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**Regular exercise prevents the development of hyperglucocorticoemia via adaptations in the brain and adrenal glands in male Zucker diabetic fatty rats**

J. E. Campbell, M. A. Kiraly, D. J. Atkinson, A. M. D'souza, M. Vranic and M. C. Riddell

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## Exercise maintains euglycemia in association with decreased activation of c-Jun NH<sub>2</sub>-terminal kinase and serine phosphorylation of IRS-1 in the liver of ZDF rats

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**Király MA, Campbell J, Park E, Bates HE, Yue JT, Rao V, Matthews SG, Bikopoulos G, Rozakis-Adcock M, Giacca A, Vranic M, Riddell MC.** Exercise maintains euglycemia in association with decreased activation of c-Jun NH<sub>2</sub>-terminal kinase and serine phosphorylation of IRS-1 in the liver of ZDF rats. *Am J Physiol Endocrinol Metab* 298: E671–E682, 2010. First published December 8, 2009; doi:10.1152/ajpendo.90575.2008.—Stress-activated systems and oxidative stress are involved in insulin resistance, which, along with  $\beta$ -cell failure, contribute to the development of type 2 diabetes mellitus (T2DM). Exercise improves insulin resistance and glucose tolerance, and these adaptations may, in part, be related to reductions in inflammation and oxidative stress. We investigated circulating and tissue-specific markers of inflammation and oxidative stress and insulin-signaling pathways in a rodent model of T2DM, the Zucker diabetic fatty rat, with and without voluntary exercise. At 5 wk of age, Zucker diabetic fatty rats ( $n = 8$ –9/group) were divided into basal (B), voluntary exercise (E), and sedentary control (S) groups. B rats were euthanized at 6 wk of age, and S and E rats were euthanized 10 wk later. E rats ran  $\sim 5$  km/day, which improved insulin sensitivity and maintained fed and fasted glucose levels and glucose tolerance. Ten weeks of exercise also decreased whole body markers of inflammation and oxidative stress in plasma and liver, including lowered circulating IL-6, haptoglobin, and malondialdehyde levels, hepatic protein oxidation, and phosphorylated JNK, the latter indicating decreased JNK activity. Hepatic phosphoenolpyruvate carboxykinase levels and Ser<sup>307</sup>-phosphorylated insulin receptor substrate-1 were also reduced in E compared with S rats. In summary, we show that, in a rodent model of T2DM, voluntary exercise decreases circulating markers of inflammation and oxidative stress and lowers hepatic JNK activation and Ser<sup>307</sup>-phosphorylated insulin receptor substrate-1. These changes in oxidative stress markers and inflammation are associated with decreased hyperglycemia and insulin resistance and reduced expression of the main gluconeogenic enzyme phosphoenolpyruvate carboxykinase.

diabetes; glycemia; insulin; hepatic; protein kinase C; insulin sensitivity; inflammation; oxidative stress

TYPE 2 DIABETES MELLITUS (T2DM) and obesity, its major predisposing factor, are the most common metabolic diseases in North America (91, 92). T2DM is characterized by a progressive decline in peripheral and hepatic insulin sensitivity and

$\beta$ -cell adaptation to insulin resistance, which eventually leads to insulin insufficiency and hyperglycemia (54).

Chronic elevations in circulating markers of inflammation, such as C-reactive protein (CRP) and haptoglobin, occur in obesity and T2DM (22). Obesity causes endoplasmic reticulum (ER) stress and leads to the activation of inflammatory signaling pathways, including JNK, contributing to insulin resistance (62–64). Indeed, JNK has recently emerged as a central metabolic modulator of insulin resistance in obesity (39). A second mechanism that initiates inflammation in obesity and T2DM is oxidative stress, which activates inflammatory signaling (14, 32, 87).

Inflammation is the primary cause of obesity-linked insulin resistance. Cytokines, such as TNF $\alpha$  and IL-6, promote the insulin resistance associated with obesity (7). The inhibition of signaling downstream of insulin receptor substrate (IRS)-1 is a primary mechanism through which inflammatory signaling leads to insulin resistance (84). Exposure of cells to TNF $\alpha$  stimulates phosphorylation of IRS-1 at Ser<sup>307</sup> (1, 40, 87), resulting in disruption of normal insulin signaling and insulin action (2, 40, 65). Serine/threonine kinases elevated in obesity (84), such as JNK, I $\kappa$ B kinase (IKK $\beta$ ), and the PKC isoforms (84, 90), are activated by inflammatory and oxidative stimuli and contribute to inhibition of insulin signaling (1, 33, 64). Modulation of hepatic JNK in rodents affects glucose metabolism (61), and JNK is stimulated by oxidative stress, increased free fatty acids (FFA), and other adipocyte-derived products, such as cytokines (12, 23, 46, 47, 59).

Other serine kinases, such as PKC and IKK $\beta$ , are implicated in the mechanisms of hepatic insulin resistance (5, 13, 15, 53, 73, 74, 77). PKC $\delta$  is activated by short-term elevations in plasma FFA (13, 53), whereas another novel isoform, PKC $\epsilon$ , appears to be involved in hepatic insulin resistance caused by high-fat feeding (73, 74). A potential pathway may involve FFA-induced activation of PKC, which impairs the insulin signaling pathway directly, by phosphorylating serine/threonine sites of insulin receptor and/or IRS-1/2, or indirectly, by activating other serine kinases such as JNK and IKK $\beta$ . Rodent models of obesity and insulin resistance, such as Zucker fatty (ZF) rats, Zucker diabetic fatty (ZDF) rats, *ob/ob* mice, and *db/db* mice, all of which lack functional leptin signaling or production, manifest increased systemic inflammation and impaired insulin signaling (26, 80).

Exercise is beneficial in the prevention and treatment of insulin resistance in humans and in rodent models of T2DM

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(11, 18, 25–27, 31, 35, 38, 68, 80). Exercise training decreases elevated obesity-related systemic markers of inflammation, such as CRP and IL-6 (22). Swim training of rats has also been shown to blunt the proinflammatory cytokine response to lipopolysaccharide (21) and to decrease CRP (3). In aging humans, when insulin resistance is prevalent, basal circulating IL-6 and CRP are lowered with increased physical activity (19, 71). IL-6, which increases during and shortly after exercise, has an inhibitory effect on TNF $\alpha$  production (75), and TNF $\alpha$  may be elevated in anti-IL-6-treated or IL-6-knockout mice (58, 60). However, the potential effects of exercise training to modulate the cellular pathways involved in inflammation and related serine phosphorylation of IRS-1, as it pertains to improved insulin sensitivity in insulin target tissues, remain unknown. The purpose of this study was to identify the signaling pathways whereby exercise inhibits inflammation and improves insulin sensitivity in a rodent model of T2DM. For this investigation, we chose to use voluntary wheel running, rather than treadmill running or swimming, inasmuch as it has a lower stress component, which also influences T2DM development (9, 50).

## MATERIALS AND METHODS

**Animals.** Male ZDF (ZDF/Gmi-*fa/fa*) rats were obtained from Charles River Laboratories (Saint-Constant, PQ, Canada) at 5 wk of age; their initial body weight was 150–175 g. Rats were housed singly in opaque microisolation cages and kept at a constant temperature of 22–23°C in humidity-controlled rooms on a standard 12:12-h light-dark cycle (lights on from 0700 to 1900). 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.** Twenty-five male ZDF rats were obtained at 5 wk of age, acclimatized for 1 wk, and then randomly divided into three groups. At 6 wk of age, one group of ZDF animals was euthanized by decapitation to serve as a basal baseline control (B;  $n = 8$ ); the remaining two groups, exercise (E,  $n = 9$ ) and sedentary controls (S,  $n = 8$ ), were incorporated into the long-term study for 10 wk and then euthanized at 16 wk of age ( $n = 9$ /group). Various markers of whole body and hepatic inflammation and key regulators of hepatic glucose production were then measured (see below).

