Swim training prevents hyperglycemia in ZDF rats: mechanisms involved in the partial maintenance of β-cell function

Michael A. Király,1 Holly E. Bates,1 Natalia A. Kaniuk,5 Jessica T. Y. Yue,1 John H. Brumell,4,5 Stephen G. Matthews,1,2,3 Michael C. Riddell,6 and Mladen Vranic1,3

Departments of 1Physiology, 2Obstetrics and Gynecology, 3Medicine, 4Molecular and Medical Genetics, University of Toronto, Toronto, Ontario; 5Cell Biology Program, Hospital for Sick Children, Toronto, Ontario; and 6School of Kinesiology and Health Science, York University, Toronto, Ontario, Canada

Submitted 20 July 2007; accepted in final form 12 November 2007


Exercise improves glucose tolerance in obese rodent models and humans; however, effects with respect to mechanisms of β-cell compensation remain unexplained. We examined exercise’s effects during the progression of hyperglycemia in male Zucker diabetic fatty (ZDF) rats until 19 wk of age. At 6 wk old, rats were assigned to 1) basal-euthanized for baseline values; 2) exercise-swam individually for 1 h/day, 5 days/wk; and 3) controls (n = 8–10/group). Exercise (13 wk) resulted in maintenance of fasted hyperinsulinemia and prevented increases in fed and fasted glucose (P < 0.05) compared with sham-exercised and sedentary controls (P < 0.05). β-Cell function calculations indicate prolonged β-cell adaptation in exercised animals alone. During an intraperitoneal glucose tolerance test (IPGTT), exercised rats had lower 2-h glucose (P < 0.05) vs. controls. Area-under-the-curve analyses from baseline for IPGTT glucose and insulin indicate improved glucose tolerance with exercise was associated with increased insulin production and/or secretion. β-Cell mass increased in exercised vs. basal animals; however, mass expansion was absent at 19 wk in controls (P < 0.05). Hypertrophy and replication contributed to expansion of β-cell mass; exercised animals had increased β-cell size and bromodeoxyuridine incorporation rates vs. controls (P < 0.05). The relative area of GLUT2 and protein kinase B was significantly elevated in exercised vs. sedentary controls (P < 0.05). Last, we show formation of ubiquitinated protein aggregates, a response to cellular/oxidative stress, occurred in nonexercised 19 wk-old ZDF rats but not in lean, 6 wk-old basal, or exercised rats. In conclusion, improved β-cell compensation through increased β-cell function and mass occurs in exercised but not sedentary ZDF rats and may be in part responsible for improved glucoregulation.

Zucker diabetic fatty; exercise; diabetes; glycemia; insulin; C-peptide; β-cell mass; β-cell function; glucose transporter 2; protein kinase B; apoptosis; proliferation; islet morphology

Obesity is the most common metabolic disease in North America (38) and along with its comorbidities is currently responsible for ~15% of the annual health care expenditure (38). One such comorbidity is type 2 diabetes mellitus (T2DM), which develops in ~35–40% of the obese adult population (38). The cause of T2DM is unknown but is predominantly associated with obesity (38), a progressive decline in peripheral insulin sensitivity, and corresponding decrements in glucose disposal in skeletal muscle, fat, and the liver (15). T2DM, however, is not defined solely by decreased hepatic and peripheral insulin action; β-cell defects with respect to insulin production and/or secretion must also be present and, in fact, are responsible for the discrepancy between insulin-resistant obese nondiabetic and type 2 diabetic patients. Studies in obese insulin-resistant but nondiabetic humans have revealed that the initial physiological response to increased body weight and its associated increase in insulin demand is a compensatory period marked by expansion of β-cell mass (4, 7, 35) and β-cell function (29), which together allow for the increased production and/or secretion of insulin. These adaptations continue uninterrupted in obese insulin-resistant hyperinsulinemic individuals. However, in subjects with T2DM, this adaptive period of β-cell compensation is short-lived and may fail altogether (56). For example, humans with T2DM have smaller islets and show a 40–60% reduction in β-cell mass compared with nondiabetic hyperinsulinemic controls (56). Why these defects occur in T2DM and not in obesity and insulin resistance alone is an issue that has not been explored sufficiently.

Because of the scarcity of available human pancreata, studies using rodent models of obesity and insulin resistance, such as the Zucker diabetic fatty (ZDF) rat, are frequently used to elucidate mechanisms responsible for the deterioration from a prediabetic state to T2DM (11, 20, 42, 52). Male Zucker fatty (ZF) rats develop a phenotype of obesity, insulin resistance, and hyperphagia due to a leptin receptor mutation, and ZDF rats also eventually develop hyperglycemia resulting in a phenotype very similar to humans with T2DM (11). In these rodents, glucose intolerance usually develops by 8 wk of age (42, 52), followed by overt hyperglycemia by 10–12 wk of age. It begins with β-cell hyperplasia and hyperinsulinemia, which compensates for the progressively increasing insulin resistance and maintains normoglycemia. After 10 wk, β-cell apoptosis first begins to exceed the rate of replication, and the β-cell mass declines. The level of insulin secretion is no longer sufficient to overcome insulin resistance and maintain normoglycemia and diabetes begins (11, 20, 42, 52).

As in humans, the prediabetic phase in ZDF rats is characterized by the maintenance of normoglycemia by a compensatory increase in β-cell function, resulting in hyperinsulinemia (7, 11, 52). This initial compensatory adaptation reflects what occurs in obese insulin-resistant subjects. However, as is the case for people with T2DM, this compensatory adaptation

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begins to fail as animals enter the “diabetic phase,” defined by a reduction in β-cell function and dramatic increases in β-cell apoptosis and a corresponding decrease in β-cell mass (42). Much work has addressed the β-cell defects responsible for the inability to maintain adaptive hyperinsulinemia and thus euglycemia (8, 11, 14, 20, 35, 42, 43, 52). Indeed, studies in ZDF rats and in humans have elucidated that reductions in β-cell function correlate strongly with decreased levels of glucose transporter-2 (GLUT2) mRNA and protein levels (24, 41, 50). Such reductions in GLUT2 levels have since been confirmed in ZDF rats (41, 54) and other models of T2DM, including the GK rat and db/db mouse (32). In addition, recent studies have demonstrated a role for molecular candidates/markers in the control of the relationship between net β-cell growth and net β-cell death. One such study indicates a role of the insulin/insulin-like growth factor I signaling pathway in the growth and net maintenance of β-cell mass expansion. Specifically, we aimed to test hypotheses that protective effects of exercise training on pancreatic β-cell function. If insulin sensitivity is increased and hyperglycemia reduced, this would by itself improve β-cell function. Together these findings indicate that regular physical activity exerts protective effects on pancreatic β-cells. However, only a paucity of such studies currently exists, and the direct effects of exercise per se on pancreatic β-cell adaptations to worsening insulin resistance remain unclear.

