Physical Exercise Enhances Hepatic Insulin Signaling and Inhibits Phosphoenolpyruvate Carboxykinase Activity in Diabetes-Prone *Psammomys obesus*

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We have shown that physical exercise enhances insulin sensitivity of skeletal muscle in diabetes-prone *Psammomys-obesus*. In this study, we examined the effect of physical exercise on the liver of these animals. Three groups of animals were exposed to a 4-week protocol; high-energy diet (CH), high-energy diet and exercising (EH), and low-energy diet (CL). Different groups were studied either in a fed state or after an overnight fast, 30 minutes after intraperitoneal (IP) injection of 1 U insulin. Hepatic phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) activity was measured. Insulin signaling response was examined after insulin injection in the fast state by analyzing tyrosine phosphorylation of insulin receptor (IR) and the association between insulin receptor substrate-1 (IRS-1) and IRS-2 with phosphatidylinositol 3 kinase (PI3-K). After 4 weeks, none of the EH animals became diabetic, whereas all the CH animals became diabetic. PEPCK activity in the fed state was higher in the CH group compared with the CL and EH groups (480 ± 28 nmol/min/mg protein, 280 ± 30 nmol/min/mg protein, and 208 ± 13 nmol/min/mg protein, respectively) ($P < .02$). G6Pase activity was higher in the CH and EH groups compared with the CL group (261 ± 54 nmol/min/mg protein, 251 ± 34 nmol/min/mg protein, and 75 ± 32 nmol/min/mg protein, respectively) ($P < .01$). After insulin administration in the fast state, tyrosine phosphorylation of IR and association of IRS-2 with PI3-K were higher in the EH and CL groups than in the CH group. We conclude that exercise improves in vivo hepatic insulin sensitivity in diabetes-prone *Psammomys-obesus*.

PHYSICAL EXERCISE HAS been shown to be effective in treatment as well as prevention of insulin resistance and type 2 diabetes mellitus.[1,2] The main mechanism underlying these phenomena appears to be an improvement in insulin sensitivity of peripheral tissues.[3-5] The direct effects of exercise were mainly analyzed in muscle[6,7] and fat tissues.[8,9] Nevertheless, although several studies have described the effects of exercise on hepatic metabolism,[10,11] no direct study, to our knowledge, has analyzed the effect of long-term exercise on hepatic insulin sensitivity and glucose metabolism and their association with the prevention of type 2 diabetes mellitus. The liver is known to be important in the regulation of blood glucose levels.[12,13] This organ also becomes insulin-resistant in type 2 diabetes mellitus.[14] Hepatic insulin resistance is expressed as reduced insulin stimulation of tyrosine phosphorylation of the insulin receptor (IR) and its major substrates[15,16] and as an increase in gluconeogenesis and hepatic glucose output[17] as a result of the inability of insulin to inhibit the activation of phosphoenolpyruvate carboxykinae (PEPCK)[18] and glucose-6-phosphatase (G6Pase).[19] We recently reported that physical exercise prevented the progression of insulin resistance to type 2 diabetes mellitus[20] and also enhanced muscle insulin sensitivity[21] in *Psammomys obesus*. This animal develops type 2 diabetes mellitus within several days to 2 weeks when transferred to a high-energy laboratory diet and is an acceptable model for studying the mechanisms of the disease in diabetes-prone populations.[22,23] Four, usually consecutive, stages (A, B, C, and D) of progression to diabetes in these animals have been described: (A) the original stage, normoglycemic and normoinsulinemic; (B) hyperinsulinemia only, which is sufficient to maintain normoglycemia; (C) hyperinsulinemia and hyperglycemia (blood glucose level >200 mg%); and (D) hyperglycemia and hypoinsulinemia, due to loss of β-cell insulin secretion capacity. Stage D is irreversible and, unless treated with insulin, the animals eventually die of severe ketoacidosis.[24] It was shown in *Psammomys obesus* that the liver has an important role in these phenomena; Ziv et al[25] showed that insulin failed to inhibit PEPCK and G6Pase activity even in the prediabetic (A) state. In another study, it was suggested that overexpression of PKCϵ in skeletal muscle and liver of *Psammomys obesus* may be connected to insulin resistance, possibly by increasing the degradation of the insulin receptors.[26] Because physical exercise prevented the progression to type 2 diabetes mellitus in *Psammomys obesus* and because the liver is known to be one of the mechanisms involved in the disease, the aim of the present study was to characterize the influence of physical exercise on hepatic insulin signaling and on the key enzymes in hepatic glucose production, PEPCK, and G6Pase.