**Volitional exercise training: wheel running.** Male ZDF rats incorporated into the exercise arm of the study were individually housed in rodent activity wheel-and-cage assemblies (Harvard Apparatus, Holliston, MA), which allow the animals to exercise voluntarily. Assemblies included a stainless steel activity wheel with low-friction Teflon bearings, which allowed quiet, smooth operation. Wheel hub supports had a cutaway to allow the food hoppers and water bottle to fit adjacent to the entire assembly, which sat on top of common polycarbonate rat cages. The cage floor space adjacent to the activity wheels met National Institutes of Health requirements for a single rodent. The wheel was  $34.5 \times 9$  cm, with a radius of 17.25 cm. Each wheel assembly was fitted with a magnetic switch counter and an LCD display, which allows recording of an animal's activity. To program the computers, we used the radius of the wheel ( $r$ ) and the following formula: circumference =  $2\pi r = 2 * 3.14159 * 17.24 = 108$  cm = 1.08 m.

Once programmed, the computers produced data for velocity (km/h) and distance run (km). S rats were subjected to the same sampling and handling procedures as the E animals. After 10 wk, all animals were euthanized in the fed state between 0900 and 1200 by decapitation.

**Running distance, food intake, and body weight measurements.** Total distance run, average and maximum velocity, and time elapsed were recorded on each morning during the 10-wk study period. Food intake (excluding the day of fast) and body weight were measured once per week (on the morning before food removal for an overnight fast). Cages were cleaned at this time to ensure that no food remained in the substrate bedding.

**Weekly/biweekly fed glucose, fasted glucose, fasted insulin, and haptoglobin concentrations.** Whole blood glucose concentration of whole blood obtained from E and S animals by tail nick was measured once per week (every Monday) by glucometer (Ascensia Elite, Bayer, Toronto, ON, Canada). In addition, fasted whole blood was sampled once per week after an 18-h fast (Thursday evenings) for determination of fasted glucose (see above), and insulin [rat insulin ELISA (Crystal Chem)] and haptoglobin [ELISA (Life Diagnostics, West Chester, PA)] concentrations were measured in plasma samples obtained by tail nick.

**Intraperitoneal glucose tolerance test with respective insulin levels.** All groups were subjected to an intraperitoneal glucose tolerance test (IPGTT) 3 days before euthanasia. For B animals, IPGTT was performed at ~6 wk of age; for E and S animals, IPGTT was performed at ~16 wk of age. After an overnight (15–18 h) fast, 50% dextrose (Abbott Laboratories, Montreal, PQ, Canada) was injected intraperitoneally at 2 g/kg body wt between 0900 and 1200. Glucose and insulin levels in blood obtained by tail nick (see above) were measured at 30-min intervals starting at *time 0* (i.e., just before injection) and continuing for 120 min.

**Resting hormone measurements at euthanasia.** For animals sampled over the 10 wk of treatment, euthanasia occurred within 5 h after the last bout of volitional running. For all hormone measurements, trunk blood was collected in 1.5-ml tubes containing EDTA and Trasylol. Immediately after decapitation, all blood samples were centrifuged at 2,500 rpm for 1 min with transferred plasma stored at –20°C. A single drop of trunk blood obtained at the time of decapitation was used to measure blood glucose with a blood glucose test strip (Ascensia Elite) and glucometer (Ascensia Elite XL blood glucose meter). Plasma insulin levels were determined using a rat insulin ELISA kit (Crystal Chem). Plasma corticosterone levels were measured by a commercially available RIA kit (Medicorp, Montreal, PQ, Canada). Plasma FFA and triglycerides were determined by an enzymatic colorimetric (acyl-CoA synthase-acyl-CoA oxidase) method (Wako Chemicals, Richmond, VA). Liver protein carbonyls were measured on cytosolic fractions using a 2,4-dinitrophenylhydrazine-reliant carbonyl assay kit (Cayman Chemicals, Ann Arbor, MI). A quantitative sandwich enzyme assay (rat IL-6 Quantikine Immunoassay, R & D Systems, Minneapolis, MN) was used to measure IL-6. Plasma haptoglobin was determined using a commercially available ELISA kit and protocol (Life Diagnostics). A thiobarbituric acid-malondialdehyde (MDA) assay was used to measure plasma MDA (44).

**Hepatocyte and myocyte homogenate preparation.** Muscle (86) and fat (10) samples were homogenized as described previously. Briefly, liver samples (150 mg) were homogenized by a hand-held glass homogenizer in *buffer A* [50 mM Tris-HCl (pH 7.5), 10 mM EGTA, 2 mM EDTA, 1 mM NaHCO<sub>3</sub>, 5 mM MgCl<sub>2</sub>, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF, 1  $\mu$ g/ml each aprotinin, leupeptin, and pepstatin, 0.1 mM PMSF, and 1  $\mu$ M microcystin]. The homogenates were centrifuged at 100,000  $g$  for 1 h at 4°C, and the supernatants were retained as the cytosolic fraction. The pellet was resuspended in *buffer B* (*buffer A* + 1% Triton X-100), homogenized by three passages through a 23-gauge needle, incubated for 15 min on ice, and centrifuged at 100,000  $g$  for 1 h at 4°C. The supernatant provided the solubilized membrane fraction. Muscle was homogenized as described previously (8, 77). Briefly, 50 mg of muscle were homogenized in a Teflon-glass homogenizer with 1 ml of buffer containing 135 mM NaCl, 25 mM  $\beta$ -glycerophosphate, 20 mM Tris (pH 7.5), 2 mM EDTA, 2 mM sodium pyrophosphate, 2 mM DTT, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10% glycerol,

1% (vol/vol) Triton X-100, 10  $\mu\text{g/ml}$  aprotinin, 1  $\mu\text{g/ml}$  okadaic acid, and 1 M PMSF. Then the samples were centrifuged at 10,000  $g$  for 10 min, and the supernatant was collected as total homogenate. The results showed that the index of purity of both fractions was  $>90\%$ .

**Western blotting of liver and muscle.** The protein concentration in all samples was determined by detergent-compatible modified Lowry microassay (Bio-Rad), with serum albumin used as the standard. For muscle (gastrocnemius) homogenates, protein concentration was determined by the Bradford method. For all samples, 50  $\mu\text{g}$  of protein were mixed with equal volumes of  $3\times$  sample-loading buffer [6.86 M urea, 4.29% SDS, 300 mM DTT, and 43 mM Tris-HCl (pH 6.8)] and left at room temperature for 30 min. The mixture was vortexed and subjected to SDS-PAGE (10% polyacrylamide). After electrophoretic separation, protein was transferred to polyvinylidene difluoride membranes, which were incubated for 1 h at room temperature in Tris-buffered saline + Tween 20 (TBST) containing 5% nonfat dry milk (pH 7.4). After the blocking step, membranes were washed in rinsing solution (TBST, pH 7.4) and then incubated overnight with the primary antibody dissolved in the blocking solution. The following antibodies were used: anti-JNK (1:1,000 dilution), anti-Thr<sup>183</sup>, Tyr<sup>185</sup>-phosphorylated JNK (anti-pJNK, 1:250 dilution), anti-I $\kappa$ B $\alpha$  (1:1,500 dilution), and anti-glucose-6-phosphatase (G6Pase)- $\alpha$  (1:1,000 dilution) obtained from Santa Cruz Biotechnology (Santa Cruz, CA); anti-PKC $\delta/\epsilon$  (1:1,000 dilution) and anti-actin (1:3,000 dilution) obtained from Sigma (St. Louis, MO); anti-IRS-1 (1:500 dilution) and anti-Ser<sup>307</sup>-phosphorylated IRS-1 (1:500 dilution) obtained from Upstate Biotechnology (Lake Placid, NY); anti-phosphoenolpyruvate carboxykinase (PEPCK, 1:5,000 dilution) obtained from Abcam (Cambridge, MA); and anti-peroxisome proliferator-activated receptor- $\gamma$  coactivator (PGC)-1 $\alpha$  obtained from Cayman Chemicals (Ann Arbor, MI). For serine-phosphorylated IRS-1, IRS-2, PEPCK, G6Pase, and PGC-1 $\alpha$ , bovine serum albumin, instead of nonfat dry milk, was used at the same concentration for preparation of blocking solution. After three washes with TBST (20 min each), membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham, Baie d'Urfe, PQ, Canada; 1:4,000 dilution). The membranes were then washed several times with TBST and developed using enhanced chemiluminescence (Zymed Laboratories, San Francisco, CA).