Therefore, the purpose of this study is to investigate the effects of long-term forced exercise training on pancreatic β-cell adaptations with respect to β-cell function and maintenance of β-cell mass expansion. Specifically, we aimed to test the hypothesis that protective effects of exercise training in vivo on β-cell function and β-cell mass compensation are associated with maintained immunodetectable levels of GLUT2 and Akt/PKB protein. We also examined the role of Akt/PKB in maintaining adaptive β-cell proliferation and ameliorating loss of β-cell mass. We show that 13 wk of swim training prevents hyperglycemia up until at least 19 wk of age and is associated with protective effects on β-cell function and compensatory mass expansion. This partial maintenance of β-cell compensation was associated with increased immunodetectable levels of GLUT2 and Akt/PKB molecules.

RESEARCH DESIGN AND METHODS

Animals. Male ZDF rats (ZDF/Gmi-fa/j) were obtained from Charles River Laboratories (Saint-Constant, Quebec, Canada) at 5 wk of age with initial body weights of 150–175 g. Rats were singly housed in opaque microisolation cages, handled daily, and kept at a constant temperature of 22–23°C in humidity-controlled rooms on a standard 12:12-h (0700–1900) 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.

Experimental design. Thirty-five male ZDF rats and 10 lean Zucker rats were obtained at 5 wk of age. Of the 35 ZDF rats, animals were randomly divided into four groups. All groups, including the lean animals, underwent the same 1-wk acclimatization period. At 6 wk of age, one group of ZDF animals was killed by decapitation to serve as a basal baseline control (n = 8), whereas the remaining three ZDF groups (exercise, sham, and sedentary controls) were incorporated into the long-term study for 13 wk, after which time they were killed at 19 wk of age (n = 9/group). Zucker lean animals (n = 10) served as a sedentary lean control group.

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 30–45 cm, once per day between 0930 and 0945, 5 days/wk for 1 h, as described previously (34). During swimming, rats wore elastic chest bands to which attachable weights could be added. Rats commenced exercising without any additional weight for the first wk; however, during the second wk of treatment, exercised rats had 3% body weight added. To maintain a constant training intensity sufficient to cause exhaustion by the end of 1 h, this weight was increased by 1% each week, reaching a maximum of 13% body weight by week 12 of the study. Swum animals were required to swim vigorously to remain afloat and did so for the duration of the hour. To minimize stress associated with cold or hot water exposure, water temperature was monitored and maintained at ~35°C. At the end of the treatment period, weights were removed, and rats were towel dried and left for ~1 h 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 exercised 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 ZDF and lean animals were subjected to the same sampling and handling procedures as exercise and sham animals, except they remained in their cages without food and water for the duration of the treatment hour. After 13 wk, exercise, sham, and sedentary control animals (ZDF and lean groups) were killed between 0900 and 1200 by decapitation.

Food intake, body weight, and postprandial glucose sampling. Food intake and body weight were measured each day before treatment. To obtain weekly average food intake values from daily measurements, food intake for animals (excluding the day of fast) was averaged over the week. To measure postprandial blood glucose concentration, blood was sampled each morning (0900) by “tail nick” using a 27-G needle and analyzed using a blood glucose monitor (ASCENDIA ELITEXL Blood Glucose Meter, Bayer, To-
To obtain weekly blood glucose values from daily measurements, daily blood glucose values (excluding the day of fast) were averaged over the week.

**Fasting blood glucose and fasting plasma insulin levels.** Once per week, the rats were fasted for 16–18 h, 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, Canada) was applied to the tails 20 min 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 microtubes (Sarstedt, Montreal, Canada). Plasma was separated from blood samples by centrifugation at 2,500 revolutions/min (rpm) for 1 min and stored at −20°C. To reduce the chance of infection, a topical germicide (BETADINE solution; Purdue Pharma, Pickering, Canada) was applied to the tail following blood collection.

**Intraperitoneal glucose tolerance test with respective insulin levels.** All groups were subjected to an intraperitoneal glucose tolerance test (IPGTT) 4 days before death. For basal animals, this test was performed at 6 wk of age, whereas exercise, sham, and control animals received an IPGTT after 13 wk of treatment (i.e., 19 wk of age). Before glucose injection, rats were fasted overnight for 16–18 h. During the normal treatment time (i.e., between 0930 and 1130), rats were administered an intraperitoneal injection of 50% dextrose (Abbott Laboratories Limited, Montreal, Canada) at a dose of 2 g/kg body wt. Blood glucose and insulin levels were measured via tail nick (see above) at 30-min intervals starting at time (t) = 0, just before injection, for 2 h.

**Resting hormone measurements made at death.** Basal animals were killed by decapitation at 6 wk of age, whereas exercise, sham, and control animals were killed at 19 wk of age, also by decapitation. For animals sampled over the 13 wk of treatment, death occurred ~24 h after their last treatment session. Trunk blood was collected in 1.5-ml tubes containing EDTA and trasylof, for all hormones. Immediately after decapitation, all blood samples were centrifuged at 2,500 rpm for 1 min with transferred plasma stored at −20°C. Postprandial insulin, glucose, C-peptide, lipids, and corticosterone were measured from samples taken at this time.

**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 ELITEXEL Blood Glucose Meter; Bayer). Plasma insulin levels were determined using a Rat Insulin Elisa Assay Kit (Crystal Chem). Plasma corticosterone and C-peptide levels were measured by commercially available RIA kits (Medicorp, Montreal, Canada). Plasma free fatty acid (FFA) and triglyceride were determined by an enzymatic colorimetric method (ACS-ACOD; Wako Chemicals, Richmond, VA).

