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Attenuation of type 2 diabetes mellitus in the male Zucker diabetic fatty rat: the effects of stress and non-volitional exercise

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Abstract

To date, a limited number of studies have investigated the effects of exercise on the maintenance of endocrine pancreatic adaptations to worsening insulin resistance. In particular, the roles of stress hormones that are associated with commonly used forced-exercise paradigms are not fully explained. To examine the effects of exercise per se in ameliorating pancreatic decompensation over time, we investigated the role of forced swimming and sham exercise stress on the development of type 2 diabetes mellitus in the Zucker diabetic fatty (ZDF) rat. Thirty-two male ZDF rats were obtained at 5 weeks of age and all went through a 1-week acclimatization period. They were then divided into 4 groups: basal (euthanized at 6 weeks of age), exercise (1 h/d; 5 d/wk), sham exercise (sham), and non-treated controls (n = 8 per group). After 6 weeks of treatment, an intraperitoneal glucose tolerance test was performed and animals were euthanized for tissue analysis. By 5 weeks of treatment, controls had elevated fed and fasted glycemia (>11.1 and 7.1 mmol/L, respectively; both $P < .05$), whereas exercise and sham rats remained euglycemic. At euthanasia, there were elevations in fed insulin levels in exercise and sham rats compared with basal animals (both $P < .05$). Despite improvements in fed and fasting glucose levels in sham rats, glucose tolerance in sham-treated rats (intraperitoneal glucose tolerance test) was similar to controls, whereas glucose levels were similar in exercised trained and basal rats. After 6 weeks, gastrocnemius glycogen content was higher in exercised rats and sham rats when compared with age-matched controls, whereas muscle glucose transporter 4 levels were similar between groups. Compared with controls, the exercise group had increased beta cell proliferation, beta cell mass, and partial maintenance of normal islet morphology. Sham rats also displayed beta cell compensation, as evidenced by increased fasting insulin levels and partial preservation of normal islet morphology. Finally, at the time of euthanasia, plasma corticosterone was increased in sham and control rats but was at basal levels in the exercise group. In summary, both exercise and sham treatment delay the progression of type 2 diabetes mellitus in the male ZDF rat by distinct mechanisms related to pancreatic function and improvements in peripheral glucose disposal.

1. Introduction

Type 2 diabetes mellitus (T2DM) has become the most common metabolic disease in North America [1]. The cause of T2DM is unknown, but is coupled to obesity and a progressive decline in insulin sensitivity with eventual insulin deficiency that results in sustained hyperglycemia [2]. Several environmental factors including physical activity, stress, and nutrition are thought to influence the development of T2DM.

Studies using rodent models of obesity and insulin resistance, such as the Zucker diabetic fatty (ZDF) rat, are frequently used to elucidate many of the mechanisms responsible for the deterioration from a prediabetic state to overt T2DM [3-6]. Male ZDF rats develop a phenotype of obesity, insulin resistance, and eventually, hyperglycemia due to a leptin receptor mutation, resulting in a phenotype very similar to humans with T2DM [4]. In these rodents, glucose intolerance usually develops by age 8 weeks, followed by overt hyperglycemia by age 10 to 12 weeks [4,5]. In the prediabetic phase, ZDF rats maintain normoglycemia by a compensatory increase in beta cell function resulting in hyperinsulinemia [4,5,7]. This compensatory

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adaptation begins to fail as animals enter the *diabetic phase*, defined by a dramatic increase in beta cell apoptosis and a corresponding decrease in beta cell mass [5]. In obese prediabetic humans, there is a similar period of adaptive beta cell expansion followed by a marked reduction in beta cell mass as the disease progresses [8-10].

In rodent models, energy restriction [11,12], a reduction in fat mass [13], and increased exercise [14-17] improve insulin sensitivity and attenuate the development of T2DM. These interventions, which increase insulin action or sensitivity, are thought to result in a decreased demand for insulin and thus attenuate beta cell exhaustion. Interestingly, in humans with T2DM, regular exercise has been shown to enhance insulin response to either hyperglycemia or arginine stimulation [18], suggesting that there may be direct effects of exercise on pancreatic function. However, a paucity of such studies exists. Remarkably, the direct effects of exercise per se in maintaining or preserving endocrine pancreatic compensation and the compensatory beta cell response to worsening insulin resistance remain unclear.

Exercise is a short-term stressor eliciting a hormonal response that is common to other types of stressors. For example, short-term exercise leads to a rise in levels of plasma glucocorticoids [19], catecholamines [20], and glucagon [21], all of which exacerbate insulin resistance. Despite aggravating insulin resistance, it is possible that periodic elevations in stress hormones, such as glucocorticoids, may exert protective effects on the beta cell. In support of this, glucocorticoid exposure stimulates beta cell expansion in rodents [22-26]. Also, the diabetogenic effects of streptozotocin, alloxan, and partial pancreatectomy can be reduced by pretreatment with glucocorticoids or mild foot shock [23,27-32]. Moreover, frequent periodic stress in Otsuka Long-Evans Tokushima fatty rats, a model of T2DM, improves glycemic control and glucose tolerance, preventing 80% of T2DM in this model [33]. It is interesting, therefore, that although glucocorticoids cause insulin resistance and increased hepatic glucose that aggravates metabolic control in diabetes, they may confer some protective effect on the development of the disease by altering beta cell function in the prediabetic animal.

The purpose of this study, therefore, is to compare the effects of forced-exercise training with non-exercise stress on pancreatic function and the development of T2DM in the male ZDF rat. We show that episodic stress that increases circulating glucocorticoids, if elevated intermittently through either exercise or stress associated with forced exercise, may have protective effects on beta cell function and the development of hyperglycemia in this animal model of T2DM.

2. Research design and methods

2.1. Animals

Male ZDF rats (ZDF/Gmi-*fa/fa*) were obtained from Charles River Laboratories (Saint-Constant, Quebec, Canada) at 5 weeks of age with initial weights of 150 to 175 g. Rats were singly housed in opaque microisolation cages, handled daily, and kept at a constant temperature of 22°C to 23°C in humidity-controlled rooms on a standard 12 hour (7:00 AM to 7:00 PM) light/dark cycle. The animals were fed water and Purina 5001 chow ad libitum throughout the experiment. All experiments were approved by the Animal Care Committee of the Faculty of Medicine at the University of Toronto in accordance with regulations set forth by the Canadian Council for Animal Care.

2.2. Experimental design

Thirty-two male ZDF rats were obtained at 5 weeks of age and randomly divided into 4 groups (n = 8 per group). All groups went through the same 1-week acclimatization period. At 6 weeks of age, 1 group was euthanized by decapitation to serve as a basal baseline control, whereas the remaining 3 groups (exercise, sham, control) were incorporated into the long-term study for 6 weeks, after which time they were euthanized at 12 weeks of age.

2.3. Treatment protocols

Each day, animals were transported to a treatment room, where exercise animals were forced to swim. The exercise group individually swam in cylindrical tanks with a diameter and height of 60 and 100 cm, respectively, in water at a depth of 30 to 45 cm, once per day between 9:30 and 11:30 AM, 5 d/wk for 1 hour, as described previously [34]. During swimming, rats wore elastic chest bands in which attachable weights could be added. Rats commenced exercising without any additional weight for the first week; however, during the second week of treatment, exercise rats had 3% body weight added to the bands. To maintain a constant training intensity sufficient to cause exhaustion by the end of 1 hour, this weight was increased by 1% each week reaching a maximum of 7% body weight by week 12 of the study. To minimize stress associated with cold or hot water exposure, water temperature was monitored and maintained at approximately 35°C. At the end of the treatment period, weights were removed and rats were towel dried and left for about 1 hour in a heated room to minimize the effects of cold exposure. In an attempt to separate the effects of exercise and the stress associated with the exercise environment, sham rats were individually placed in identical swimming tanks and wore the same chest bands, but sat in shallow water (~5 cm) at the same temperature, duration, and frequency as exercise rats. This sham treatment provided the closest similarity to the swim environment as possible and has previously been shown by us to cause a transient increase in glucocorticoid levels, similar to what is observed during swimming exercise [34]. Sedentary control animals were subjected to the same sampling and handling procedures as exercise and sham exercise animals, except remained in their cages without food and water for the duration of the

treatment hour. After 6 weeks, exercise, sham, and control animals were euthanized between 9:00 AM and 12:00 noon by decapitation.