**Western blot analysis.** A Kodak cassette was loaded with membranes that were anchored in place using acetate film and tape. Film was exposed for 5–300 s, depending on the strength of the enhanced chemiluminescence reaction. Negative optical densities were then analyzed using a computerized image analysis system (Imaging Research Software, St. Catherine's, ON, Canada).

**Statistical analysis.** For all measurements made over time, a two-way (treatment group  $\times$  time interval) repeated-measures ANOVA was used. To examine the effects of treatment at specific times, the ANOVA was collapsed, decomposing the ANOVA and allowing testing for differences between groups. For parameters measured at euthanasia, a one-way (treatment) ANOVA was used. Duncan's post hoc analysis was used to determine differences between relevant mean values. Values are means  $\pm$  SE. Probability was determined using Statistica software (version 6.0, Tulsa, OK);  $P \leq 0.05$  was considered the limit for statistical significance.

## RESULTS

**Weekly run distance, body mass, and food intake.** Figure 1A shows the running distances observed over the 10 wk of study. Daily running distance peaked at  $6.5 \pm 0.47$  km/day at week 5 and then declined gradually to  $4.5 \pm 0.65$  km/day by week 10. At the start of the study, body mass was not different between groups (B, E, and S) but increased significantly more in S than in E rats ( $P < 0.05$ ; Fig. 1B). By the end of the study, S rats weighed  $\sim 6\%$  more than E rats. Epididymal fat pad mass was

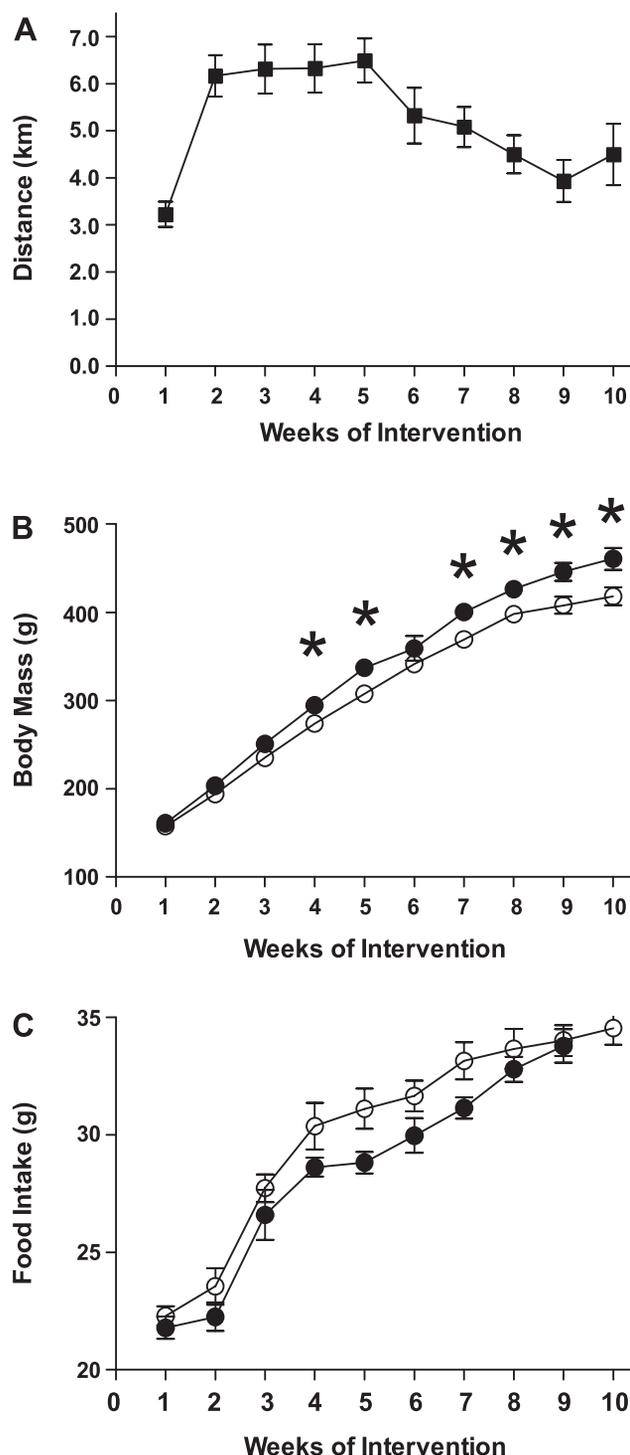


Fig. 1. Daily running distance (A), body mass (B), and food intake (C) during the intervention period. Body mass and food intake were measured within a 3-h period of lights-on (i.e., 0700–1000).  $\circ$ , Exercise (E) group ( $n = 9$ );  $\bullet$ , sedentary control (S) group ( $n = 8$ ). Values are means  $\pm$  SE. \* $P < 0.05$ , S vs. E. E tended to eat more than S, but the difference did not achieve statistical significance ( $P = 0.08$ ).

similar between E and S rats and nearly twofold higher than in B rats (Table 1). Daily food intake was not significantly different between S and E rats throughout the course of the 10-wk measurement period ( $P > 0.05$ ; Fig. 1C).

Table 1. Plasma metabolites, hormone concentrations, and epididymal adipose mass at euthanasia

	Basal (n = 8)	Sedentary (n = 8)	Exercise (n = 9)
FFA, meq/l	313.38 ± 15.2	816.35 ± 78.4*	324.89 ± 21.7†
TG, mM	1.51 ± 0.1	5.61 ± 0.4*	3.73 ± 0.3*†
Glucose, mM	6.5 ± 0.2	16.6 ± 2.9*	6.6 ± 0.2†
Insulin, ng/ml	3.05 ± 0.6	17.1 ± 2.5*	28.1 ± 2.6*†
Corticosterone, ng/ml	25.6 ± 5.9	129.9 ± 43.7*	33.3 ± 7.3†
Epididymal fat mass, g/kg body wt	5.29 ± 0.1	10.55 ± 1.6*	9.59 ± 0.4*

Values are means ± SE. FFA, free fatty acid; TG, triglyceride. \* $P < 0.01$  vs. basal. † $P < 0.01$  vs. sedentary.

Plasma glucose, plasma insulin, homeostasis model assessment of insulin resistance, and haptoglobin levels. A change in the glycemic profile is an indication of a change in insulin sensitivity and/or insulin production. Therefore, we measured fed and fasted blood glucose levels weekly and plotted these data over the course of the study (Fig. 2, A and B). At the start of the study, fed glucose levels were similar among B ( $6.5 \pm 0.2$  mM), S ( $6.5 \pm 0.5$  mM), and E ( $6.6 \pm 0.4$  mM) rats. By week 4 and throughout the rest of the experimental period, fed glucose levels were elevated in S compared with E rats ( $P < 0.05$ ; Fig. 2A). Similarly, by week 6 and beyond, fasted glucose levels were elevated in S compared with E rats (Fig. 2B).