**Pancreas studies: tissue preparation.** Before removal of the pancreas (6 h), the animals were injected intraperitoneally with 100 mg/kg bromodeoxyuridine (BrdU; Sigma Aldrich Canada, Oakville, ON, Canada), a thymidine analog incorporated into newly synthesized DNA (20, 34). Within 10 min of decapitation, the pancreas was removed and blotted, and extraneous fat and lymph nodes were removed (20, 34). The pancreas was then weighed before being placed in Bock’s fixative. After fixation, tissue samples were cut into ~20 small pieces (depending on the animal’s age and size of pancreas) and randomly placed in 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. Slices (4 μm) were cut on an Olympus microtome (Carsen Group, Markham, ON, Canada) from paraffin blocks and mounted on 25 × 75 mm slides. Slides were stored at room temperature until time of analysis.

**Double immunohistochemical bright-field staining for insulin/BrdU.** Paraffin sections were dewaxed with xylene, 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 Laboratories) and then washed in PBS. Following antigen retrieval, blocking of avidin/biotin blocking kit (Vector Laboratories, Burlington, ON). Slides were then washed with PBS, blocked with normal goat serum (Vector labs), drained, and then incubated with rabbit anti-insulin IgG (Dako, Mississauga, ON) primary antibody at 1:100 for 1 h 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 h 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 (DAB; 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 incubated with normal horse serum. The slides were then drained and incubated with mouse anti-BrdU primary antibody (In- vitrogen, Burlington, ON) at 1:1,000 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 (Vec- tor Laboratories), at a concentration of 1:500 for 1 h at room temperature, washed with PBS, and then incubated with peroxidase-conjugated ultrastreptavidin labeling reagent (Vector Laboratories). Color development for β-cells incorporating BrdU (BrdU + cells) was done with freshly prepared diaminobenzidine (DAB; Dako), 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.

**Data analysis: measurement of β-cell mass and β-cell replication.** β-Cell mass was determined from the insulin antibody-stained sections by scanning and capturing tiled images of the entire cross-sectional area of the pancreas for each animal. Such images contained, on average, between 350 and 500 islets that were all used in subsequent calculations. 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 β-cells was divided by the cross-sectional area of all the pancreatic tissue (20, 34) over the area occupied by all ~20 sections of each slide. Tissue areas were objectively quantified, using one common preset positive pixel count algorithm, available with APERIO SCANSCOPE software (Vista). β-Cell mass per animal was estimated as the product of the total cross-sectional area of β-cells/total tissue and the weight of the isolated pancreas before fixation (20, 34). Measurement of β-cell replication was performed by capturing no less than 30 random images of areas of interest outlined from the large tiled images of the entire pancreatic cross-sectional area. BrdU + nuclei were determined at ×40 magnification in an experimentally blinded fashion by two individuals (34). No less than 3,000 β-cells/animal (a minimum 24,000/treatment group) were counted and classified as BrdU + or negative, and only those β-cells with a clearly defined nucleus were measured.

**Data analysis: mean β-cell size measurements, islet size measurements, and indicators of neogenesis.** Mean β-cell size or β-cell hypertrophy was calculated by scanning islet areas that were used for β-cell replication studies (see above). For these calculations, the relative cross-sectional area of insulin-stained tissue was divided by the number nuclei counted within that same insulin-stained area to yield mean β-cell size. Only those β-cells with a clearly defined nucleus were measured. Using the data generated from scanning entire slide areas used for our β-cell mass studies (see above), the number of islets for each animal (calculated as average number/total slide area) was determined. With this same data set, we also determined the relative sizes, or cross-sectional surface areas, of individual islets by converting pixel values to micrometers squared and tabulating for each animal to yield islet distribution characteristics. Using the same insulin-stained sections that were used for islet β-cell mass measure-
ments, the total number of β-cells as singles and clusters as doublets, triplets, quadruplets, and up to five cell diameters (very small islets) were counted for each animal. Single and clustered β-cells, regardless of their location, show no signs of replication and therefore were considered neogenic and may aggregate to form new islets (27). Neogenic areas were measured by adding the areas represented by these small neogenic clusters, which were less than six cells. The possibility of measuring islets two times was eliminated by association of a particular islet with an object number assigned by computers during the scanning process.