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MATERIALS AND METHODS

Animals

A total of 48 male *Psammomys obesus* aged 6 weeks (3 weeks after weaning) from the Hebrew University-Hadassah Medical School Animal Farm were used in the present study. The animals were housed in suitable cages (5 animals per cage) in a temperature- (22°C to 25°C) and light- (12:12-hour light-dark cycle) controlled room. The animals were randomly assigned to 6 groups of animals (8 animals per group);

exercising animals consuming a high-energy diet (EH) (12.27 kJ/g; Weizmann Institute, Rehovot, Israel), control animals (CH) consuming the same high-energy diet, and control animals consuming a low-energy diet (CL) (9.97 kJ/g; Weizmann Institute), which does not induce diabetes.[24] The high-energy diet was composed of 23.6% protein, 2.4% fat, 68% carbohydrate, and 6% ash; 12.27 kJ/g (Weizmann Institute). The low-energy diet was composed of 16.7% protein, 3.1% fat, 70% carbohydrate, and 10.2% ash; 9.97 kJ/g (Weizmann Institute). The other 3 groups went through the same 4-week protocol, but were killed after an overnight fast (12 hours), 30 minutes after insulin injection. Food and water were supplied ad libitum. All experimental procedures were authorized by the Institutional Animal Care Committee (protocol no. 11/147/00).

To calculate the animals' energy consumption, food consumption in each cage of 5 animals was measured daily. The average food consumed by 1 animal was then calculated by dividing this value by 5 and then multiplied by the suitable energy value (kJ/g) according to the type of food.

Materials

Antiphosphotyrosine was obtained from Upstate Biotechnology, Lake Placid, NY. Anti-insulin receptor (IR) and antiphosphatidylinositol 3-kinase (PI3-K) was obtained from Santa Cruz (Santa Cruz, CA). Anti-insulin receptor substrate-2 (IRS-2) was obtained from Transduction Laboratories, Lexington, KY. KH14C03 was obtained from NEN, Boston, MA. All other chemicals were purchased from Sigma-Aldrich, St Louis, MO.

Experimental Protocol

The exercise training protocol was previously described.[27] Briefly, during the 4-week protocol, the animals ran on a treadmill (Quinton Q55, Seattle WA; 2.25 km/h, 6% slope) 5 days a week, 90 minutes a day (45 minutes in the morning and 45 minutes in the afternoon). The other groups, CH and CL, were held sedentary consuming high-energy or low-energy diets, respectively. After 4 weeks (24 hours after the last exercise in the EH group) the animals were anesthetized (sodium pentobarbital 0.03 mg/g intraperitoneal [IP]), the liver was excised, immediately frozen in liquid nitrogen, and then stored at −70°C. A total of 2 mL blood were taken directly from the left ventricle cavity, centrifuged for 15 minutes, and the serum was kept at −70°C for later analysis. The animals were then killed by an overdose of sodium pentobarbital.

In the animals that went through an overnight fast, blood glucose levels were measured before and 30 minutes after 1 U insulin injection IP, and the animals were killed as described above. We analyzed the response to insulin after 30 minutes, because in our preliminary experiments, we have found that maximal response was achieved after this period of time. In preliminary studies saline injection in the same protocol had no effect on blood glucose levels, as well as on the expression, phosphorylation, and activity of the various proteins. Thus, in the present study, similary to our previous studies,[20,21] we did not include saline groups.