Figure 2C shows changes in fasting insulin values over the course of the study. Fasting insulin levels at weeks 6 and 8 were higher in S than in E rats, suggesting an increase in  $\beta$ -cell compensation and adaptive hyperinsulinemia ( $P < 0.05$ ). At week 10, however, fasting insulin levels were similar between E and S rats. At euthanasia, fed insulin levels were higher in E and S than in B rats (both  $P < 0.05$ ; Table 1) and higher in E than in S rats ( $P < 0.05$ ; Table 1). As an index of insulin sensitivity, we performed weekly homeostasis model assessment of insulin resistance calculations (Fig. 2D), which showed higher insulin resistance in S than in E rats by week 6 and throughout the rest of the experimental period ( $P < 0.05$ ). Levels of haptoglobin, a protein made in hepatocytes and adipocytes in response to systemic inflammation, were not significantly different between E and S rats at the start of treatment (Fig. 2E). By week 4, however, haptoglobin levels were higher in S than in E rats, and this difference remained throughout the treatment protocol (Fig. 2E;  $P < 0.05$ ).

IPGTT plasma glucose and plasma insulin levels. An IPGTT was performed in B rats (6 wk of age) and after 10 wk of intervention in E and S rats. As expected, glucose tolerance was lower in S than in B rats, indicating a deterioration in insulin sensitivity with time ( $P < 0.05$ ; Fig. 3A). Before glucose injection, fasting glucose was elevated in S compared with E rats, whereas it was similar in E and B rats ( $P < 0.05$ ). At 2 h after glucose injection, E rats recovered from hyperglycemia, whereas glucose levels remained elevated in S rats ( $P < 0.05$ ; Fig. 3A). Glucose values were lower in E than in S rats during IPGTT at all time points ( $P < 0.05$ ). Glucose levels were significantly higher in E than in B rats only at 60 min ( $P < 0.05$ ). Plasma insulin levels during an oral glucose tolerance test did not differ between E and S rats, except at 90 min ( $P < 0.05$ ; Fig. 3B). During IPGTT, insulin levels were significantly higher in E than in B rats at all time points ( $P < 0.05$ ; Fig. 3B).

Other plasma markers of metabolism and inflammation at euthanasia. At the time of euthanasia, we sampled mixed trunk blood and measured a number of factors involved in glucose homeostasis, inflammation, and oxidative stress (Table 1).

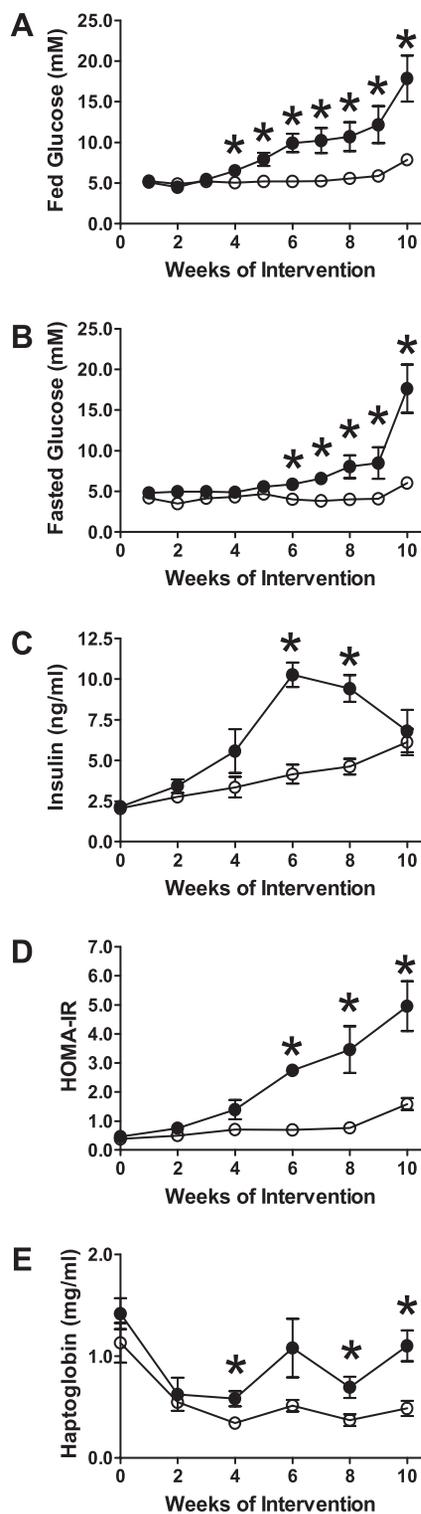


Fig. 2. Fed glucose (A), fasting glucose (B), fasting insulin (C), homeostasis model assessment of insulin resistance (HOMA-IR, D), and haptoglobin levels (E) during the intervention period. Fasting glucose and insulin were measured weekly after an 18-h fast.  $\circ$ , E group (n = 9);  $\bullet$ , S group (n = 8). Values are means ± SE. \* $P < 0.05$ , S vs. E.

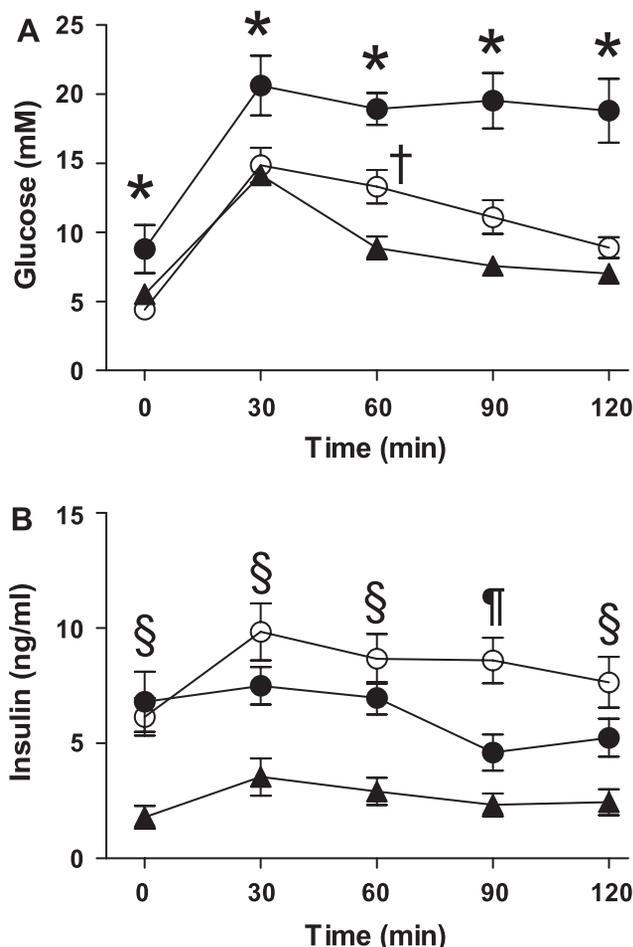


Fig. 3. Intra-peritoneal glucose tolerance test (IPGTT) in basal (B,  $n = 8$  ▲), S ( $n = 8$ , ●), and E ( $n = 9$ , ○) rats. Glucose (A) and insulin (B) levels are shown over a 2 h period. Values are means  $\pm$  SE. \* $P < 0.05$ , S vs. B and E. † $P < 0.05$ , E vs. B. § $P < 0.05$ , S and E vs. B. ¶ $P < 0.05$ , E vs. S and B.

