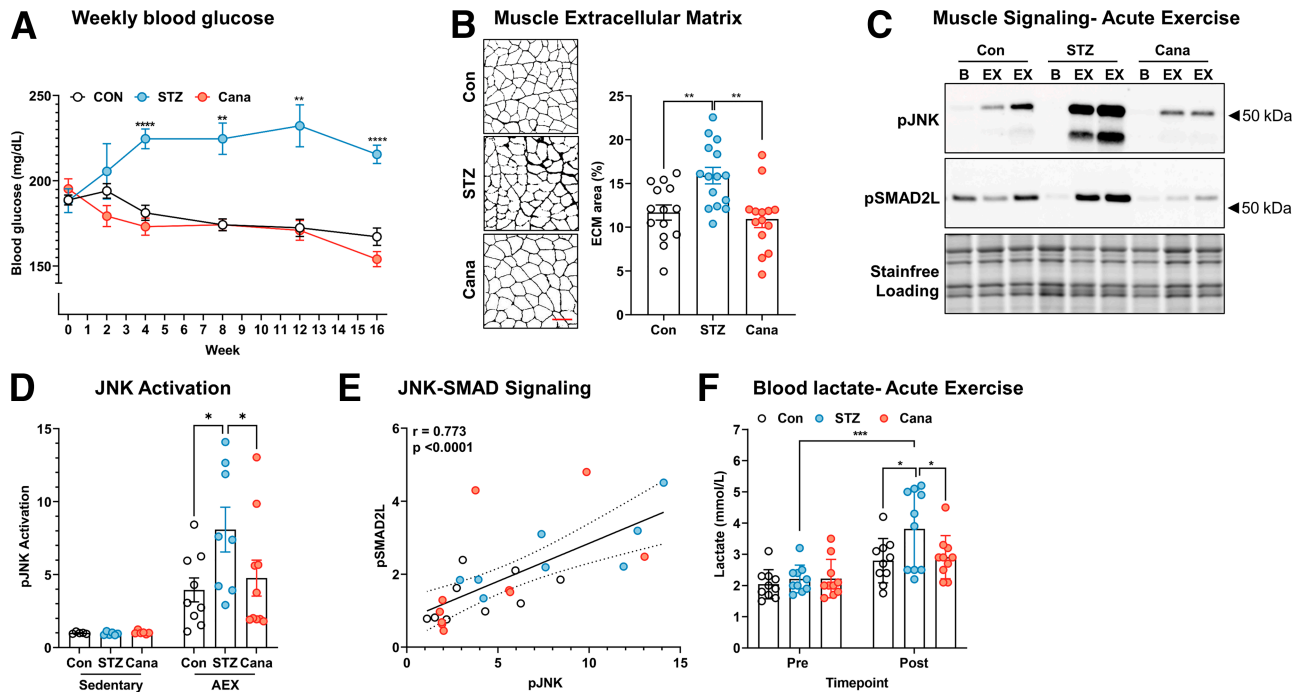


Figure 6—Hyperglycemia-induced ECM accumulation and JNK hyperactivation with acute exercise are normalized by Cana. *A*: Blood glucose was measured over 16 weeks in a separate cohort of Con and STZ- and Cana-treated mice. *B*: Muscle ECM was visualized with WGA, and the percentage area was quantified for each mouse. Scale bar = 50 μ m. *C*: Prior to tissue collection, mice underwent 30 min of moderate treadmill exercise (EX) or remained as sedentary controls. B, basal. *D*: JNK activation was measured by Western blotting and quantified. AEX, acute exercise. *E*: JNK activation was significantly associated with phosphorylation of its downstream effector, SMAD2 in its linker region (pSMAD2L). Dotted lines represent the 95% confidence bands of the best-fit line. *F*: Blood lactate was higher following acute exercise in STZ, but this effect was normalized by Cana. $n = 8$ –18 per group. Points and columns with error bars represent mean \pm SEM. Group differences were assessed by one-way ANOVA in *A* and *B* or by two-way ANOVA in *D* and *F*, followed by Holm-Šidák post hoc testing. A nonparametric Spearman coefficient was calculated in *E*. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