Physiologic Measurements

The animals were weighed at baseline and once a week thereafter for the duration of the study. Blood for glucose determinations was taken at baseline and once a week from the tail vein using the glucometer Elite (Bayer, Japan) (in the EH group, blood was taken 24 hours after the preceding exercise). Serum insulin concentration was measured by radioimmunoassay (RIA) using the standard 18-hour incubation double antibody assay. Primary (guinea pig) and secondary (goat antiguinea pig) antisera were from Linco Research (St Charles, MO). Human insulin standard (Novo Nordisk, Bagsvaerd, Denmark) was used for *Psammomys obesus* insulin RIA; cross-reactivity and dilution linearity were previously determined.[28] The minimum detectable concentration was 11 pmol/L; routine intra-assay coefficient of variation (CV) was 4% to 6%, and interassay CV was 6% to 10%.

Preparation of Liver Tissue for Insulin Signaling Analysis

Liver tissue samples were washed with Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS) to remove excess blood cells and then mechanically lysed in RIPA buffer (Tris HCl pH 7.4 50 mmol/L; NaCl, 150 mmol/L; EDTA 1 mmol/L; NaF, 10 mmol/L; Triton × 100, 1%; sodium dodecyl sulfate [SDS], 0.1%; Na deoxycholate, 1%) containing a cocktail of protease inhibitors (leupeptin, 20 μg/mL; aprotinin, 10 μg/mL; phenylmethylsulfonyl fluoride [PMSF], 0.1 mmol/L; dithiothreitol [DTT], 1 mmol/L) and phosphatase inhibitors (orthovanadate, 200 μmol/L; pepstatin, 2 μg/mL) (Sigma). After a 30-second homogenization, the preparation was centrifuged at 20,000 × *g* for 20 minutes at 4°C. The supernatant, containing all the tissue's proteins, was then stored at −70°C for later analysis.

Protein Content

The protein content in each sample was measured using Bio-Rad protein assay according to the manufacturer's instructions (Bio-Rad, Richmond, CA).

Immunoprecipitation

A total of 25 μL of protein A/G Sepharose was added to 0.3 mL of the lysate (40 μg of protein), and the suspension was rotated continuously for 30 minutes at 4°C. The preparation was then centrifuged at 20,000 × *g* at 4°C for 10 minutes, and 30 μL of A/G Sepharose was added to the supernatant along with specific monoclonal or polyclonal antibodies to various antigens. The suspension was rotated overnight at 4°C. The suspension was then centrifuged at 20,000 × *g* for 10 minutes at 4°C, and the pellet was washed twice as above with RIPA buffer. The beads were deluted with 25 μL of sample buffer (0.5 mol/L Tris HCl pH 6.8; 10% SDS; 10% glycerol; 4% 2-β-mercaptoethanol; 0.05% bromophenol blue). The suspension was again centrifuged at 15,000 × *g* (4°C for 10 minutes) and washed 4 times in Tris-buffered saline Tween (TBST). Sample buffer was added and the samples were boiled for 5 minutes and then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Western Blotting

Protein, 20 to 25 μg, was electrophoresed through SDS-polyacrylamide gels (7.5% or 10%) and electrophoretically transferred onto Immobilon-P (Millipore, Bedford, MA) membranes. Following transfer, the membranes were subjected to standard blocking and incubation procedures and were incubated with specific monoclonal or polyclonal antibodies to the various proteins. The membranes were washed 4 times for 15 minutes in TBST and then further incubated for 20 minutes at room temperature with horseradish peroxidase (HRP)-labeled secondary antibody (goat antirabbit or mouse IgG) diluted 1:10,000 in blocking buffer. Following 3 washes (1 × 15 minutes and 2 × 5 minutes) in TBST, the membranes were treated with ECL reagent for 1 minute, then exposed on x-ray film (Kodak, Rochester, NY) for the required times (5 to 30 seconds), developed, and quantified by densitometry.