Plasma FFA and triglyceride levels were higher in S than in B rats ( $P < 0.05$ ), and these values were attenuated in E rats (Table 1). Corticosterone, the main glucocorticoid in rats, was elevated in S compared with B rats but was similar between E and B rats (Table 1).

At the time of euthanasia, plasma haptoglobin was elevated by  $\sim 70\%$  in S compared with B and E rats ( $P < 0.05$ ; Fig. 4A). IL-6, a cytokine/myokine that is produced by the immune system and by skeletal muscle, is thought to play a role in mediating insulin resistance, possibly by increasing hepatic gluconeogenesis. Interestingly, IL-6 levels were similar in S and B rats ( $P < 0.05$ ; Fig. 4B) and  $\sim 40\%$  lower in E than in B or S rats ( $P < 0.05$ ; Fig. 4B). Levels of MDA, a widely accepted marker of lipid peroxidation (i.e., oxidative stress), were slightly, but significantly, higher in S than in B rats, but not in E rats ( $P < 0.05$ ; Fig. 4C).

**Hepatic protein carbonyl and Western blot data: pJNK, I $\kappa$ B $\alpha$ , PKC $\epsilon$ , PKC $\delta$ , and serine-phosphorylated IRS-1.** We next analyzed the signaling pathways involved in inflammation and insulin resistance in the liver, which is known to be a major insulin target tissue and an organ responsible, at least in part, for the hyperglycemia associated with T2DM. Hepatic protein carbonyl content, a marker of protein oxidation, tended to be

elevated in S compared with B rats, although this increase failed to reach statistical significance ( $P = 0.15$ ; Fig. 5A). Hepatic protein carbonyl content was lower in E than in S rats ( $P = 0.03$ ; Fig. 5A). Oxidative stress activates multiple serine/threonine kinases, including PKC, IKK $\beta$ , and JNK. PKC activation, as indicated by membrane translocation, is responsible for serine phosphorylation of IRS and related impairment of insulin signaling in vitro (36). More specifically, PKC $\delta$  and PKC $\epsilon$  have been shown to be altered in hepatic insulin resistance (13, 53, 74). We found no significant differences in PKC $\epsilon$  values among the three groups ( $P = 0.29$ ; Fig. 5B). In contrast to PKC $\epsilon$ , we found that the PKC $\delta$  membrane-to-cytosol ratio tended to be lower in E than in B rats ( $P = 0.06$ ; Fig. 5C). Activation of IKK $\beta$  has been implicated in the pathogenesis of hepatic insulin resistance via serine phosphor-

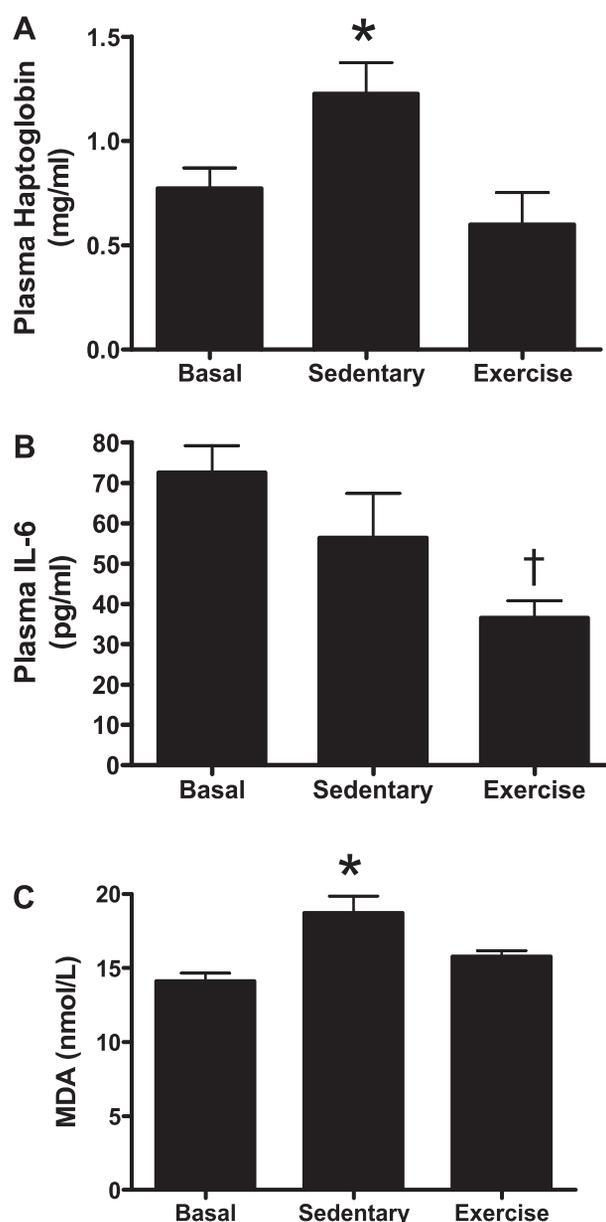


Fig. 4. Plasma haptoglobin (A), plasma IL-6 (B), and plasma malondialdehyde (MDA, C) at euthanasia in B ( $n = 8$ ), S ( $n = 8$ ), and E ( $n = 9$ ) rats. Values are means  $\pm$  SE. \* $P < 0.05$ , S vs. E and B. † $P < 0.05$ , E vs. B.