Double immunohistochemical fluorescent staining for insulin/GLUT2, insulin/Akt/PKB, insulin/FK2, and insulin/DAPI/TUNEL: multiple-labeling immunofluorescent microscopy. Paraffin sections stained during fluorescent studies were dewaxed and hydrated exactly as described above. Antigen unmasking/epitope retrieval was performed by irradiating hydrated sections with microwaves while in a 10 mM NaOH solution of sodium citrate buffer, pH 6.0. The temperature of citrate buffer was kept above 95°C for 20 min in this fashion and then allowed to cool at room temperature until the solution and sections were ~20°C. Following antigen unmasking, slides stained for insulin/Akt/PKB and insulin/FK2 were washed in PBS (0.1% Tween), and then blocked for 3 h at room temperature in a solution of 1% BSA and 5% donkey serum in 0.2% saponin PBS. Slides stained for GLUT2 were handled similarly, with the exception that blocking solution did not contain saponin. Sections were then immunostained simultaneously in antibody mixtures of either guinea pig anti-insulin IgG (Dako) (1:150) and rabbit-derived anti-GLUT2 1:1,000 polyclonal antibody (Chemicon) or guinea pig anti-insulin IgG (Dako) (1:150) and a mouse-derived anti-phospho-Akt-Ser473 (1:200) monoclonal antibody (Cell Signaling Technology) overnight at 4°C. Staining for aggresome-like induced structures (ALIS) was performed with a antibody mixture of guinea pig anti-insulin IgG (Dako) (1:150) and a mouse-derived anti-FK2 (1:200) monoclonal antibody (Biomol) overnight at 4°C. On the following day, slides were washed copiously in PBST and then incubated in a secondary antibody mixture that contained donkey anti-species-specific IgG conjugated to fluorescein isothiocyanate (FITC, 1:200), or cyanine-3 (CY3, 1:200; both from Jackson ImmunoResearch). Following secondary antibody/fluoroophore conjugate incubation, sections were counterstained in 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) for 5 min and washed with PBS and three quick successive baths of double-distilled water followed by mounting with a water-based mounting medium (AQUA POLYMount, Polysciences). Controls for primary antibody specificity (anti-insulin IgG) were performed in spleen sections that were harvested, fixed, and processed identically. Secondary antibody specificity was performed by processing pancreatic sections identically to that explained above while omitting the overnight primary antibody incubation. For determination of apoptotic β-cells, a TUNEL-TMR-red kit (Roche Diagnostics) was used to fluoresceinly mark cells with fragmented DNA strands. In these sections, pancreatic tissue was cleared of wax in three successive baths of xylene and rehydrated in graded ethanol, as described above. Sections were then digested with Proteinase K at a concentration of 20 μg/ml in 10 mM Tris·HCl for 15 min at 37°C. Slides were then washed in TBS, incubated with TUNEL-TMR-red for 1 h at 37°C, and washed in TBS for 1 h at room temperature in a solution of 3% BSA and 1% Triton X-100 in TBS. Following blocking, slides were incubated overnight at 4°C with primary insulin antibody (same as above, 1:150). On the following day, slides were washed in TBS (0.1% Tween) and then incubated for 1 h at room temperature with FITC-conjugated donkey anti-guinea pig (1:200). Slides were then washed five times for 5 min in TBS and then stained with DAPI as described above, followed by mounting of cover slips. All slides stained for fluorescent studies were kept in a ~20°C freezer until analysis (1–5 days later).

Image analysis. Quantitative imaging of GLUT2 and Akt/PKB was performed by capturing monochromatic images of a minimum of 200 sequential fields of view for each pancreatic section. This process of tiling and image gathering was performed for each of the wavelengths corresponding to the fluorophores under study and worked out to analysis of a minimum of 20 random islets per animal at a magnification of ×40 (minimum of 160 islets/treatment condition). Images corresponding to a particular islet and filter set were then merged and pseudocolored using IMAGE PRO PLUS software (Media Cybernetics). A “masking” feature available in Image Pro Plus allowed for the accurate quantification of staining, which was specific to β-cells alone. For identification of ALIS, a minimum of 60 random islets/treatment condition were imaged at ×40 and pseudocolored as described above. Sections were imaged using an upright Olympus BX50 microscope equipped with Olympus UPlanS Apo 10x/0.40, UPlanS Apo 20x/0.75, and Olympus UPlanFl 40x/0.75 lenses and an automated tiling platform. Illumination was provided by a 100-Watt HBO Mercury energy source and was filtered with DAPI 377/50 nm, FITC 482/35 nm, and TRITC (CY3) 543/22 nm filter sets. Images were captured using a Photometrics COOLSnap HQ2 (Roper Biosciences) camera and a computer running Media Cybernetics IN VIVO software (Bethesda, MD). All images were saved in a lossless TIFF format.

Statistical analysis. For all measurements made over time, a two-way (treatment group × time interval) repeated measures 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 death, a one-way (treatment) ANOVA was used. Duncan’s post hoc analysis was used to determine differences between relevant mean values. All values are reported as means ± SE and were obtained using STATISTICA (Tulsa, OK) software (version 6.0) with P ≤ 0.05 as the limit for statistical significance.

RESULTS

Weekly body mass and food intake. At the commencement of the study, body mass was not different between the three ZDF treatment groups and increased equally in all three groups over the study (P < 0.05, Fig. 1A). Lean Zucker rats weighed significantly less than the three ZDF groups at all points during the duration of the study. Daily food intake increased in all treatment groups (P < 0.05, Fig. 1B) and was similar between exercise and sedentary controls over the duration of the study. Sham-treated animals ate less than sedentary controls at weeks 3–5 and less than exercised rats for weeks 2–4 and weeks 10 and 11 of the study. There were no differences in food intake between exercised and sedentary control rats over the last 2 mo of the study. For the entire period of the study, lean animals ate significantly less than exercised, sham-treated, or sedentary control rats (P < 0.05).

Glucose measurements. Daily fed blood glucose measurements for each animal were summated, averaged over the 4 week days, and plotted over the course of the study (Fig. 2A). Preintervention fed glucose values were similar between exercised and control groups but were both elevated compared with sham-treated rats at this time (P < 0.05; Fig. 2A). All three groups displayed elevated fed glucose concentrations compared with lean ZDF rats at this time (P < 0.05). At week 7 of the intervention period, fed glucose increased sharply in control and sham rats and remained elevated with respect to exercised rats for the duration of the study (P < 0.05). In contrast to sedentary control and sham-treated ZDF rats, exercised animals did not show a marked increase in fed glucose throughout the duration of the study, although they were significantly higher than the lean controls (P < 0.05). Relative to exercised animals, sham animals became hyperglycemic
In sham and sedentary controls, adaptive hyperinsulinemia increased gradually until the 8th (sham) and 9th (sedentary control) wk of intervention and then dropped drastically. As expected, declining insulinemia coincided closely with the sharp increase in fed and fasting glucose. In contrast to sham and sedentary control rats, exercised rats displayed an adaptive hyperinsulinemia that resulted in fasting insulin values that were significantly higher than sham and sedentary controls by week 11 of intervention and for the remaining 3 wk ($P < 0.05$).