2.4. Food intake, body weight, and postprandial glucose sampling

Food intake and body weight were measured each day before treatment by using a scale accurate to 2 g. To obtain weekly average food intake values from daily measurements, daily food intakes for animals (excluding the day of fast) were averaged over the week. Total food intake over the course of the study was also determined for each animal by summing the weekly averages. We also calculated food intake spanning the time between treatment (9:00-10:00 AM) and food removal (4:00-6:00 PM) on the days of fast (Thursdays). These values for each week were then summated over the 6 weeks of treatment. To measure postprandial blood glucose concentration, blood was sampled each morning (9:00 AM) by "tail nick" using a 27 G needle and analyzed by using a blood glucose monitor (Ascensia Elite XL Blood Glucose Meter, Bayer, Toronto, ON, Canada). To obtain weekly blood glucose values from daily measurements, daily blood glucose values (excluding the day of fast) were averaged over the week.

2.5. Fasting blood glucose and fasting plasma insulin levels

Once per week, the rats were fasted for 16 to 18 hours after which blood samples were taken via a nick to the nub of the tail (see above). To minimize the stress induced by this method of sampling, a topical anesthetic (EMLA cream, AstraZeneca, Mississauga, ON, Canada) was applied to the tails 20 minutes before blood sampling. Samples taken to measure fasting insulin levels (~10 μL) were done at the same time as measurement of fasting blood glucose samples and collected into heparinized microvettes (Starstedt Heparinized Microvettes, Montreal, Quebec, Canada). Plasma was separated from blood samples by centrifugation at 2500 rpm for 1 minute and stored at -20°C. To reduce the chance of infection, a topical germicide (Betadine solution, Purdue Pharma, Pickering, ON, Canada) was applied to the tail after blood collection.

2.6. Intraperitoneal glucose tolerance test with respective insulin levels

All groups received an intraperitoneal glucose tolerance test (IPGTT) 4 days before euthanasia. For basal animals, this test was performed at 6 weeks of age, whereas exercise, sham, and control animals received an IPGTT after 6 weeks of treatment (ie, 12 weeks of age). Before glucose injection, rats were fasted overnight for 16 to 18 hours. During the normal treatment time (ie, between 9:30 and 11:30 AM), rats were administered an intraperitoneal injection of 50% dextrose (Abbott Laboratories Limited, Montreal, Quebec, Canada) at a dose of 2 g/kg body weight. Blood glucose and insulin levels were measured via tail nick (see above) at 30-minute intervals starting at $T = 0$, just before injection, for 2 hours.

2.7. Corticosterone sampling

Plasma corticosterone levels of all rats were measured on the first day of treatment of each week (ie, Monday) both before and after treatment. After administration of topical anesthetic and a wait period of 20 minutes, approximately 20 μL of blood was collected into heparinized microvettes via the tail nick method (described above). Sampling was consistently performed within an approximately 30-second period to minimize the effect of the sampling procedure on measured corticosterone levels. Pretreatment corticosterone levels represent circulating corticosterone levels in rats after a brief handling procedure, common to all rats in all treatment groups. Posttreatment corticosterone levels represent the stress response elicited by treatment and/or animal handling [34]. We performed pilot studies in 6- and 12-week-old ZDF rats that underwent carotid artery and jugular vein cannulations to investigate the corticosterone response to our tail nick methods of sampling blood. We found that corticosterone concentrations in tail nick blood collected within approximately 1 minute of first disturbing the rats did not differ significantly from corticosterone samples obtained from jugular vein or carotid artery cannulae (M. A. Király, unpublished observations). Plasma was separated from blood samples by centrifugation at 2500 rpm for 1 minute and stored at -20°C. We were able to consistently sample approximately 20 μL of blood in less than 30 seconds, minimizing the stress of the sampling procedure on corticosterone measurements.

2.8. Resting hormone measurements made at euthanasia

Basal animals were euthanized by decapitation at 6 weeks of age, whereas exercise, sham, and control animals were euthanized at 12 weeks of age, also by decapitation. For animals sampled over the 6 weeks of treatment, euthanasia occurred approximately 24 hours after their last treatment session. Trunk blood was collected in 1.5-mL tubes containing EDTA and Trasylol for all hormones except corticosterone, which was collected in heparin-treated tubes, and lipids, which were placed in tubes with a lipase inhibitor. Immediately after decapitation, all blood samples were centrifuged at 2500 rpm for 1 minute with transferred plasma stored at -20°C. Postprandial insulin, glucose, corticosterone, lipids, adiponectin, and glucagon were measured from samples taken at this time.

2.9. Analytical procedures

Blood glucose was measured using a single drop of tail capillary blood (~5 μL) with a blood glucose test strip (Ascensia Elite, Bayer) and glucometer (Ascensia Elite XL Blood Glucose Meter, Bayer). Plasma insulin levels were determined by using a Rat Insulin Elisa Assay Kit (Crystal Chem, Downer's Grove, IL). Plasma corticosterone was measured by a corticosterone radioimmunoassay (RIA) kit (Medicorp, Montreal, QC, Canada). Plasma free fatty acid (FFA) and triglyceride (TG) were determined by an

enzymatic colorimetric method (ACS-ACOD, Wako Chemicals, Richmond, VA). Plasma glucagon and adiponectin were measured by using RIA kits (Cerdarlane Laboratories, Hornby, ON, Canada).

2.10. Glycogen content assay

To access the glycogen content of white and red gastrocnemius muscle, 20 to 25 mg of muscle tissue was digested in 0.5 mL of 1 mol/L KOH at 65°C. For analysis of glycogen content, the pH of muscle digest was titrated to 4.8 before the addition of acetate buffer (pH 4.8) and 0.5 mg/mL of amyloglucosidase. Subsequently, glycogen was hydrolyzed at 40°C for 2 hours and glucose was analyzed enzymatically and the absorbance read on a spectrophotometer (Ultraspec 2100 pro, G.E. Healthcare, Baie d'Urfe, Quebec, Canada) at 340 nm wavelength. The protein concentration in each sample was determined by the Bradford method [35,36].