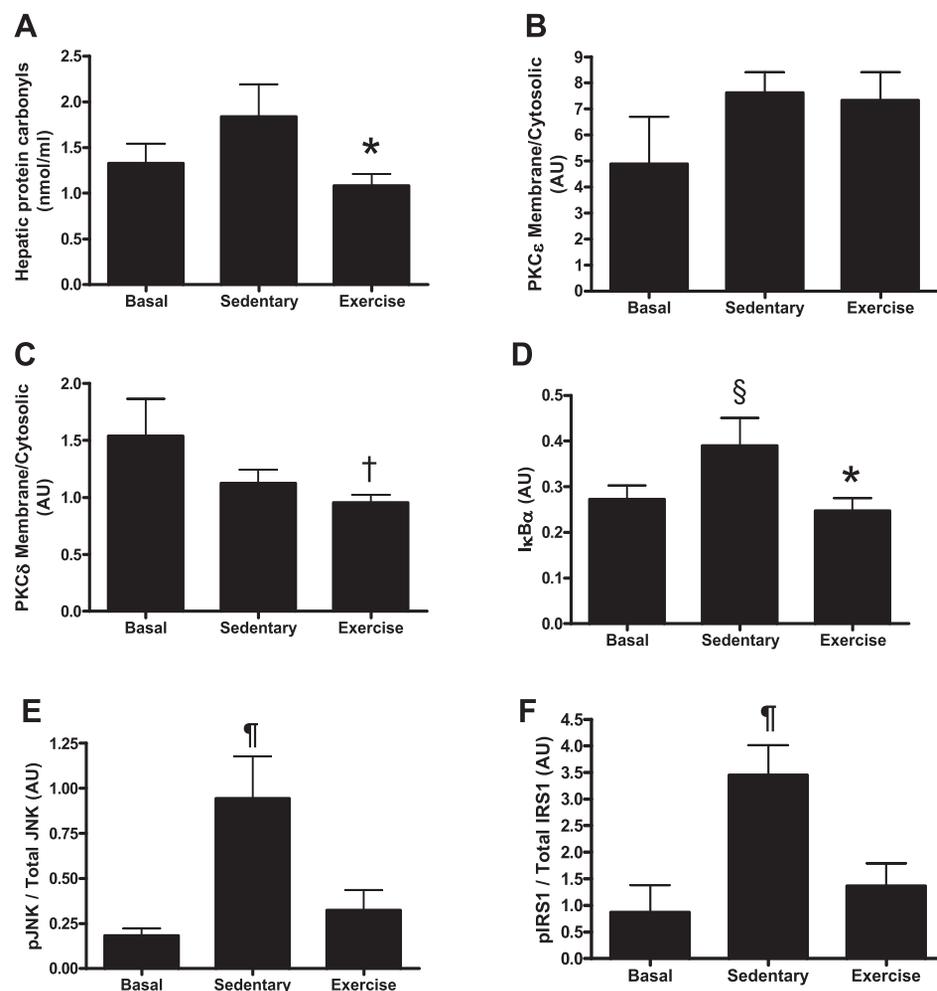


Fig. 5. Inflammatory markers in plasma (A) and hepatic tissue (B–F) of B ( $n = 8$ ), S ( $n = 8$ ), and E ( $n = 9$ ) rats. pJNK, phosphorylated JNK; pIRS1, phosphorylated insulin receptor substrate-1; AU, arbitrary units. Values are means  $\pm$  SE. \* $P < 0.05$ , E vs. S. † $P < 0.05$ , E vs. B. § $P = 0.06$ , S vs. B. ¶ $P < 0.05$ , S vs. E and B.

ylation of IRS. We used I $\kappa$ B $\alpha$  content as an indirect measure of IKK $\beta$  activity in this study and found similar levels in E and B rats (Fig. 5D). Surprisingly, I $\kappa$ B $\alpha$  was increased in S compared with B and E rats, suggesting decreased IKK $\beta$  activity ( $P < 0.05$ ; Fig. 5D). JNK activation has also been implicated in serine phosphorylation of IRS and hepatic insulin resistance. Therefore, we measured pJNK and total JNK. Total JNK protein expression was not different between either of the groups (not shown). Exercise prevented the increase in phosphorylation of JNK compared with S rats ( $P < 0.05$ ; Fig. 5E). Because JNK can phosphorylate IRS, including IRS-1, at Ser<sup>307</sup>, which is known to attenuate the insulin signal (1, 2), we next measured hepatic Ser<sup>307</sup>-phosphorylated and total IRS-1. Total IRS-1 protein was not significantly changed (not shown), but Ser<sup>307</sup>-phosphorylated IRS-1 was elevated in S compared with E and B rats ( $P < 0.05$ ; Fig. 5F). In gastrocnemius muscle, serine-phosphorylated IRS-1-to-total IRS-1 (Fig. 6A) and serine-phosphorylated JNK-to-total JNK (Fig. 6B) ratios were not significantly different among the three groups.

**Hepatic gluconeogenic markers.** Since increased glucose production in T2DM is known to be caused by increased rates of hepatic gluconeogenesis (57), we probed for protein levels of two enzymes associated with this dysregulation, PEPCK and G6Pase, and for PGC-1 $\alpha$ , which plays a pivotal role in hepatic gluconeogenesis regulation and PEPCK gene transcription (88). Protein expression of PEPCK was higher in S than in B

rats ( $P < 0.05$ ; Fig. 7A) but did not differ between E and B rats ( $P > 0.05$ ). G6Pase expression was higher in E than in B rats ( $P < 0.01$ ), and a trend was found for higher expression in S than in B rats ( $P = 0.07$ ; Fig. 7B). Finally, PGC-1 $\alpha$  expression did not differ among the three groups (Fig. 7C).

## DISCUSSION

This study demonstrates that, compared with sedentary behavior, 10 wk of volitional running maintains euglycemia and insulin sensitivity in association with reduced systemic and hepatic markers of inflammation (IL-6 and haptoglobin) and oxidative stress (MDA and protein carbonyls). Furthermore, we show reduced JNK activation and reduced serine phosphorylation of IRS-1 in hepatic tissues of exercised male ZDF rats. These improvements in inflammatory mediators and glucose metabolism are associated with reductions in circulating lipids and hepatic PEPCK protein levels, which may have contributed to the improved glycemia via reductions in hepatic gluconeogenesis. An overall schema of our findings is shown in Fig. 8.

Use of a voluntary exercise model is important to disassociate the effects of exercise and stress, inasmuch as nonexercise forms of stress have been shown to exert some protection against hyperglycemia/glucose intolerance in ZDF rats and other models of obesity and insulin resistance (8, 9, 45, 49, 50).

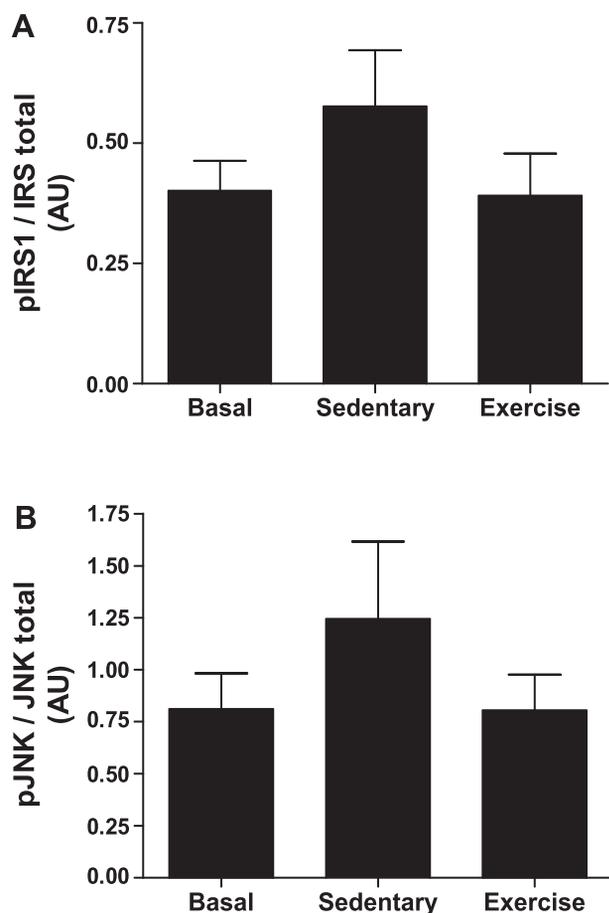


Fig. 6. Ratio of serine-phosphorylated IRS-1 to total IRS-1 (A) and serine-phosphorylated JNK to total JNK (B) expression in mixed gastrocnemius. Values are means  $\pm$  SE. No significant difference was found between groups.

In the present study, wheel running at  $\sim$ 5 km/day (Fig. 1) maintained fasting, fed glycemia, and whole body insulin sensitivity and preserved glucose tolerance in the male ZDF rat (Figs. 2 and 3, Table 1), as has been shown previously with forced treadmill running (68) and daily swimming (49, 50) in this rodent model of T2DM.