mechanisms may also be relevant in humans, we measured muscle ECM area in participants with a range of glucose tolerances. We then determined whether muscle ECM area is associated with 1) JNK activation with acute exercise, 2) VO_{2peak} , and 3) metabolic health. We demonstrate a significant correlation between ECM area in human skeletal muscle and the degree of JNK activation with exercise (Fig. 7A and B). In addition, we demonstrate that ECM area is negatively associated with VO_{2peak} and the siOGTT index in these participants (Fig. 7C and D). siOGTT is a marker of metabolic health calculated from an OGTT that strongly predicts VO_{2peak} in human populations (10). Thus, our data from humans and animal models support the hypothesis that ECM accumulation and JNK hyperactivation are novel mechanisms that can contribute to low VO_{2peak} associated with hyperglycemia. A working model detailing these mechanisms is presented in Fig. 8.

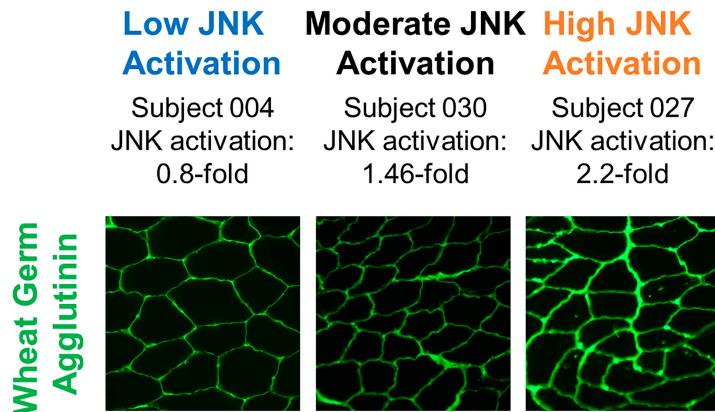
DISCUSSION

The use of antidiabetes medications, including SGLT2i, to enhance VO_{2peak} has been tested with mixed results, with some studies showing benefits and others demonstrating no improvement (28,29,33–36). A common side effect of SGLT2i is weight loss, which can make it difficult to

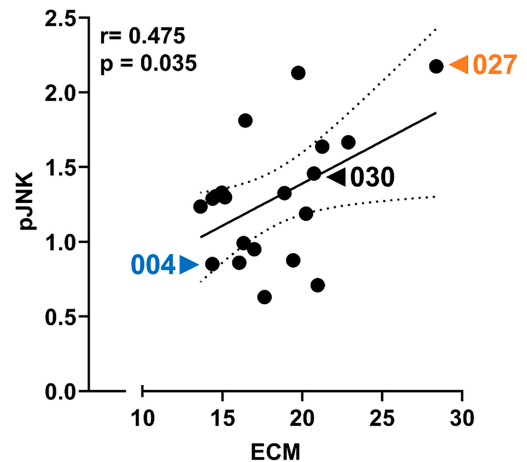
assess whether improvements in VO_{2peak} associated with treatment are due to improved aerobic capacity or an artifact attributed to normalization of VO_{2peak} for body weight. Our results suggest that in the absence of exercise training, SGLT2i-induced improvements in VO_{2peak} may be attributed to loss of fat mass. Importantly, we also demonstrate adiposity-independent effects of SGLT2i to improve VO_{2peak} when combined with aerobic training.

Our data indicate that glucose lowering is a key mechanism for prevention of LRT, as SGLT2i treatment can prevent hyperglycemia-associated impairments in muscle remodeling and signal transduction that contribute to blunted improvements in VO_{2peak} . SGLT2 inhibitors are a relatively new class of drugs; thus, few clinical studies to date have examined their effects in combination with exercise. In one investigation, Newman et al. (27) (2019) demonstrated that Cana did not impact changes in VO_{2peak} with aerobic training in humans. However, participants in that study did not have hyperglycemia, and therefore, did not experience metabolic improvements with Cana treatment. Thus, the results of Newman et al. (27) support our contention that glucose-lowering is one mechanism by which Cana can improve aerobic capacity when combined with exercise.