2.11. Western blot determination of glucose transporter 4

Immediately after all treatments, muscles were frozen in liquid nitrogen and stored at −80°C until analysis. For preparation of muscle lysates, 50 to 60 mg of red and white gastrocnemius muscles were homogenized in buffer containing 135 mmol/L NaCl, 1 mmol/L $MgCl_2$, 2.7 mmol/L KCl, 20 mmol/L Tris (pH 8.0), 1% Triton, 10% glycerol, and protease and phosphatase inhibitors (0.5 mmol/L Na_3VO_4, 10 mmol/L NaF, 1 μmol/L leupeptin, 1 μmol/L pepstatin, 1 μmol/L okadaic acid, and 0.2 mmol/L phenylmethylsulfonyl fluoride), and heated (65°C for 5 minutes). An aliquot of the homogenate was used to determine the protein concentration in each sample by the Bradford method. Before loading onto sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels, the samples were diluted 1:1 (vol/vol) with 2× Laemmli sample buffer (62.5 mmol/L Tris-HCl, pH 6.8, 2% [wt/vol] SDS, 50 mmol/L dithiothreitol, and 0.01% [wt/vol] bromphenol blue). Aliquots of muscle homogenates containing 50 μg of protein were run through SDS-PAGE gels (12%) and then transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Burlington, ON, Canada). The glucose transporter 4 (GLUT4) content was determined by using a GLUT4-specific antibody. Equal loading of all gels was confirmed by Coomassie staining of all gels. All antibodies were applied in a 1:1000 dilution and were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.12. Pancreas studies: tissue preparation and measurement of beta cell replication

Six hours before removal of the pancreas, the animals were injected intraperitoneally with 100 mg/kg BrdU (Sigma Aldrich Canada, Oakville, ON, Canada), a thymidine analogue incorporated into newly synthesized DNA [6,37]. Within 10 minutes of decapitation the pancreas was removed, blotted, and extraneous fat and lymph nodes were removed [6,37]. The pancreas was then weighed before being placed in Bock's fixative. After fixation, tissue samples were cut into approximately 20 small pieces (depending on animal's age and size of pancreas), and randomly placed into tissue cassettes to ensure an equal representation of head and tail segments. The cassettes were then placed in 70% ethanol until time of paraffin embedding. Four-micrometer slices were cut on an Olympus microtome (Carsen Group, Markham, ON, Canada) from paraffin blocks and mounted onto 25 × 75-mm slides. Slides were stored at room temperature until time of analysis.

2.13. Double immunohistochemical staining for insulin/BrdU

Paraffin sections were dewaxed and hydrated through graded alcohols and brought to water. After hydration, endogenous peroxidase activity was blocked with 3% hydrogen peroxide and washed in water. Antigen unmasking was then carried out by enzyme digestion using pepsin (Sigma, Aldrich, Oakville, Canada) and then washed in phosphate-buffered saline (PBS). After antigen retrieval, blocking of avidin/biotin activity was performed with the Avidin/Biotin blocking kit (Vector Laboratories, Burlington, ON, Canada). Slides were then washed with PBS, blocked with normal goat serum (Vector Laboratories), drained, and then incubated with rabbit anti–insulin IgG (Dako, Mis-

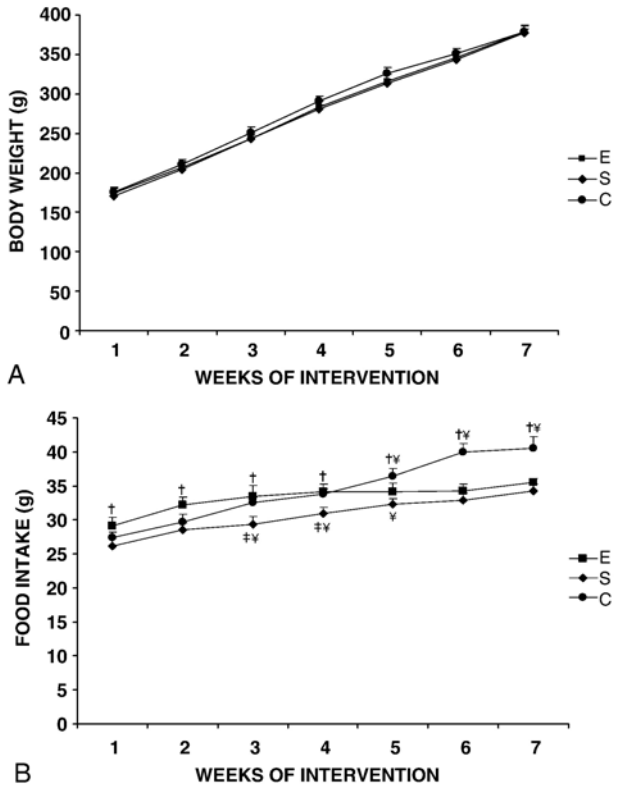

Fig. 1. Body weight (A) and food intake (B) were measured daily. Average body weight and food intake per rat per day is presented for the weeks during the intervention period. Body weight and food intake measurements were made within a 3-hour period of lights on (7:00 AM) and before treatment. ■ indicates exercise group; ♦, sham group; ●, untreated group. Data are presented as means ± SEM, n = 8 rats per group. $^{¥}P < .05$ vs exercise (E) groups; $^{†}P < .05$ vs sham (S) groups; $^{‡}P < .05$ vs control (C) groups.

Table 1
Metabolic characteristics and pancreatic cell mass at the time of euthanization in all treatment groups

Measurements made at euthanasia	Basal (n = 8)	Exercise (n = 8)	Sham (n = 8)	Control (n = 8)
Glucose (mmol/L)	6.1 ± 0.26*	7.41 ± 0.50*	9.33 ± 1.92*	17.7 ± 2.07
Insulin (pg/mL)	1112.06 ± 133.48	2395.95 ± 536.39†	2542.19 ± 477.35†	1644.51 ± 379.33
Adiponectin (pg/mL)	8.51 ± 0.29	4.89 ± 0.15†	4.87 ± 0.35†	4.17 ± 0.35†
Glucagon (ng/mL)	56.62 ± 4.35	109.84 ± 4.44†	93.96 ± 3.87*†	113.64 ± 8.50†
Corticosterone (ng/mL)	169.7 ± 38.39	107.9 ± 32.61	241.6 ± 36.41‡	234.7 ± 47.4‡
TG (mmol/L)	2.24 ± 0.20	7.33 ± 0.46†	8.28 ± 1.24†	7.71 ± 0.75†
NEFA (mmol/L)	0.30 ± 0.02	0.60 ± 0.06†	0.78 ± 0.12†	0.71 ± 0.06†
Beta cell mass (mg/kg)	54.43 ± 6.37	46.47 ± 1.86†	35.98 ± 1.85†‡	34.94 ± 2.90†‡
Alpha cell mass (mg/kg)	2.01 ± 0.19	0.81 ± 0.07†	1.15 ± 0.06†‡	0.99 ± 0.06†
Delta cell mass (mg/kg)	0.16 ± 0.006	0.28 ± 0.016†	0.36 ± 0.039†‡	0.34 ± 0.018†
Total food consumption (g)				
Fast day sum (9:00 AM to 6:00 PM) (g)	N/A	34 ± 2	28 ± 4	35 ± 4
Total sum (24 h) (g)	N/A	1634 ± 43	1555 ± 170‡‡	1721 ± 175

Data are means ± SEM. NEFA = nonesterified fatty acids; N/A = not available.
* $P < .05$ vs control (C) groups.
† $P < .05$ vs basal (B) groups.
‡ $P < .05$ vs exercise (E) groups.

sissauga, ON, Canada) primary antibody at 1:100 for 1 hour at room temperature in a moist chamber. Slides were then washed with PBS and incubated with the secondary antibody, biotinylated goat anti–rabbit IgG (Vector Laboratories) at 1:500 for 1 hour at room temperature, washed with PBS and then incubated with peroxidase-conjugated ultrastreptavidin labeling reagent (Vector Laboratories). Color development for insulin was done with freshly prepared diaminobenzidine (Dako), which yields a brown color. The slides were washed in water, blocked again with 3% hydrogen peroxide, washed in water again, and then rinsed in PBS, followed by incubation with normal horse serum. The slides were then drained and incubated with mouse anti–BrdU primary antibody (Invitrogen, Burlington, ON, Canada) at 1:1000 overnight at room temperature, in a moist chamber. The slides were washed with PBS and incubated with the secondary antibody, biotinylated horse anti–mouse IgG (Vector Laboratories) at a concentration of 1:500 for 1 hour at room temperature, were washed with PBS and then incubated with peroxidase-conjugated ultrastreptavidin labeling reagent (Vector Laboratories). Color development for beta cells incorporating BrdU ($BrdU^+$ cells) was done with freshly prepared nickel diaminobenzidine, which yields a dark blue/black color.