The mechanisms for the prevention of hyperglycemia by regular exercise are likely multifactorial. Indeed, daily treadmill running of ZDF rats (20 m/min, 1 h/day, for 5 wk) reduced the glucagon-to-insulin ratio, lowered plasma FFA and glucose levels, and was associated with changes in the expression of multiple genes in skeletal muscle and liver tissue, as measured by gene chip array (20). Changes in body weight or body composition may also play a role in the prevention of hyperglycemia caused by regular physical activity, although this, in itself, is not likely to be the only explanation. In this study, we found a small, but significant, attenuation in body weight gain in E compared with S male ZDF rats, which was apparent by 4 wk of training (Fig. 1B). This relative reduction in body mass in E rats may have contributed, at least in part, to their improved insulin sensitivity. Interestingly, we observed gradual increases in plasma insulin levels in E rats throughout the 10 wk of intervention. In contrast, a marked and transient increase in insulin levels was observed in S rats from 4 to 8 wk followed by a dramatic reduction of insulin levels, likely as a result of  $\beta$ -cell decompensation (Fig. 2C). The concept of

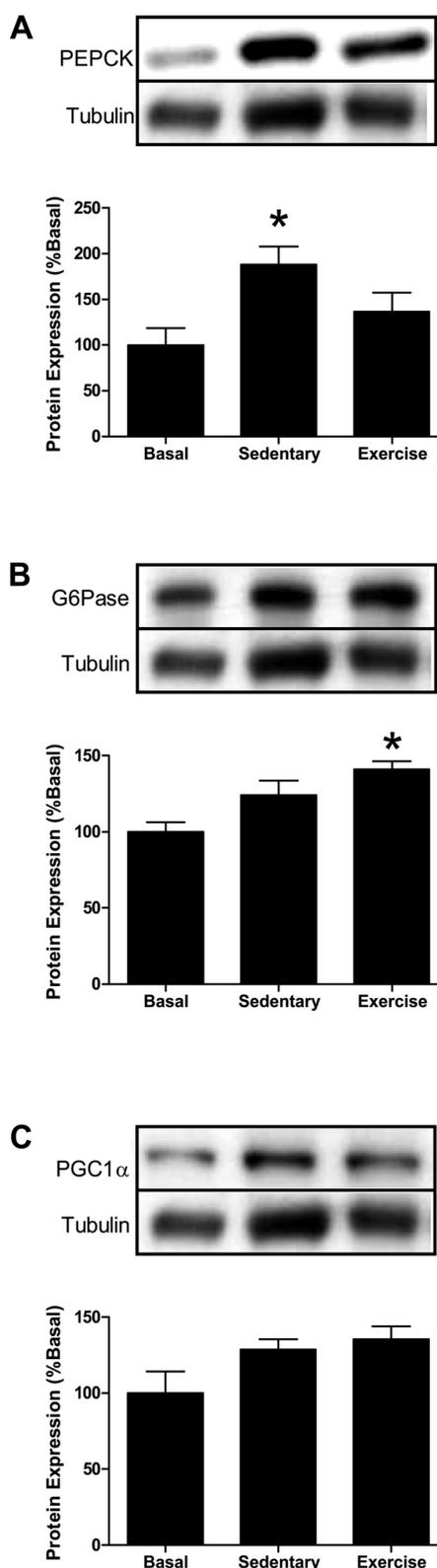


Fig. 7. Regulators of hepatic gluconeogenesis in B ( $n = 8$ ), E ( $n = 9$ ), and S ( $n = 8$ ) rats. A: phosphoenolpyruvate carboxykinase (PEPCK); B: glucose-6-phosphatase (G6Pase); C: peroxisome proliferator-activated receptor- $\gamma$  coactivator (PGC)-1 $\alpha$ . Values are means  $\pm$  SE. \* $P < 0.05$  vs. B.

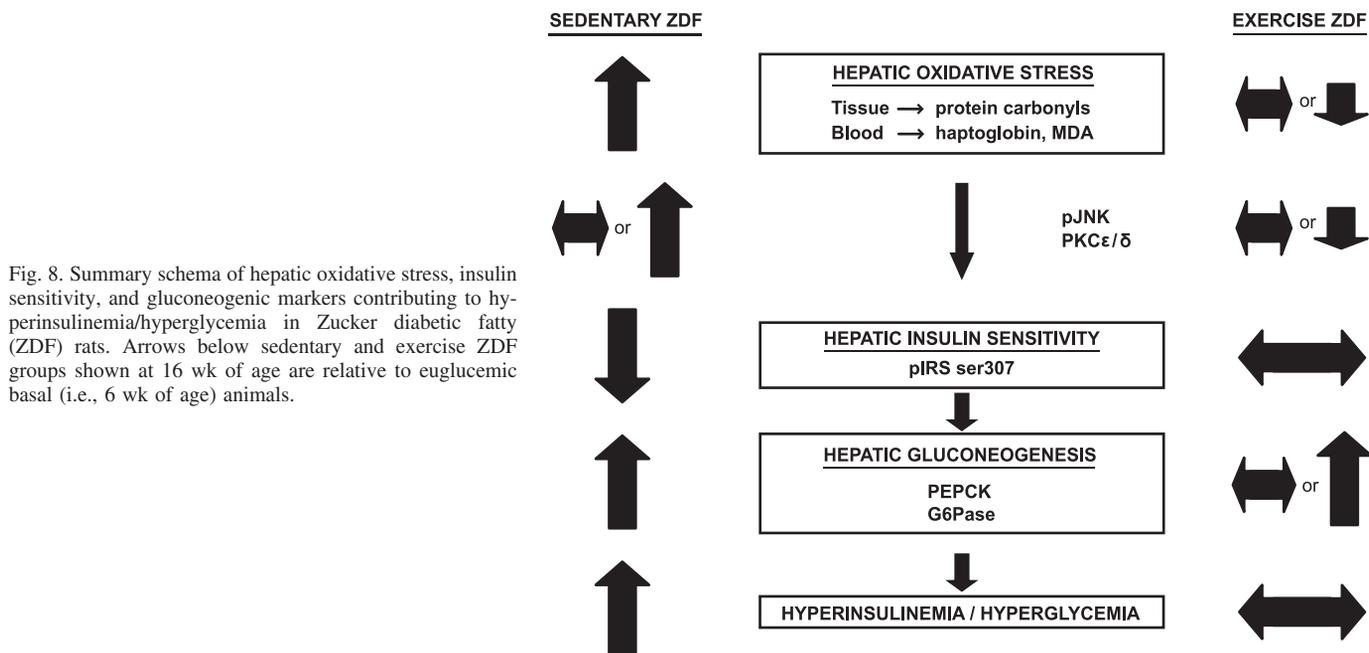


Fig. 8. Summary schema of hepatic oxidative stress, insulin sensitivity, and gluconeogenic markers contributing to hyperinsulinemia/hyperglycemia in Zucker diabetic fatty (ZDF) rats. Arrows below sedentary and exercise ZDF groups shown at 16 wk of age are relative to euglycemic basal (i.e., 6 wk of age) animals.

$\beta$ -cell failure in T2DM is thought to be critical in the development of hyperglycemia, although the mechanisms for this occurrence is not entirely clear (67–70, 80, 81). The failure to observe any reduction in insulin levels in the E group is unclear but may be related to reduction in hyperglycemia, reduction in hyperlipidemia, a combination of the two (i.e., less glucolipotoxicity), and/or decreased stress on  $\beta$ -cells due to improved insulin sensitivity (49, 68).

**Inflammatory markers.** In obesity, insulin resistance is, at least in part, mediated by cytokines produced in adipose tissue, such as TNF $\alpha$  and IL-6 (28, 41, 48, 76). TNF $\alpha$  is a proinflammatory cytokine that induces the production of other cytokines, such as IL-6 and the acute-phase reactants CRP and haptoglobin (66). We measured IL-6, but not TNF $\alpha$ , because TNF $\alpha$  is difficult to detect in plasma and because IL-6 is increased in obesity and T2DM. We found that circulating basal levels of IL-6 are decreased by voluntary exercise (Fig. 4B), which is of interest because elevated circulating levels of systemic markers of inflammation, such as IL-6, do, in part, contribute to hepatic insulin resistance in obesity (52). There is disagreement about the response of IL-6 and its role in exercise and insulin resistance, however. Acute exercise is known to induce IL-6 release from skeletal muscle, which has anti-inflammatory and insulin-sensitizing effects via AMP-activated protein kinase in skeletal muscle and adipose tissue (72). It may be that different reports with respect to the effect of exercise on IL-6 levels may reflect altered levels of production from muscle and/or from adipose tissue-macrophage consortiums, which produce IL-6 in response to TNF $\alpha$ .