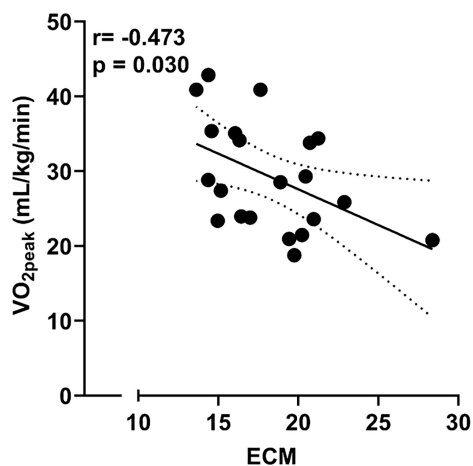
A Human Skeletal Muscle ECM



B ECM x JNK Activation



C ECM x VO_{2peak}



D ECM x siOGTT

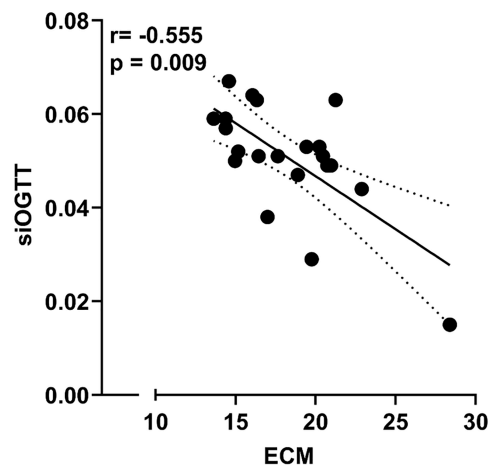


Figure 7—Muscle ECM correlates with JNK activation during acute exercise, VO_{2peak}, and metabolic health in humans. *A*: Muscle ECM was stained with WGA in vastus lateralis muscle collected from human participants. Representative images are shown for subjects with low, moderate, or high JNK activation in response to 30 min of moderate-intensity cycling exercise. JNK activation in these participants was originally reported in (15). *B*: A significant positive correlation was observed between muscle ECM content and JNK activation with acute exercise. Muscle ECM content was negatively associated with VO_{2peak} (*C*) and siOGTT (*D*). $n = 21$ for ECM, VO_{2peak}, and siOGTT measurements; $n = 20$ for pJNK. Nonparametric Spearman coefficients were calculated in *B–D*. Dotted lines in *B–D* represent the 95% confidence bands of the best-fit line.

SGLT2 is predominantly expressed in the kidney, and enhanced renal glucose excretion is a primary physiological consequence of SGLT2 inhibition (37). However, it is possible that mechanisms other than glucose lowering could contribute to the beneficial effects we observed with exercise/SGLT2i cotherapy. We demonstrate elevated ketones in sedentary and trained mice treated with Cana, and increased ketone oxidation has been suggested as a potential mechanism for some benefits of SGLT2i (38,39). Improved cardiovascular parameters have also been linked with SGLT2i treatment (40). Consistent with this, we observed improved cardiac capillary density, which can enhance cardiac function, in mice treated with Cana and

exercise. We also show that blood lactate accumulation with submaximal exercise is elevated in hyperglycemic STZ mice compared with Con, but this effect is prevented by Cana treatment. Thus, ameliorating the metabolic response to acute exercise may be another benefit imparted by exercise and SGLT2i cotherapy.