Finally, the sections were counterstained with Mayer's hematoxylin. Sections were then washed in water, dehydrated in graded alcohol, cleared with xylene and mounted.

2.14. Data analysis: measurement of beta cell mass and beta cell replication

Beta cell mass was determined from the insulin antibody-stained sections by using an image analysis system (Aperio Scanscope system, Vista, CA) capable of capturing high-resolution digital images at 20× and 40× optical magnification. Digitally, these images could be further increased in magnification to 400× without a significant loss in image quality. For each animal, at a magnification of 20×, the relative cross-sectional area of insulin-stained beta cells was divided by the cross-sectional area of all the pancreatic tissue [6,37] over the area occupied by all approximately 20 sections of each slide. Tissue areas were objectively quantified, using one common preset positive pixel count

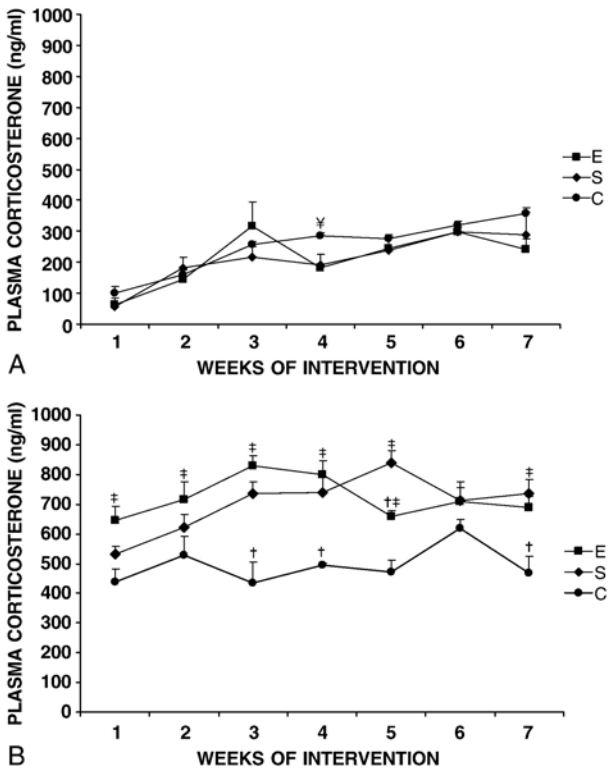

Fig. 2. Pretreatment (A) and posttreatment (B) corticosterone were measured weekly. Pretreatment corticosterone samples were obtained within a 3-hour period of lights on (7:00 AM) and before treatment. Posttreatment corticosterone values were made immediately upon completion of the 1-hour treatment period. ■ indicates exercise group; ♦, sham group; ●, untreated group. Data are presented as means ± SEM, n = 8 rats per group. ¥$P < .05$ vs exercise (E) groups; †$P < .05$ vs sham (S) groups; ‡$P < .05$ vs control (C) groups.

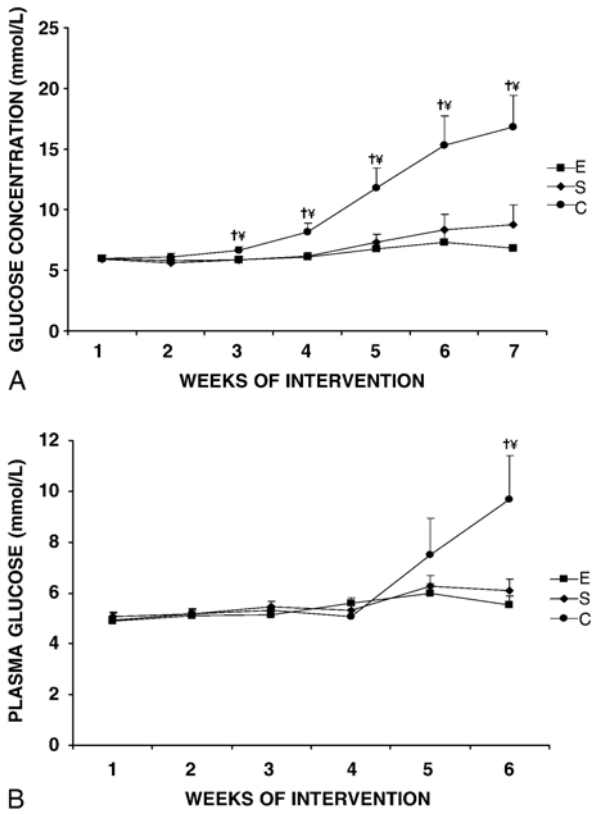

Fig. 3. Postprandial glucose (A) and fasting glucose (B) measurements were made daily within 3 hours of lights on (7:00 AM) before the administration of treatment. Average postprandial glucose per rat per day is presented for the weeks during the intervention period. Fasting glucose was measured weekly after an 18-hour fast. ■ indicates exercise group; ♦, sham group; ●, untreated group. Data are presented as means ± SEM, n = 8 rats per group. $^{¥}P < .05$ vs exercise (E) groups; $^{†}P < .05$ vs sham (S) groups; $^{‡}P < .05$ vs control (C) groups.

algorithm, available with Aperio Scanscope software. Beta cell mass per animal was estimated as the product of the total cross-sectional area of beta cells/total tissue and the weight of the isolated pancreas before fixation [6,37]. Beta cell replication was determined at 40× magnification in an experimentally blinded fashion by one individual by counting $BrdU^{+}$ nuclei [6,37]. No fewer than 1000 beta cells per animal (a minimum of 8000 per treatment group) were counted and classified as $BrdU^{+}$ or $BrdU^{-}$.

2.15. Immunohistochemical staining for somatostatin and glucagon

Paraffin sections used to stain for somatostatin and glucagon were processed identically to those described in our insulin/BrdU studies until the point when primary antibodies are added. The primary antibodies used were polyclonal rabbit antisomatostatin (Zymed, Burlington, ON, Canada) and rabbit antiglucagon (Novacastra, Norwell, MA) at 1:300 dilution for 1 hour in a moist chamber. Slides were washed with PBS and incubated with the secondary antibody, biotinylated goat anti–rabbit IgG (Vector Laboratories), at room temperature for 1 hour at a concentration of 1:500, washed with PBS, and then incubated with peroxidase-conjugated ultrastreptavidin labeling reagent (Vector Laboratories). Color development was done with freshly prepared Nova red (Vector Laboratories). Finally, the sections were counterstained with Mayer's hematoxylin. Sections were then washed in water, dehydrated in graded alcohol, cleared with xylene and mounted. Alpha cell and delta cell mass determinations were performed objectively by one common preset algorithm in an identical fashion to beta cell mass calculations (see "Research design and methods" for beta cell mass quantification).