As another inflammatory marker, we measured haptoglobin, rather than CRP, because CRP is not an ideal measurement of the liver's response to inflammatory status in rats (34). Plasma haptoglobin levels were significantly less in E than in S rats by 4 wk of intervention and remained below levels in S rats throughout the rest of the study (Fig. 2E). We

also observed that haptoglobin values at the time of euthanasia were similar between E and B rats and were elevated by  $\sim$ 40% in S rats (Fig. 4A). The normalization of haptoglobin levels in E rats was likely related to the decreased hepatic and systemic oxidative stress that we also observed in these animals (i.e., protein carbonyl and MDA levels; Figs. 5A and 4C, respectively). Importantly, in the E rats, reductions in haptoglobin preceded relative improvements in glucose, which suggests that early reduction of inflammation with exercise may have improved glucoregulation. However, the systemic antioxidant and anti-inflammatory effects of exercise were also likely secondary to the decreases in plasma lipid and glucose levels caused by increased insulin sensitivity and muscle substrate disposal.

**Signaling.** Exercise is well known to improve insulin signaling in skeletal muscle (51) and liver (37). In response to stimuli such as ER stress, oxidative stress, cytokines, and FFA and glucose, JNK is activated and phosphorylates IRS-1 on Ser<sup>307</sup>, thereby impairing insulin action (1, 33, 64). Oxidative stress in hepatocytes is increased by FFA and glucose directly and/or through the action of PKC (43, 78, 79) and in response to TNF $\alpha$  signaling. Removal of inflammatory mediators such as TNF $\alpha$  or downregulation of inflammatory signals such as JNK and IKK $\beta$  protects against insulin resistance in obese rodent models and in humans (39, 41, 42, 82, 89). Indeed, evidence supports the role of JNK phosphorylation in inducing hepatic insulin resistance. For example, in obesity, JNK activity is elevated in liver, muscle, and fat tissues, and loss of JNK prevents the development of T2DM in genetic and dietary mouse models of obesity (39).

To better understand the mechanisms by which exercise improves glucoregulation, we prepared liver homogenate and immunoblotted for factors known to govern hepatic insulin sensitivity. PKC $\delta$  and PKC $\epsilon$  activities have been shown to be altered in hepatic insulin resistance (13, 53, 74). Specifically, PKC $\epsilon$  is causally involved in hepatic insulin

resistance in response to a high-fat diet (73) and was previously found to be activated in ZF and ZDF models (73, 74). Although we observed a tendency for increased PKC $\epsilon$  (a marker of activation) membrane translocation in E and S compared with B rats in this study (Fig. 5B), this difference did not reach statistical significance. On the other hand, PKC $\delta$  membrane translocation (Fig. 5C) was shown to be reduced in E compared with B rats, although levels did not differ significantly between S and E rats. We measured JNK phosphorylation and I $\kappa$ B $\alpha$  content (whose decreased levels indicated increased IKK $\beta$  activity), inasmuch as JNK and IKK $\beta$  are involved in the mechanism of fat-induced hepatic insulin resistance (5, 13, 15, 53, 73, 74, 77). Paradoxically, I $\kappa$ B $\alpha$  was lower in E than in S rats (Fig. 5D), but phosphorylated JNK was normalized in parallel with a relative reduction in serine phosphorylation of IRS-1 (Fig. 5, E and F). Taken together, neither PKC isoform nor IKK $\beta$  activity appeared to reflect the pattern of hepatic Ser<sup>307</sup> phosphorylation of IRS-1 in our study; however, hepatic JNK phosphorylation did.

The mechanisms of activation of JNK in sedentary ZDF rats and its restoration by exercise remain a contentious issue, but oxidative stress, as well as ER stress, are potent activators of JNK (61, 77). Hepatic JNK activation is also induced by circulating proinflammatory cytokines (56, 83, 85). To examine the possibility that decreases in JNK activation in E rats were related to changes in hepatic oxidative stress, we measured hepatic protein carbonyl content, which indicates protein oxidation (55). Protein carbonyl levels were reduced in E compared with S rats (Fig. 5A). Our finding of reduced hepatic protein carbonyl content in E compared with S rats is consistent with the lower levels of plasma MDA (Fig. 4C), which is a marker of systemic lipid peroxidation.

Surprisingly, we failed to observe significant improvements in muscle Ser<sup>307</sup> phosphorylation of IRS-1 or in muscle JNK phosphorylation with voluntary wheel running, although both of these parameters were  $\sim$ 30% lower in E than in S rats (Figs. 6). It may be that the overall distance run by these animals [ $\sim$ 5 km/day compared with 10–15 km/day for healthy rodents (16, 29)] and the variability in animals for these parameters prevent us from observing these expected adaptations.

**Expression of PEPCK and G6Pase proteins.** Gluconeogenesis is controlled at the transcriptional level primarily by hepatic expression and/or activity of PEPCK and G6Pase. In healthy, nondiabetic rodents, PEPCK and G6Pase activities increase during acute exercise (24). Consistent with improved glucoregulation in exercising ZDF animals, we report decreased expression of hepatic PEPCK in E rats compared with their S counterparts (Fig. 7A). Similarly, Chang et al. (17) observed a hyperglycemia-related elevation in mRNA and protein content of hepatic PEPCK in sedentary ZF rats and a marked reduction in these levels by exercise training. Interestingly, acute exercise increases hepatic PEPCK mRNA levels and enzyme activity via increases in glucocorticoids (30). It may be that the reduction in PEPCK levels in the E compared with S rats was a direct result of the lower levels of circulating glucocorticoids observed in the former group in addition to improved hepatic insulin sensitivity. This is particularly important,

because increased glucocorticoids decrease hepatic and peripheral insulin sensitivity and influence the immune system (4).

PGC-1 $\alpha$  has been shown to play a pivotal role in hepatic gluconeogenic regulation and PEPCK gene transcription (88). The effects of regular exercise on PGC-1 $\alpha$  are unclear. However, acute exercise has recently been shown to increase PGC-1 $\alpha$  mRNA levels in concert with increased gene expression of PEPCK and G6Pase in healthy rodents (6). Despite differences in PEPCK levels, we found no changes in PGC-1 $\alpha$  levels among the three groups in this study (Fig. 7C).

The functional significance of decreased PEPCK expression in this study is unclear, in view of the similar (or possibly even higher) expression of G6Pase in E and S rats (Fig. 7B). Since G6Pase catalyzes the last step of glucose production, this enzyme is common to gluconeogenesis and glycogenolysis. It may be, therefore, that exercise training in this model lowers gluconeogenesis, but not glycogenolysis, or perhaps even hepatic glucose production. Similar to our observation here, regular exercise has been shown to increase G6Pase activity in healthy rats (37). Although there may be a number of contributing factors, we suspect that the dramatically improved glucoregulation observed in the E rats compared with S rats, is a result of a combination of enhanced hepatic insulin sensitivity, improved peripheral insulin sensitivity, and enhancement of  $\beta$ -cell function.

**Summary.** We show, for the first time, that volitional wheel running effectively attenuates loss of glucoregulation in male ZDF rats until  $\geq$ 16 wk of age. Such improvements in glycemia are associated with decreases in whole body and hepatic markers of inflammation and oxidative stress, improvements in hepatic insulin signaling, and lower PEPCK expression compared with sedentary, diabetic animals. Our investigation identifies the anti-inflammatory actions of exercise as new potential mechanisms for the beneficial effects of exercise on T2DM prevention.

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