This maintenance of compensatory basal hyperinsulinemia in relation to fasting glucose levels can be represented by dividing fasting insulin by fasting glucose levels (Fig. 3B). As seen with fasting insulinemia values, increases in β-cell function ceased and began to decline in sham-treated and sedentary control animals toward the end of the study period. In contrast, exercised animals continued to show adaptive increases in β-cell function with time and function remained higher relative to sham and control rats after and including week 11 of the intervention period ($P < 0.05$). This maintenance of compensatory hyperinsulinemia in exercised but not in sham-treated and sedentary control rats is further exemplified with fed insulin measurements at the time of death (Table 1). Last, as a
Table 1. Glucose measurements

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Basal (n = 8)</th>
<th>Lean (n = 10)</th>
<th>Exercise (n = 9)</th>
<th>Sham (n = 9)</th>
<th>Control (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglycerides, mM</td>
<td>2.24 ± 0.20</td>
<td>1.73 ± 0.20</td>
<td>9.51 ± 0.90</td>
<td>8.30 ± 0.89</td>
<td>10.06 ± 0.97</td>
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<tr>
<td>Free fatty acids, meq/l</td>
<td>0.23 ± 0.02</td>
<td>0.25 ± 0.10</td>
<td>0.73 ± 0.05</td>
<td>0.68 ± 0.08</td>
<td>0.61 ± 0.03</td>
</tr>
<tr>
<td>Euthanasia corticosterone, ng/ml</td>
<td>169.71 ± 38.40</td>
<td>186.71 ± 23.50</td>
<td>158.99 ± 37.98</td>
<td>154.25 ± 47.46</td>
<td>379.20 ± 38.20</td>
</tr>
<tr>
<td>Euthanasia glucose, mmol/l</td>
<td>6.1 ± 0.26</td>
<td>5.69 ± 0.20</td>
<td>7.41 ± 0.56</td>
<td>16.84 ± 2.58</td>
<td>21.76 ± 0.95</td>
</tr>
<tr>
<td>Euthanasia insulin, pM</td>
<td>6.39 ± 0.77</td>
<td>2.52 ± 0.23</td>
<td>35.04 ± 6.39</td>
<td>19.10 ± 3.81</td>
<td>20.70 ± 3.78</td>
</tr>
<tr>
<td>IPGTT glucose, AUC</td>
<td>14.27 ± 0.72</td>
<td>23.69 ± 2.64</td>
<td>38.21 ± 3.52</td>
<td>41.04 ± 3.79</td>
<td>44.69 ± 4.03</td>
</tr>
<tr>
<td>IPGTT insulin, AUC</td>
<td>8.91 ± 1.78</td>
<td>NA</td>
<td>72.62 ± 5.14</td>
<td>54.05 ± 5.33</td>
<td>58.98 ± 9.58</td>
</tr>
</tbody>
</table>

Data are means ± SE; n, no. of animals. IPGTT, ip glucose tolerance test; AUC, area under the curve. P < 0.05, basal vs. sham (a), basal versus exercise (b), basal vs. control (c), lean vs. basal (d), lean vs. exercise (e), lean vs. sham (f), lean vs. control (g), exercise vs. sham (h), exercise vs. control (i), and sham vs. controls (j). NA, not available.

The more accurate measure of insulin secretion, plasma C-peptide (Table 1) concentrations were elevated in all ZDF rats relative to lean rats (P < 0.05). At the time of death (19 wk of age), exercised animals had significantly higher plasma C-peptide levels than either basal, sham, or sedentary control animals (Table 1, P < 0.05), whereas basal animals were not different from sham or sedentary controls, which did not have adaptive increase in β-cell insulin secretion/function (P < 0.05).

**IPGTT plasma glucose and IPGTT plasma insulin levels.** An IPGTT was performed in basal animals (6 wk of age) and after 13 wk of intervention in lean controls and in ZDF control, exercise, and sham-treated rats at 19 wk of age. As expected, all obese ZDF groups showed impaired glucose tolerance compared with lean animals at 19 wk of age (P < 0.05). Before glucose injection, sham and control rats had elevated fasting glucose compared with exercised and 6-wk-old basal rats (P < 0.05), which were not different. Throughout the 120 min following glucose bolus, glucose excursions in shams and controls were similar, but glycemia was reduced at all time points in exercised rats relative to sedentary controls (P < 0.05) and at all time points, excluding t = 30, compared with shams (P < 0.05). As an overall indicator of the postload excursion, Δglucose values at each time point (t = 0 or baseline glucose level subtracted from actual glucose values) were summed for each animal (Table 1). At 19 wk of age, all ZDF groups showed similar glucose excursions and were significantly less glucose tolerant than 6-wk-old basal ZDF animals or 19-wk-old lean ZDF, as expected (P < 0.05). Similar calculations were performed on insulin data (Table 1), revealing increased β-cell responsiveness to a glucose stimulus in all 19-wk-old ZDF rats compared with basal animals at 6 wk of age (P < 0.05), as expected. At 19 wk of age, however, exercised animals displayed increased insulin secretion over this 2-h time period vs. sham-treated and sedentary control animals (P < 0.05) (Fig. 4).

**Lipid and corticosterone measurements made at death.** Obesity and T2DM are linked to increased circulating plasma lipids and free corticosterone; therefore, we measured these parameters at the time of death. We found that, at 19 wk of age, plasma triglycerides and nonesterified fatty acids were elevated in the three obese ZDF groups compared with basal and lean animals; however, there were no differences between exercise, sham-treated, and sedentary control animals (Table 1). Corticosterone measurements made at death indicate that large increases in resting corticosterone levels occur over time in sedentary control ZDF rats compared with 6-wk-old basal animals and lean Zucker rats (P < 0.05). Both exercise training and sham treatment were effective to normalize this age- and/or obesity-associated increase in circulating corticosterone levels compared with sedentary control ZDF animals (P < 0.05).
In efforts to better understand the contributors to changes in β-cell mass seen in our study, we determined the number of islets/β-cell clusters that fell within a certain size range spanning 750 to 50,000 μm², where mean β-cell size/area was based on preceding hypertrophy measurements (Fig. 5F). As expected, β-cell clusters were more numerous in each of the 19-wk-old ZDF treatment groups relative to 6-wk-old basal animals and 19-wk-old lean ZDF rats (P < 0.05). Exercise and sham-exercise treatment resulted in a greater number of β-cell clusters for each size range (excluding the 5,000–10,000 range) compared with sedentary control animals (P < 0.05).