2.16. Statistical analysis

For all measurements made over time, a 2-way (treatment group × time interval) repeated-measures analysis of variance (ANOVA) was used. To examine the effects of treatment at specific times, the ANOVA was collapsed, decomposing the ANOVA, allowing one to test differences between groups. For parameters measured at euthanasia, a 1-way (treatment) ANOVA was used. Duncan post hoc analysis was used to determine differences between relevant mean values. All values are reported as a mean ± SEM and were obtained using STATISTICA

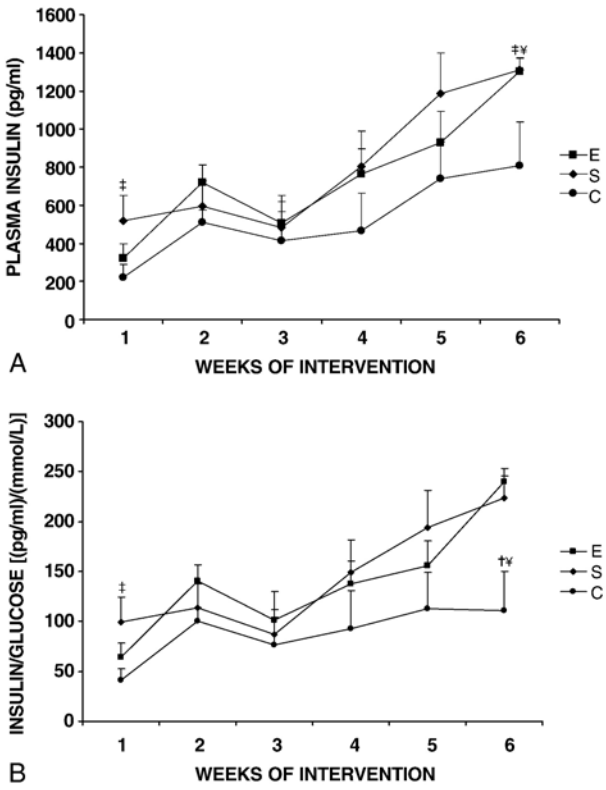

Fig. 4. A, Fasting plasma insulin was measured weekly after an 18-hour fast. B, Weekly beta cell function values are obtained by dividing fasting insulin by fasting glucose values. Measurement of fasting insulin and fasting glucose values were made within 3 hours of lights on (7:00 AM) and before treatment. ■ indicates exercise group; ♦, sham group; ●, untreated group. Data are presented as means ± SEM, n = 8 rats per group. $^{¥}P < .05$ vs exercise (E) groups; $^{†}P < .05$ vs sham (S) groups; $^{‡}P < .05$ vs control (C) groups.

software (StatSoft Inc, v.6.0, Tulsa, OK) with $P \leq .05$ as the limit for statistical significance.

3. Results

3.1. Weekly body weight and food intake

At the commencement of the study, body weight was not different between the treatment groups (Fig. 1A). Body weight increased equally in all 3 groups over the study ($P < .05$). Daily food intake (Fig. 1B) increased in all treatment groups over the course of intervention ($P < .05$). Food intake in sham rats was lower than in exercise rats over the first 5 weeks of intervention ($P < .05$); however, there was no difference between these 2 groups over the final 2 weeks of the study. For the first 4 weeks, food intake was not different between exercise and control rats. Over the last 3 weeks of intervention, however, exercised rats ate less than controls ($P < .05$). Total summated food intake indicates that food consumption spanning the study period was similar between exercise and control rats, although sham rats ate less than exercise ($P < .05$) and control ($P < .05$) animals (Table 1).

3.2. Corticosterone levels

Pretreatment corticosterone (Fig. 2A) increased over time in all 3 treatment groups ($P < .05$) and did not differ between the groups except at week 4, where values were higher in controls compared with the other 2 groups. Posttreatment corticosterone (Fig. 2B) was elevated in exercise when compared with controls at all weeks ($P < .05$), except for week 6. For data collapsed over time, posttreatment corticosterone levels were similar between exercise and sham groups.

3.3. Plasma glucose levels

Daily fed blood glucose measurements for each animal were summated, averaged over 4 week days, and plotted over the course of the study (fasting blood glucose measurements were made on Friday and were not included into this fed blood glucose calculation) (Fig. 3A). Fed blood glucose concentrations were similar among the groups at the start of the experiment. Fed blood glucose levels increased dramatically in control rats over the course of the study and by the third week of intervention were higher than in exercised and sham rats ($P < .05$). Fed blood glucose levels between exercise and sham rats did not differ from each other for the entire duration of the study. By 10 weeks of age (week 5 of intervention), control rats were diabetic based on the human criteria for postprandial hyperglycemia of greater than 11.1 mmol/L, whereas the other 2 groups remained euglycemic in the fed state. Fasted blood glucose (Fig. 3B) did not differ between exercise and sham groups during the study and remained euglycemic during the 6 weeks of

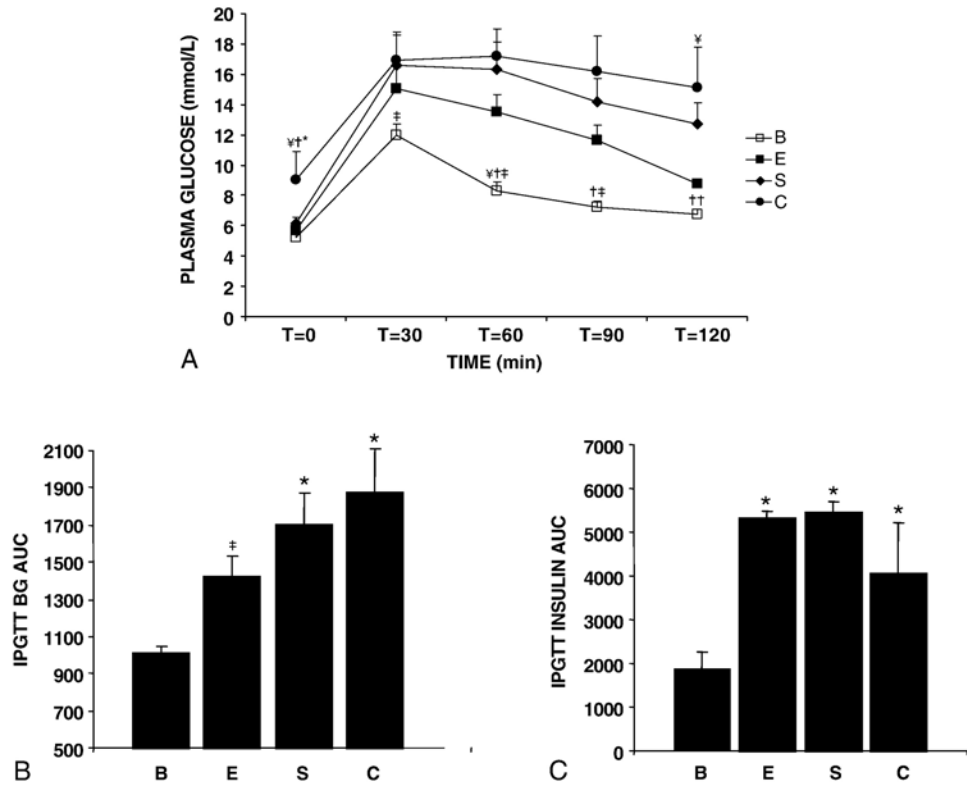

Fig. 5. Four days before euthanasia and after an 18-hour fast, an IPGTT was performed. Plasma was sampled every 30 minutes over a 2-hour period. Glucose: A, AUC analysis of glucose profiles (B). Insulin: C, AUC analysis of insulin profiles. Data are presented as means ± SEM, n = 8 rats per group. *$P < .05$ vs basal (B) groups; ‡$P < .05$ vs exercise (E) groups; $P < .05$ vs sham (S) groups; $P < .05$ vs control (C) groups.

treatment. By the sixth week, however, fasted blood glucose in controls was elevated compared with exercise and sham rats (both $P < .05$) and had exceeded the diabetic human criteria for fasting normoglycemia (>7.1 mmol/L).