Homogenate Preparation for PEPCK and G6Pase Analysis

Liver homogenates were prepared in STE buffer (10 mL sucrose, 0.5 mmol/L EDTA, 10 mmol/L hepes, pH 7.4). The homogenates were

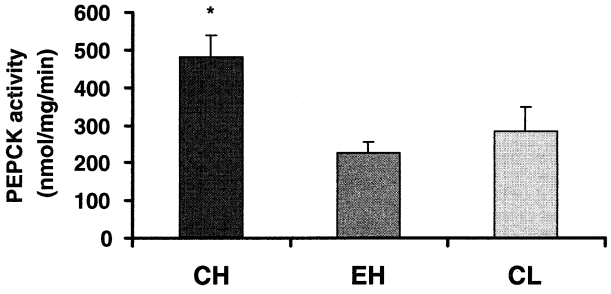

Fig 1. Hepatic PEPCK activity in the CH, EH and CL groups of *Psammomys obesus* after 4 weeks; *>EH and CL ($P < .02$).

centrifuged for 10 minutes at 8,000 × *g*, and the supernatants were centrifuged for 45 minutes at 105,000 × *g*. The obtained microsomal fraction and the supernatant were saved for assay of G6Pase and PEPCK activities, respectively.

G6Pase activity was measured according to Burchell et al[29] with slight modification. The microsomal fraction was diluted to the 2 mg protein/mL and preincubated for 10 minutes at 5°C with 0.2% deoxycholate (DOC). At the end of the preincubation 25 μg DOC-treated microsomes were added to the reaction mixture containing 50 mmol/L Tris/cacodylate buffer, pH 6.15, 15 mmol/L albumin, 20 mmol/L glucose-6-phosphate. After a 10-minute incubation in 30°C, the assay was terminated as previously described.[29] PEPCK activity was determined according to the method of Chang and Lane[19] using the rate of exchange between $KH^{14}CO_3$ and unlabeled oxaloacetate.

Statistical Analysis

Results were analyzed using analysis of variance (ANOVA) followed by Tukey's pairwise comparisons. *P* values less than .05 were considered significant. Data are presented as mean ± SE.

RESULTS

Physiological Profile of the Animals During the Study in the Fed State

The physiological characteristics of the animals in the different groups 4 weeks after initiation of the study were previously described.[21] After 4 weeks none of the animals in the EH group became diabetic (average blood glucose level, 4.6 ± 0.3 mmol/L; average blood insulin level, 4,600 ± 1,000 pmol/L), whereas all the animals in the CH group became diabetic (average blood glucose level, 21 ± 0.4 mmol/L; average blood insulin level, 4,900 ± 1,200 pmol/L). No animal in the CL group became diabetic (average blood glucose level, 4.3 ± 0.4 mmol/L; average blood insulin level, 300 ± 100 pmol/L). In agreement with earlier studies, the physiological results clearly point to the fact that the animals in the CH group were in the hyperinsulinemic-hyperglycemic stage (C) of the disease, while those in the EH group remained in the hyperinsulinemic-normoglycemic stage (B) of the disease.

Average energy consumption was highest in the EH group compared with the CH and CL groups (150 ± 10 kJ/animal/d, 130 ± 9 kJ/animal/d and 135 ± 8 kJ/animal/d, respectively; $P < .05$).

Hepatic PEPCK Activity

As summarized in Fig 1, PEPCK activity was significantly higher ($P < .02$) in the CH group (480 ± 28 nmol/min/mg protein) compared with either the EH and the CL groups (208 ± 13 nmol/min/mg protein and 280 ± 30 nmol/min/mg protein). No differences were found between the groups in PEPCK protein expression levels.

Hepatic G6Pase Activity

G6Pase activity was higher in the EH (251 ± 34 mmol/min/mg protein) and CH (261 ± 54 nmol/min/mg protein) groups compared with the CL group (75 ± 32 nmol/min/mg protein). These differences were statistically significant ($P < .05$). The difference between the EH and CH groups was not significant (Fig 2). No differences were found between the groups in G6Pase protein expression levels.

Hepatic Insulin Signaling Response

After an overnight fast, blood glucose levels of the various groups were not significantly different (4.6 ± 0.7 mmol/L in the CH group, 4 ± 0.2 mmol/L in the EH group and 4.2 ± 0.3 mmol/L in the CL group). Thirty minutes after insulin injection to the fasted animals, the reduction in blood glucose levels was significantly higher in the EH and CH groups compared with the CL group (by 1.5 ± 0.1 mmol/L, 1.5 ± 0.2mmol/L, and 0.6 ± 0.1mmol/L, respectively) ($P < .02$). Tyrosine phosphorylation of IR and association of IRS-2 with PI3-K were higher in the EH and CL groups compared with the CH group (Figs 3 and 4). No differences were found in the association between IRS-1 and PI3-K. No differences were found in the baseline levels of these proteins expression and phosphorylation.