Fluorescent immunohistochemical β-cell studies: morphology, insulin intensity, GLUT2, Akt/PKB, ALIS formation (FK2), and death. At death, β-cell morphology (Fig. 6A) and β-cell insulin content, as assessed by intensity of fluorescence, in pancreata of exercised animals were similar to 6-wk-old ZDF rats and 19-wk-old lean animals (Fig. 6A). With respect to GLUT2 detected by immunohistochemistry, there were pronounced differences in the levels of GLUT2 (Fig. 6B). At 6 wk of age (basal animals) detectable GLUT2 was decreased relative to lean controls that were 13 wk older (P < 0.05; Fig. 6B). Drastic decreases in GLUT2 also occurred in sham-treated and sedentary control rats at 19 wk of age compared with lean rats (P < 0.05; Fig. 6B). Exercise training attenuated the reduction of GLUT2 over time (P < 0.05).

Because the Akt/PKB pathway has been implicated in normal β-cell survival and compensatory mass adaptation, we performed immunohistochemical staining to identify and locate Akt/PKB. Staining for Akt/PKB shows decreased detection in 19-wk-old ZDF rats, including lean rats, relative to 6-wk-old basal animals (P < 0.05; Fig. 6C). Between the four groups of animals, detectable Akt/PKB was maintained at lean levels in exercised rats such that levels were higher than in sham-treated and sedentary control rats (P < 0.05). At termination of the experiment, Akt/PKB levels were not different between exercised and lean nondiabetic rats.

Oxidative stress associated with hyperglycemia is known to induce protein ubiquitination and their accumulation in cytosolic structures known as ALIS (aggresome-like induced structures) (31). To detect ALIS, we stained tissues with a monoclonal antibody (FK2) that recognizes mono- and polyubiquitinated proteins (21). As expected, sedentary control animals demonstrated many ALIS in pancreatic β-cells indicative of oxidative stress caused by hyperglycemia (31). In contrast, the presence of ALIS was not detected in basal, lean, or exercised animals. Sham-exercised animals were characterized by the presence of a paucity of ALIS, whereas sedentary control animals demonstrated many ALIS; the numbers of such structures were many more than any of the other groups incorporated into the study (Fig. 6D). These studies demonstrate that β-cell hypertrophy may be measured. All ZDF rats, including basal animals, had β-cells with approximately fivefold higher mean areas than the lean phenotype (P < 0.05; Fig. 5D). After 13 wk of treatment, only sham-treated and exercised rats demonstrated β-cell hypertrophy compared with basal animals (P < 0.05) and had further increases in mean β-cell areas compared with sedentary controls (both P < 0.05). When looking at indicators of neogenesis, both exercise and sham-exercise treatment resulted in an increased number of β-cell clusters (P < 0.05; Fig. 5E).

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Fig. 5. A: β-cell morphology. B: representative pancreatic sections at 6 wk of age and after 13 wk of treatment period (E, S, C, and L). Insulin-stained cells appear reddish-brown while non-insulin-stained tissue appears purple. B: β-cell mass. C: %β-cell proliferation/bromodeoxyuridine positive (BrdU+) incorporation. Examples of BrdU+ nuclei are identified with pointers. β-Cell hypertrophy (D), β-cell neogenesis (E), and mean islet size distribution (F). ⊥, basal group (n = 8); ■, exercise group (n = 9); ●, sham group (n = 9); ○, untreated group (n = 9); □, untreated lean (n = 10). Data are presented as means ± SE. P < 0.05, basal vs. sham (*), basal vs. exercise (†), basal vs. control (‡), lean vs. basal ($), lean vs. exercise (§), lean vs. sham (¶), lean vs. control (‖), exercise vs. sham (**), exercise vs. control (††), and sham vs. controls (§§).
Fig. 6. A: fluorescent studies: β-cell insulin (fluorescein isothiocyanate) intensity. B: immunodetectable β-cell GLUT2 (CY3) and relative GLUT2 pixel area. C: immunodetectable β-cell protein kinase B (Akt/PKB) (CY3) and relative Akt/PKB pixel area. D: β-cell aggresome-like induced structures (ALIS) (CY3) formation.  ◦, basal group (n = 8); ■, exercise group (n = 9); ▲, sham group (n = 9); ●, untreated group (n = 9); □, untreated leans (n = 10). Data are presented as means ± SE. P < 0.05, basal vs. sham (*), basal vs. exercise (†), basal vs. control (‡), lean vs. basal (§), lean vs. exercise (¶), lean vs. sham (‖), lean vs. control (¶), exercise vs. sham (***), exercise vs. control (††), and sham vs. controls (§§).
pancreatic β-cells are exposed to less oxidative stress when exercised.

DISCUSSION

This study demonstrates that 13 wk of swim exercise results in the maintenance of euglycemia at least in part through attenuation of the loss of β-cell mass, which was related to increased β-cell proliferation, increased budding of new insulin positive clusters (neogenesis), and augmented β-cell hypertrophy. Furthermore, the reduction of hyperglycemia with regular exercise is characterized by attenuated loss of β-cell function and higher detectable levels of insulin staining intensity, GLUT2, and Akt/PKB protein. These improvements are not a result of reduced food intake, or body weight. Additionally, we show for the first time that exercise is associated with reductions in the protein-ubiquitination-degradation pathway.

Exercise maintained fed and fasting euglycemia. We did not cannulate animals and perform a hyperinsulinemic-euglycemic clamp, since the recovery period following surgery would have likely nullified the training effect. We were also concerned that chronically cannulated animals would be susceptible to infection. Other studies in ZF and ZDF rats indicate that drastic deterioration in insulin sensitivity occurs overtime (26, 43). Recently, Pold et al. (43) showed, in ZDF rats, there was a marked decrease in total GLUT4 protein in white and red gastrocnemius muscle compared with lean controls. Exercise increased GLUT4 content markedly in red gastrocnemius muscle and moderately in white gastrocnemius muscle. Measurements of basal glucose turnover during a hyperinsulinemic euglycemic clamp also indicated increased insulin sensitivity with training in these animals (43).