While our preclinical data suggest prevention of LRT is possible, one key question that remains is whether SGLT2i can restore the response to exercise training in individuals with longstanding hyperglycemia. The time course for reversal of established LRT associated with hyperglycemia and whether other glucose-lowering strategies (e.g., dietary interventions or insulin) can restore

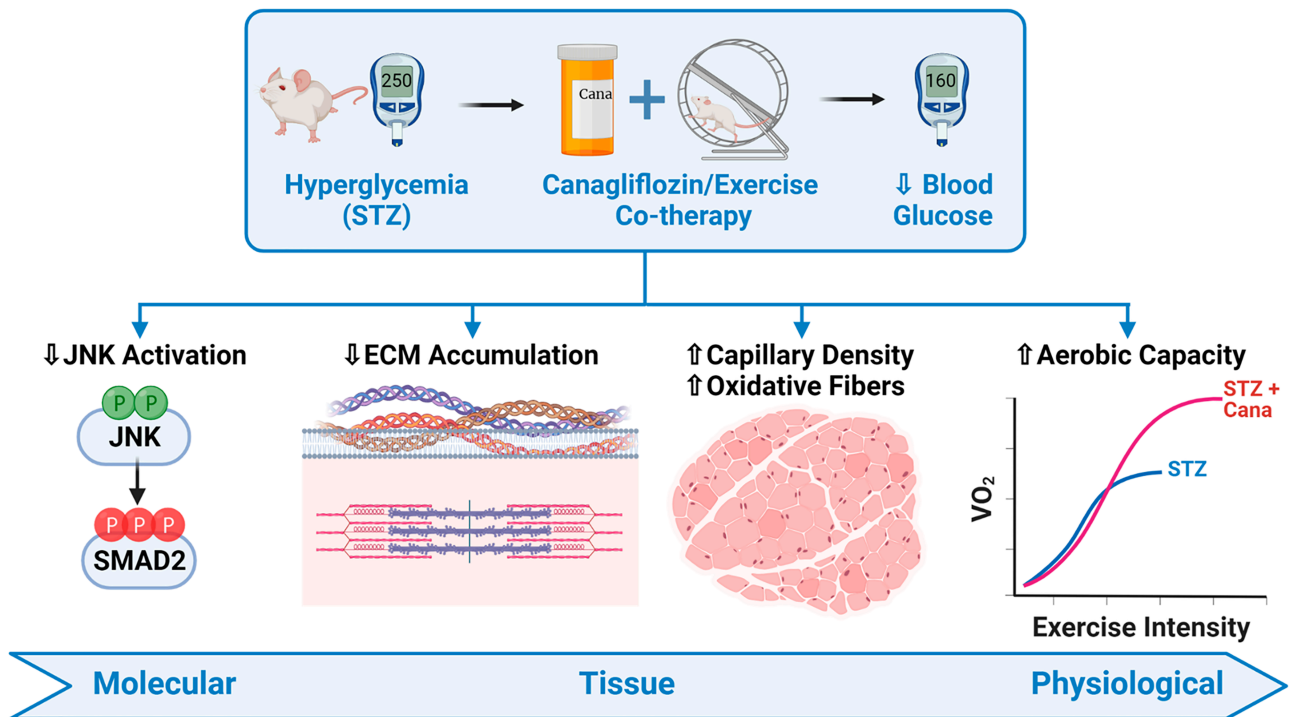


Figure 8—Hypothesized mechanisms by which Cana improves exercise response in a model of hyperglycemia. STZ treatment induced hyperglycemia in CD-1 mice, which was reversed by Cana treatment. Blood glucose lowering with Cana attenuated muscle ECM accumulation that normally occurs with hyperglycemia. At the molecular level, reduced mechanical signaling via JNK was associated with lower ECM accumulation in muscle. In muscle tissue, these changes in signaling were associated with increased capillary density and a more oxidative fiber type following exercise training. At the physiological level, we propose Cana-induced changes in the acute and chronic responses to exercise contribute to improved aerobic exercise capacity (VO_{2peak}). In support of this model, muscle ECM accumulation was also associated with elevated JNK signaling and low VO_{2peak} in human participants. Figure was created with Biorender.com.

exercise response are key next steps. Importantly, despite having blunted improvements in some aspects of the adaptive response, STZ mice still had multiple positive adaptations to exercise, including increased muscle GLUT4 and mitochondrial proteins, and improved whole-body metabolism. Therefore, it appears that LRT impacts selective exercise adaptations related to VO_{2peak} , while other key benefits remain intact, suggesting that regular exercise is of great clinical value, even in the presence of chronic hyperglycemia.