DISCUSSION

Type 2 diabetes mellitus is the most common endocrine disease in the western world.[30] The disease is mainly characterized by severe insulin resistance in peripheral tissues, as well as the liver.[13] Physical exercise is an accepted treatment and preventative method for insulin resistance and type 2 diabetes mellitus.[1,2,20] However, while the effects of exercise on muscle and fat have been widely studied, less direct information exists on the hepatic response to long-term exercise and its association with the prevention of the type 2 diabetes mellitus. One study, however, pointed to the possible inhibitory effect of

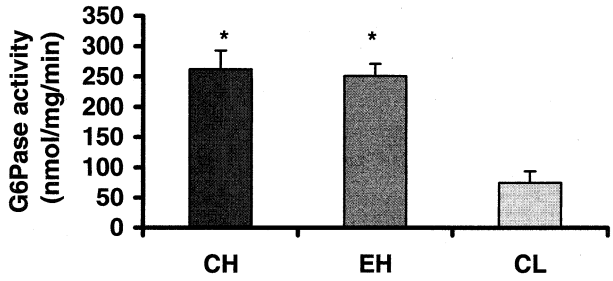

Fig 2. Hepatic G6Pase activity in the CH, EH, and CL groups of *Psammomys obesus* after 4 weeks; *>CL ($P < .05$).

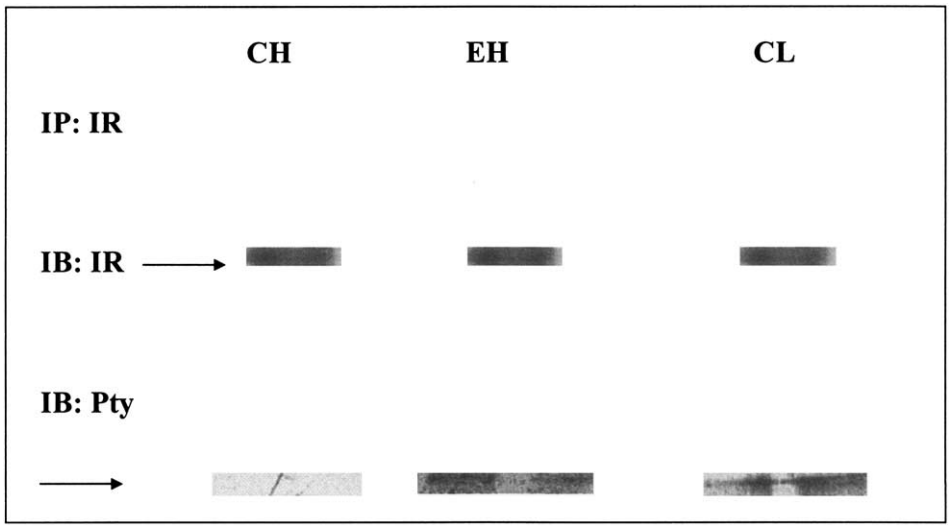

Fig 3. Hepatic IR expression and tyrosine phosphorylation (Pty) 30 minutes after insulin injection in the CH, EH, and CL groups of *Psammomys obesus* after an overnight fast. Each lane represents 1 animal from each group of animals.