3.4. Insulin levels and beta cell function

Fasted insulin levels increased gradually in all groups. By week 6, control rats had lower levels ($P < .05$) than the other 2 groups, suggesting that they had become insulin deficient (Fig. 4A), as their fasted glucose was elevated at this time (see above). At euthanasia, there were higher postprandial insulin concentrations in exercise and sham rats when compared with basal animals ($P < .05$, Table 1). Postprandial insulin concentrations in control animals and basal rats were not different, therefore indicating compensatory hyperinsulinemia was transient in control rats, as expected, but maintained in exercise and sham groups. This maintenance of compensatory hyperinsulinemia in relation to fasting glucose levels can be represented as a simple test of beta cell function by dividing fasting insulin by fasting glucose levels (Fig. 4B). At the first week of treatment (week 1), beta cell function was higher in shams compared with controls. However, beta cell function was not different between exercise and control groups at this time. Beta cell function was similar among all groups over weeks 2 to 5, but was lower in controls than in the other 2 groups at week 6. Thus, greater quantities of insulin could be produced and/or secreted in response to a glucose stimulus in exercise and sham animals, but not in controls.

3.5. Intraperitoneal glucose tolerance test plasma glucose and plasma insulin levels

As mentioned above, an IPGTT was performed at week 1 in basal animals (6 weeks of age) and after 6 weeks of treatment in controls, exercise, and sham groups (all 12 weeks of age). Before glucose injection, basal, exercise,

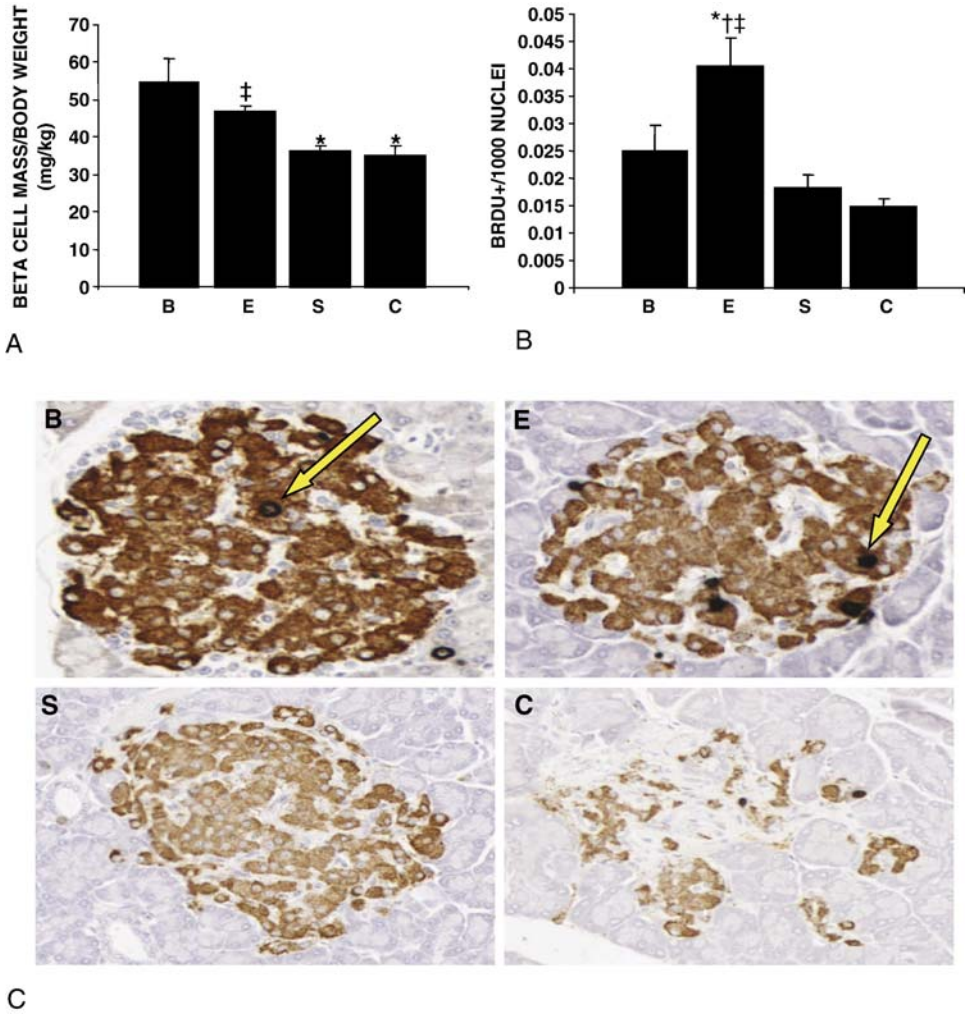

Fig. 6. Beta cell mass (A) and percent beta cell proliferation/BrdU$^+$ incorporation (B). Data are presented as means ± SEM, n = 8 rats per group. *$P < .05$ vs basal (B) groups; $^{¥}P < .05$ vs exercise (E) groups; $^{†}P < .05$ vs sham (S) groups; $^{‡}P < .05$ vs control (C) groups. Islet morphology: representative sections of pancreas at 6 weeks of age before the treatment period (B), and at 12 weeks of age after the treatment period (E, SE, NTC). Islet morphology and examples of BrdU$^+$ cells (C). Insulin-stained cells appear reddish-brown, whereas non–insulin stained tissue appears purple. E animals show more intense insulin staining compared to control animals. E and SE rats have improved islet morphology compared with NTC animals. Examples of BrdU$^+$ nuclei are identified by arrows (C).

and sham rats had lower fasting glucose compared with control rats ($P < .05$). Blood glucose in controls were higher than in basal rats at all time points during the IPGTT ($P < .05$), indicating a deterioration in glucose tolerance with time (Fig. 5A). Sham and control rats also had increased blood glucose area under the curve (AUC) relative to basal rats (Fig. 5B). Two-hour postload glucose was higher in sham and control rats (both $P < .05$) compared with basal rats (Fig. 5A). Insulin AUC during the IPGTT was higher in exercise, sham, and controls compared with basal rats (Fig. 5C, $P < .05$).

3.6. Islet studies and immunohistochemistry of alpha, beta, and delta cell mass

Beta cell mass, adjusted for body weight, was reduced in sham and control rats when compared with basal rats ($P < .05$). In the exercise group, however, beta cell mass was similar to that of basal animals and approximately 25% higher than in controls ($P < .05$). Beta cell proliferation, as assessed by BrdU incorporation into proliferating cells, was increased in exercise rats compared with the other groups ($P < .05$, Fig. 6B). Fig. 6C shows examples of $BrdU^+$ nuclei. Immunohistochemical staining for islet glucagon indicated that alpha cell mass was elevated in sham compared with exercise rats ($P < .05$), and decreased in all 3 groups compared with the basal group ($P < .05$, Table 1). Compared with basal rats, delta cell mass was elevated at 12 weeks of age in exercise, sham, and controls ($P < .05$, Table 1). However, delta cell mass was lower in exercise compared with sham rats at this time ($P < .05$, Table 1). Compared with controls, islet cell morphology of the exercised and sham treatment rats was significantly improved, with increased density and darker insulin staining, and normal rounded islet appearance, similar to what was observed in basal animals. Control rats showed significant fibrosis and less intense staining for insulin compared with the other 3 groups.