In contrast to their unexercised counterparts, swim animals maintained compensatory increases in β-cell adaptation and sustained adaptive hyperinsulinemia for the duration of the study in contrast to sham-treated and sedentary controls, which were unable to secrete sufficient insulin to keep glucose levels below hyperglycemic values. Thus training in ZDF animals is effective in attenuating the loss of compensatory hyperinsulinemia that occurs with worsening insulin resistance and obesity in ZF rats, and in obese nondiabetic humans. In vivo basal β-cell function, described as a quotient of weekly fasted insulin divided by weekly fasted glucose, gives some indication of the β-cell’s response to a basal glucose stimulus. This index increased in a compensatory fashion in ZDF rats during the prediabetic phase. However, as animals entered the diabetic phase, β-cell function decreased considerably in sham-treated and sedentary rats, but not in exercised rats.

Glucose tolerance deteriorated in all 13-wk-old ZDF animals. Interestingly, although all groups manifested impaired glucose tolerance, exercised rats displayed significant improvements at 2 h postglucose load. Our present findings that exercised animals recover from their glucose excursions after glucose bolus likely reflects increased total insulin release. Interestingly, we show with this current study that such improvements in glucose tolerance are independent of changes in weight gain or food intake. The absence of changes in body weight in Zucker rats with swim training is confirmed by a recent study (17). Interestingly, a study that employed forced running as the exercise regime showed that body weight increased in exercised animals despite a lack of differences in muscle mass or fat pad mass (43). The authors of this study attributed such body mass differences to observations that 25–30% of the caloric intake by untreated ZDF rats is excreted due to glucosuria (43). We did not measure glucosuria in this present study, but changes in glucose excretion may have played a role.

Further evidence of maintained β-cell compensation in exercised ZDF rats is provided by fed insulin concentrations as well as circulating C-peptide levels measured at death. Exercised animals displayed elevated fed insulin levels compared with nonexercised animals. C-peptide is a better indication of insulin secretion because it is not metabolized by the liver (16). Thus these data indicate adequate β-cell function following an overnight feeding stimulus in exercised rats, but not in sedentary and sham-treated rats. These findings are associated with exercise-induced improvements in β-cell morphology in ZDF rats (43) and are in line with studies in subjects with T2DM, suggesting that exercise increases the insulin response to either hyperglycemia or arginine (16). Indeed, there is a positive correlation between the maintenance of normal islet morphology and β-cell function (25).

Exercise is not only associated with sustained β-cell compensation and function but also with attenuated loss of β-cell mass. At 6 wk of age, during their prediabetic phase, basal animals already manifested increased β-cell proliferation compared with lean animals. Daily swim exercise partially maintains this adaptive proliferation, which in contrast was decreased in sham-treated and sedentary control animals at 19 wk. This is in agreement with our previous study at 12 wk of age in ZDF rats (34). We show that neogenesis and β-cell hypertrophy are increased in exercised rats compared with control rats. Proliferation, neogenesis, and hypertrophy data support our findings of increased β-cell mass, which has previously been shown in ZDF rats with exercise training (34) and with forced running (43). However, we extended the exercise duration by a minimum of 6 wk compared with other studies (34, 43). Interestingly, we also confirm our previous findings that chronic intermittent stress associated with either sham exercise treatment (34) or restraint stress (2) results in sustained β-cell mass compensation in ZDF rats. Glucocorticoids can inhibit insulin secretion (36), and it is possible that decreased basal glucocorticoids in exercised and sham-exercised rats could, in addition to changes in glycemia, contribute to partial preservation of β-cell mass.

We did not measure fat pad mass, since we were most concerned with the timely removal of the pancreas before autodigestion. We recognize that reductions in visceral fat pad mass may have occurred in exercised and sham-treated rats.