The phenomenon of individual variance in response to aerobic training was given prominence by the HERITAGE (HEalth, RIsk factors, exercise Training And GENetics) Family Study (11) and has since been the subject of numerous investigations (13,41–44). Efforts to pinpoint a mechanism for LRT illustrate that the phenotype is complex and attributed to a combination of inherited and environmental factors (45). Hyperglycemia, which can have both genetic and environmental underpinnings, has emerged as a potential negative regulator of training response (9,10,15). Several lines of evidence indicate that chronic hyperglycemia leads to accumulation of the skeletal muscle ECM and that this is associated with LRT (15). The LRT phenotype is also associated with differential expression of ECM genes in human skeletal muscle (46),

and reversing ECM accretion may partly mediate exercise-induced improvements in insulin sensitivity (47). We now demonstrate that Cana can prevent muscle ECM accumulation and prevent LRT in a mouse model of hyperglycemia. We also show that in humans, muscle ECM accretion is associated with low VO_{2peak} . Thus, data from animal and human investigations indicate that aberrant ECM remodeling secondary to hyperglycemia may contribute to LRT. How specific changes to muscle ECM induced by hyperglycemia impact muscle remodeling and improved VO_{2peak} with exercise should be addressed by future studies.

Molecular screening of rats genetically selected for LRT identified JNK activation during exercise as a potential regulator of impaired aerobic remodeling of skeletal muscle with training (14). Follow-up studies of muscle-specific JNK-knockout mice demonstrated that JNK activation is sufficient to prevent aerobic remodeling of muscle, including exercise-induced angiogenesis and a switch to a more oxidative fiber type. JNK activation in muscle was also sufficient to blunt improvements in aerobic exercise capacity with training (31), confirming this signaling pathway as a molecular mechanism for LRT. The precise mechanisms for JNK hyperactivation during exercise in the context of hyperglycemia are unknown. However, our data indicate that ECM accumulation is consistently

associated with increased JNK signaling in muscle during aerobic exercise in multiple animal models (14,15) and human participants (Fig. 7), suggesting a potential link between these two mechanisms. We now demonstrate that Cana can prevent hyperactivation of JNK with acute aerobic exercise in a model of hyperglycemia. These data provide a molecular signaling mechanism by which Cana may improve muscle remodeling and VO_{2peak} in response to aerobic training.

In summary, our data demonstrate that Cana can prevent hyperglycemia-associated impairments to the acute and chronic responses to aerobic exercise, leading to improved aerobic exercise capacity. In addition, we provide evidence of novel mechanisms that may lead to hyperglycemia-associated declines in VO_{2peak} in humans and animals. Our results suggest that cotherapy with exercise and SGLT2 inhibition may work to enhance VO_{2peak} in people living with hyperglycemia, which represents a steadily increasing population. Given the strong associations between low VO_{2peak} and diabetes onset, complications, and mortality, these results may have important clinical implications.

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Author Contributions. T.L.M., P.P., E.M.C., R.C.N., and S.J.L. performed mouse experiments. T.L.M., P.P., E.M.C., R.C.N., and S.J.L. analyzed and interpreted the data. T.L.M., R.C.N., and S.J.L. designed the experiments. T.L.M., J.M., S.H., and S.J.L. performed studies with human subjects. T.L.M. and S.J.L. conceived the initial study. T.L.M. and S.J.L. drafted the manuscript. P.P., E.M.C., R.C.N., J.M., and S.H. read and approved the manuscript. S.J.L. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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