3.7. Plasma and tissue data collected at euthanasia

Postprandial metabolites, hormones, and other measurements made at euthanasia are shown in Table 1. Blood glucose levels at euthanasia were similar between basal, exercise, and sham groups, all of which were lower than in control animals ($P < .05$). In exercise and sham rats, insulin levels were higher than in basal rats ($P < .05$), whereas the levels in controls and basal animals were similar. Corticosterone level was lower in the exercise group compared with sham and control groups ($P < .05$). Plasma adiponectin levels were reduced in all treatment groups relative to basal ($P < .05$). Plasma glucagon levels were higher in exercise, sham, and control rats compared with the basal group ($P < .05$); however, sham rats had a lower plasma glucagon than controls ($P < .05$). Although there were no significant differences in postprandial FFA or TG concentrations

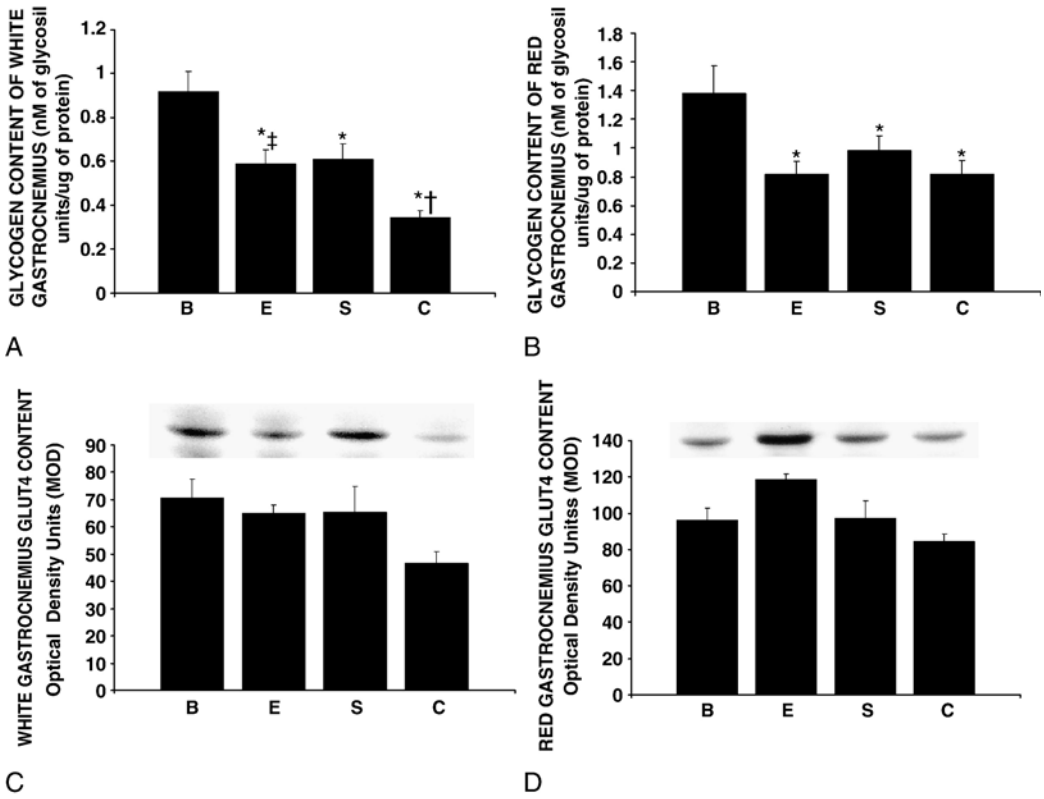

Fig. 7. Glycogen content of white (A) and red (B) gastrocnemius muscles. C and D, Total GLUT4 content of white gastrocnemius and red gastrocnemius muscles, respectively.

between treatment groups, levels were higher than in basal animals ($P < .05$).

3.8. Muscle GLUT4 expression and glycogen content

White gastrocnemius glycogen content was lower in all treatment groups compared with basal animals ($P < .05$, Fig. 7A); however, compared with controls, levels were higher in both exercise and sham animals ($P < .05$). Red gastrocnemius muscle (Fig. 7B) glycogen content was lower in all 3 treatment groups relative to basal animals ($P < .05$), but did not differ between the 3 treatment groups. Total GLUT4 content in both white and red gastrocnemius muscles did not differ between treatment groups (Fig. 7C and D).

4. Discussion

This study demonstrates that stressful stimuli that are episodic in nature, such as those associated with forced-exercise swim training, result in the attenuation of T2DM in the male ZDF rat. The main findings are that both swim exercise and sham treatment result in the maintenance of euglycemia through adaptive attenuation in the loss of beta cell function. Specifically, attenuation of T2DM with regular exercise is characterized by increased rates of beta cell proliferation and beta cell mass compared with sedentary control animals. Furthermore, in this model, improvement in glucose tolerance with exercise is not a result of reduced food intake, a reduction in body weight, or differences in plasma lipid profiles. Chronic, intermittent, non-exercise stress also attenuates the progression of hyperglycemia in the ZDF rat. However, unlike exercise training, the protective effect of non-exercise stress is not mediated by preservation of beta cell mass but rather preservation of beta cell function. It appears, therefore, that a number of potential mechanisms exist to delay or prevent the development of diabetes in this animal model.

Six weeks of swim training resulted in significant improvements in both postprandial (Fig. 3A, Table 1) and fasted glucose (Fig. 3B) concentrations, such that by the end of the intervention period, glucose in exercised rats was approximately 60% and approximately 40% lower than in controls for postprandial and fasted glucose, respectively. Based on IPGTT, exercised animals have improved peripheral insulin action (Fig. 5), as has been shown using treadmill training in ZDF rats [14] and other forms of exercise in rat models of insulin resistance [15-17]. In our study, exercise training resulted in blood glucose levels, 2 hours after glucose administration in the IPGTT, that were below the human diabetic threshold of T2DM (11.1 mmol/L), although control animals became overtly diabetic. Throughout the 2 hours after intraperitoneal glucose injection, total insulin levels (AUC) were not different between the 3 treatment groups, whereas blood glucose AUC (Fig. 5B) and 2-hour postload glucose levels were considerably lower in exercise vs control rats, indicating increased peripheral insulin action occurred in the former group (Fig. 5A and C). Our findings of improved insulin action in exercised ZDF rats is in agreement with other studies showing that training improves insulin sensitivity in obese Zucker fatty rats [16,17,38,39].

Enhanced peripheral insulin-stimulated glucose disposal with exercise training may be related to an up-regulation of GLUT4 protein expression [16,38,40-45], GLUT4 translocation [41], and cell surface GLUT4 content after insulin stimulation [38]. For example, with our current study we show a tendency ($P = .095$) for a reduction in white gastrocnemius GLUT4 content in sedentary controls over time. Interestingly, with both sham exercise ($P = .17$) and exercise ($P = .18$) treatment there was a tendency to preserve GLUT4 levels when compared with sedentary controls. Similarly, swim training resulted in a tendency for a 30% increase in red gastrocnemius muscle GLUT4 protein expression relative to sedentary controls. A possible explanation regarding why statistical significance was not obtained with this present study is likely related to the high variability seen within and between our treatment groups. Thus, our present findings partially support results from other swim studies with rodents, reporting a short-lived 30% increase in GLUT4 messenger RNA (mRNA) levels [46] with training.

Other factors are also likely involved in improving insulin sensitivity. For example, we found that swim-trained rats had higher white gastrocnemius glycogen content compared with controls (Fig. 7A), which may help to explain their increased insulin sensitivity. Indeed, greater insulin sensitivity is associated with increased muscle glycogen synthesis in humans [46-49]. Others have also shown that skeletal muscle adenosine monophosphate–activated protein kinase levels increase with training and are associated with improved insulin sensitivity and a delay in the development of T2DM in male ZDF rats [14]. Taken together, it is likely that a number of peripheral adaptations occur with exercise that improve insulin sensitivity, which may in turn cause a reduced reliance on pancreatic function.