Recently, the role of β-cell insulin signaling and downstream Akt/PKB activation (3, 23, 48, 53, 57), as well as the maintenance of GLUT2 protein (28, 51) expression, have been implicated in the role of maintaining β-cell mass compensation and function. In ZF rats, Akt/PKB has been implicated in regulating duct-derived neogenesis and β-cell proliferation, cell size, differentiation, and survival (1, 3, 18, 23, 48, 53, 55). Normal Akt/PKB is also critically important for controlling β-cell mass (39, 44). To the best of our knowledge, we show for the first time that exercise is associated with phospho-Akt-473 activation and the partial maintenance of immunodetectable levels of β-cell GLUT2 protein expression. Increased Akt/PKB activation in β-cells of exercised animals offers an
explanation for increased mean β-cell hypertrophy, since Akt/ 
PKB activity correlates with protein synthesis and β-cell hy-
pertrophy (9). It does not, however, explain increases in the 
rates of β-cell proliferation, since mitogenesis is under the 
control of the ERK1/2 pathway (5). The fact that Akt/PKB and 
ERK1/2 are both under the control of IRS-2 tyrosine phosphory-
sation suggests that increased signaling via this intermediate is 
taking place. GLUT2 is partially maintained in exercised 
animals, and this is likely related to increased β-cell function. 
Exercise prevented fed or fasted hyperglycemia, which induces 
oxidative stress and causes a subsequent loss of GLUT2 (24).
Indeed, prevention of hyperglycemia with agents that improve 
glucose clearance attenuates the loss of GLUT2. Antioxidants 
such as N-acetylcysteine (NAC) also resulted in the mainte-
nance of adaptive insulin content (30). Prevention of hyper-
glycemia combined with an increase of pancreatic antioxidant 
defenses (12) may have resulted in decreased loss of GLUT2 
protein. Previously, it has been shown that GLUT2 was im-
proved by caloric restriction in ZDF rats (41). However, 
preservation of GLUT2 with exercise was not related to de-
creased food intake in our exercised animals. It is known that 
antecedent elevated plasma glucose levels are an important 
factor for the loss of β-cell phenotype in ZDF rats (24) and in 
humans (13, 19). We show that loss of β-cell GLUT2 is not 
secondary to β-cell damage. GLUT2 levels in basal ZDF rats 
were significantly reduced relative to lean rats before reduc-
tions in β-cell mass, marked deteriorations in normal islet 
morphology, and adaptive increases in proliferation. Alterna-
tively, we propose that GLUT2 loss occurs as a result of 
maintained hyperglycemia and as a consequence of glucotox-
icity and oxidative stress. Further support that oxidative stress 
is reduced in β-cells of exercised animals is the presence of 
ALIS in islets of hyperglycemic but not trained ZDF rats. 
Glucose toxicity is associated with the formation of reactive 
oxygen species (45), which induce ALIS formation in different 
cell types (49). Oxidative stress damages long-lived proteins, 
and these are targeted to ALIS for ubiquitination, and we 
suggested that clearance of ubiquitinated protein aggregates 
is regulated by autophagy (31). When the production of mis-
folded proteins exceeds its degradation, proteins aggregate 
intracellularly. This type of chronic endoplasmic reticulum 
stress can result in apoptotic cell death. (6). Insoluble proteins 
(ALIS) are present in the islets of 19-wk old ZDF rats but not in 
young ZDF rats or lean phenotypes (31). Additional in vitro 
ones with β-cell lines confirmed a dose-response relation-
ship between increasing hyperglycemia and ALIS formation 
(31). Formation of ALIS could be prevented by administering 
antioxidants such as NAC and taurine, which prevent oxidative 
stress in β-cells (31). We postulate that prevention of ALIS 
formation in trained rats is due to prevention of hyperglycemia 
and subsequent oxidative stress. In sham-treated rats, we saw a 
minimal effect to prevent ALIS formation despite marked 
hyperglycemia, suggesting that severity of hyperglycemia 
alone is the main, but not the only, factor responsible for ALIS 
formation. It is possible that the duration of islet exposure 
in vivo to hyperglycemia contributes to the ALIS formation. 
Although we cannot exclude the contribution of intracellular 
lipid metabolites and lipotoxicity to induce oxidative stress, we 
did not see any differences between the ZDF groups with 
respect to plasma triglycerides or FFA. It is possible that 
reduced oxidative stress in β-cells (reduced protein ubiquiti-
nation) could be related to a reduction in lipid accumulation. 
However, because de novo lipid synthesis in pancreatic β-cells 
has been shown to depend on plasma lipids and is in direct 
relation to elevated glucose levels (40), we speculated that 
reductions in β-cell oxidative stress are related to maintenance 
of euglycemia in trained animals. It is important to note that we 
(34) and others (43) demonstrated that lipids were increased 
already at 12 and 13 wk of age. However, we cannot exclude 
the possibility that normoglycemia affects lipid profiles in the 
islet irrespective of treatment. Because of the way in which we 
removed and fixed entire pancreas, we could not measure islet 
lipids. However, two recent studies (33, 37) showed that swim 
exercise and running had no effects on islet FFA oxidation (33, 
37) or islet expression of key transcription factors and enzymes 
of lipid metabolism (37). Therefore, we suggest that decreased 
protein ubiquitination, prevented with decreased oxidative 
stress (31) and during exercise, is the result of maintained 
euglycemia and subsequent attenuation of oxidative stress. 
This is supported by our findings of reduced oxidative stress-
induced protein ubiquitination in the pancreatic β-cells studied. 
We therefore suggest that decreased oxidative stress in β-
cells of trained ZDF rats prevents loss of GLUT2 protein, β-cell 
function, and β-cell mass.

We attempted to measure apoptosis by TUNEL and cleaved 
caspase 3 staining and using nuclear stains such as DAPI and 
propidium iodide, which are used to identify intensified fluo-
rescence associated with DNA fragmentation (46). With the 
current study, however, extensive background staining of 
cleaved caspase 3 made it impossible to definitively identify 
positive staining. TUNEL-positive cells in pancreatic islets 
could also not be identified quantitatively. In addition, nuclear 
staining with DAPI and propidium iodide did not stain clearly 
in the nucleus in the older ZDF islets at any variation in 
concentration or duration of dye staining. We believe that this 
is related to the difficulty in locating TUNEL-positive nuclei in 
the islets of older hyperglycemic animals despite evidence of 
increased apoptosis in paraffin-embedded tissues in sections 
from male ZDF rats (27, 42). We speculate that trained animals 
had decreased apoptosis and/or necrosis because of reduced 
hyperglycemia and therefore oxidative stress. 

In summary, we show for the first time that swim training in 
male ZDF rats is associated with preserved β-cell compensa-
tion with respect to insulin secretion and β-cell mass up until 
19 wk of age when ZDF rats are expected to be severely 
hypoinsulinemic. Although we were unable to quantify apop-
tosis, we speculate that, because of the degree of islet fibrosis 
and disorganization, apoptosis and/or necrosis played a con-
siderable role in the loss of compensatory β-cell mass in the 
diabetic control group. Our findings of maintained adaptive 
hyperinsulinemia in exercised animals is associated with par-
tial maintenance of β-cell GLUT2 protein, proper β-cell insu-
lin signaling, and prevention of the formation of ALIS, which 
is linked to β-cell growth and survival. We conclude that 
improved β-cell function is mainly related to maintenance of 
normoglycemia and is the result of the effect of training to 
ameliorate hepatic and peripheral insulin resistance and con-
sequently glucose regulation. We cannot, however, exclude 
the possibility that maintenance of normoglycemia also lessened 
lipid accumulation in the islet, which could have had beneficial 
effects in addition to normalization of glycemia. These studies
reveal novel mechanisms for the protective role of exercise on β-cell compensation during the development of T2DM.

ACKNOWLEDGMENTS

We thank Drs. Owen Chan and Karen Inouye for stimulating conversation and support.

GRANTS

This work was generously supported by research grants from the Canadian Institutes of Health Research (CIHR-MT2197: M. Vranic, S. G. Matthews, and M. Riddel) and Natural Science and Engineering Research Council of Canada (NSERC). J. H. Brumell holds an Investigator in Pathogenesis of Infectious Disease Award from the Burroughs Welcome Fund. N. A. Kanik is the recipient of a CAG/CIHR/Axcan Pharma postdoctoral fellowship administered by the Canadian Association of Gastroenterology. M. A. Király is a recipient of the Ontario Graduate Scholarship, the Banting and Best Diabetes Centre Novo-Nordisk Scholarship, the NSERC PGS-A award, and the University of Toronto’s Department of Physiology Scholarship. H. E. Bates and J. T. Y. Yue are recipients of CIHR doctoral studentships. J. T. Y. Yue is also a recipient of the Banting and Best Diabetes Centre Novo-Nordisk Scholarship.

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