In agreement with the improved glycemia and enhanced beta cell mass relative to sedentary controls, we found that exercise training partially maintains compensatory beta cell adaptations and is associated with maintenance of normal islet morphology (Fig. 6). Islets from control rats had significant fibrosis and less intense staining for insulin compared with the other groups after 6 weeks of treatment. In exercised animals, islets were dense and circular with insulin staining characteristics that were similar to that in basal animals. In fact, islet morphology between 12-week-old exercised and 6-week-old basal animals was not noticeably different. These findings confirm recent observations that exercise protects beta cell morphology in ZDF rats [14] and are in line with human studies suggesting that regular exercise increases insulin secretion to either hyperglycemia or arginine stimulation [18]. Thus, it appears that moderate exercise training improves beta cell function either directly by increasing beta cell mass or as a result of "sparing" insulin needs by increasing peripheral insulin

action. Indeed, a strong positive correlation exists between the maintenance of normal islet morphology and beta cell function [50]. Our immunohistochemical analysis of the pancreas showed that in exercised animals there was a significant attenuation in the reduction of beta cell mass when compared with age-matched controls (Fig. 6), similar to observations made in insulin-resistant Otsuka Long-Evans Tokushima fatty rats who undergo volitional wheel running [15] and male ZDF rats exposed to treadmill training [14]. Taken together, it appears that both volitional and forced-exercise paradigms delay the progression of hyperglycemia in insulin-resistant rats by partially maintaining beta cell mass. Moreover we show, for the first time, that preservation of beta cell mass with exercise is associated with increases in beta cell proliferation in the ZDF rat. To investigate beta cell apoptosis in this model, we used immunohistochemical staining with the terminal transferase uridyl nick end labeling of sectioned tissues and found no detectable apoptosis. Subsequent 4′,6-diamidino-2-phenylindole (DAPI) staining also did not yield any detectable levels of DNA fragmentation or cell death. Although we were unable to quantify apoptosis in this study, we speculate that based on the degree of islet fibrosis and disorganization of islet morphology, apoptosis and/or necrosis played a considerable role in a loss of compensatory beta cell mass in the control group. The exact mechanisms of how exercise training attenuates the loss of beta cell mass remains to be elucidated, but we believe that by improving insulin sensitivity peripherally there may be less strain on beta cell function and subsequent maintenance of normoglycemia. Interestingly, antecedent hyperglycemia has been shown to be responsible for reductions in insulin mRNA and increases in beta cell apoptosis preceding beta cell dysfunction in the male ZDF rat [51,52]. Thus, the partial preservation of beta cell function in exercise and sham rats in this study may be related to a lower glycemic strain because both groups remained euglycemic compared with controls. Our data do not support the notion that hyperlipidemia played a major role in beta cell failure, as plasma FFA and TG concentrations increased similarly in all groups during the progression of the disease (Table 1).

Previous studies have shown an increase in delta cell mass and presomatostatin mRNA in diabetic dogs, monkeys, rats, and humans [53,54]. We show for the first time in ZDF rats that alpha cell mass decreases, whereas delta cell mass increases, over time. Because somatostatin inhibits glucagon synthesis and secretion, it is not surprising that alpha cell mass was decreased [54]. Interestingly, however, plasma glucagon increased over time in exercise, sham, and control rats despite the reduction in alpha cell mass. This increase in glucagon, however, was less pronounced in sham rats, which exhibited lower circulating glucagon than controls or exercised animals ($P = .05$). It is unknown whether this reflects enhanced glucagon secretion or decreased glucagon disposal. Prudence should be used when interpreting glucagon data, as the specificity of commercially available glucagon assays is debatable. We recognize that the specificity of the assay used is not 100% and may be affected by related peptides, such as glucagon-like peptide 1. The general consensus is that glucagon assays (when C-terminal) do not bind with glucagon-like peptide 1, but may combine nonspecifically up to 60% with proteins different from glucagon. This may offer an explanation for the discordance in our findings with respect to our alpha cell mass and glucagon data. These results, however, may explain, in part, the beneficial effect of sham treatment on glucoregulation.

By 12 weeks of age, control animals had become overtly hyperglycemic with respect to postprandial and fasted glucose concentrations and at the same time showed reduced insulin levels relative to exercise- and sham-treated groups. These data suggest that exercise and sham animals are able to compensate for their insulin resistance by increasing insulin production and/or secretion in response to a sustained glucose stimulus. We estimated beta cell function by dividing fasted insulin by fasted glucose levels (Fig. 4B). Based on this ratio, we found that beta cell function increased over time in all 3 groups when compared with basal animals. By the end of the study, however, exercise and sham animals had greater beta cell function than controls, offering a plausible explanation for the maintenance of postprandial and fasted glucose values below the human diabetic criteria of greater than 11.1 and greater than 7.1 mmol/L, respectively.

Perhaps the most surprising finding of this study is that intermittent sham stress, which is associated with elevations in glucocorticoid levels, also limits the progression of overt T2DM in the ZDF rat. The improvements in fed and fasted glucose profiles in sham rats may be related to the fact that food consumption in sham rats was slightly reduced when compared with exercise or control rats. This observation, however, is an unlikely explanation for improved glycemia in these sham animals, as food intake during the lights-on period (7:00 AM to 7:00 PM) and body weight were not reduced in this group despite their reduced total food consumption over the 6 weeks of treatment. Furthermore, sham animals were not observed to be any more active than sedentary controls during the daily treatment portion of the study. This observation is supported by the fact that body weight in sham rats was not reduced when compared with controls despite a reduced food intake. Despite marked improvements in fed and fasted glucose profiles in sham rats, significant improvements in glucose tolerance did not occur. Surprisingly, similar to that observed in exercised rats, sham treatment significantly increased white gastrocnemius glycogen content when compared with sedentary controls lacking intervention. We recognize that favorable changes in body composition, such as reductions in visceral fat pad mass, may have occurred in sham rats in response to episodic stress. Unfortunately, we did not measure fat pad mass for this study, as we were most concerned with the timely removal of the pancreas before autodigestion.

Furthermore, we did not have access to any other methodology to access body composition (ie, dual x-ray absorptiometry, magnetic resonance imaging, or computed tomography). It may be, therefore, that altered body composition that improves glucose disposal or small reductions in food intake may explain the apparent improvements in glycemia in intermittently stressed animals. These observations are paradoxical, however, as glucocorticoid administration is associated with increased insulin resistance in liver [55], skeletal muscle, and adipose tissue [55,56]. Our finding that intermittent stress is protective in the development of T2DM is supported by additional studies performed in our laboratory showing that chronic intermittent restraint stress effectively attenuates hyperglycemia and beta cell decompensation in male ZDF rats [57].

The effects of episodic elevations in glucocorticoid exposure on the pancreas remain unclear. In contrast to the diabetogenic effects of glucocorticoids, the diabetogenic effects of streptozotocin, alloxan, and partial pancreatectomy can be reduced by pretreatment with glucocorticoids or mild foot shock [23,27-32]. Finally, treatments that periodically lower insulin secretion in the face of increased insulin resistance have been effective at attenuating or even preventing T2DM in both humans [58] and in male ZDF rats [59]. Because brief periods of beta cell rest protract the progression of T2DM in ZDF rats, we speculate that brief and intermittent exposures to elevated glucocorticoids and/or catecholamines occurring during times of acute stress may confer a protective effect to the beta cell by temporarily inhibiting insulin secretion and, therefore, attenuating pancreatic beta cell decompensation [60,61].

In summary, glucose tolerance improves and prevention of T2DM occurs with forced swimming in male ZDF rats. In addition, it appears that the stress accompanying non-volitional exercise paradigms also resists the development of T2DM in this animal model, likely via distinct mechanisms. We speculate that exercise exerts protective effects on the attenuation of hyperglycemia in male ZDF rats by improving insulin sensitivity, which helps to partially maintain beta cell mass by increased beta cell proliferation. In contrast to the benefits of exercise on beta cell function and insulin sensitivity, we also reveal that non-exercise stress does not result in improvements in glucose tolerance or in beta cell mass but lowers fed and fasted glucose levels, perhaps by reducing food intake and by lowering glucagon secretion from alpha cells.

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