exercise on hepatic glucose production in obese people.[31] We recently reported that physical exercise prevented the deterioration to type 2 diabetes mellitus in the exercising *Psammomys obesus*[20] and enhanced their muscle insulin responsiveness.[21] The aim of the present study was therefore to characterize the influence of physical exercise on hepatic insulin signaling and on the activity of 2 key enzymes in the glucose production process, PEPCK and G6Pase, in this animal model. These mechanisms have been reported to be defective in this animal model[25,26] and are considered an important part in insulin resistance and type 2 diabetes mellitus.[13,25,26] Our results showed that a high-energy diet significantly induced PEPCK and G6Pase activities (CH group). The increase in PEPCK activity was however, prevented when high-energy diet consumption was combined with physical exercise (EH group). Insulin is known to inhibit PEPCK and G6Pase activity,[27] but because blood insulin levels were similar in the CH and EH groups, the different activities of PEPCK could not be directly related to the amount of circulating insulin. A possible explanation, however, for this result is that physical exercise caused an increase in the hepatic insulin sensitivity, which inhibits PEPCK. We hypothesized that this would be consistent with the higher insulin signaling response in the EH group compared with the CH group. Indeed, tyrosine phosphorylation of IR and the association of IRS-2 with PI3-K were significantly higher in the EH group. It should also be noted that the effect of physical exercise on PEPCK activity mimics the pharmacologic effect of vanadate, an accepted pharmacologic treatment for type 2 diabetes mellitus.[32]

G6Pase activity, on the other hand, was higher in the CH and EH groups compared with the CL group, with no effect of exercise on its activity. G6Pase activity is known to increase in type 2 diabetes mellitus.[33] This is a critical enzyme during the last step in the production of glucose by both the glycogenolytic and gluconeogenetic pathways.[11] The elevated activity of G6Pase in the CH and EH groups was not affected by hyper-

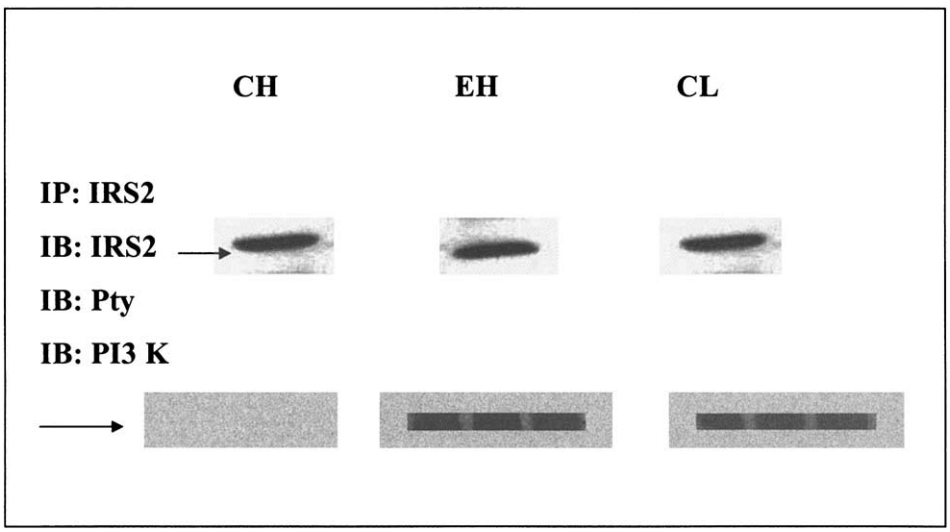

Fig 4. Hepatic association of IRS-2 and PI3-K 30 minutes after insulin injection in the CH, EH, and CL groups of *Psammomys obesus* after an overnight fast. Each lane represents 1 animal from each group of animals.

insulinemia with or without concomitant-hyperglycemia, although both hyperglycemia and hyperinsulinemia are known to inhibit G6Pase activity.[11] This result may be part of the characterization of the EH group that, after all, was in the B stage of the disease (hyperinsulinemic-normoglycemic) and therefore was more insulin-resistant than the CL group.

As for the insulin signaling response, tyrosine phosphorylation of IR and the association of IRS-2 with PI3-K were significantly higher in the EH group. Previous studies have pointed to a direct effect of these insulin signaling key proteins on the hepatic insulin resistance.[16] We suggest, therefore, that the maintenance of their responsiveness to insulin by physical exercise has an important role in the prevention of hyperglycemia, and therefore, in the deterioration to type 2 diabetes mellitus. No differences were found, however, in the IRS-1. This can be explained by the assumption that IRS-2 is dominant in hepatic insulin receptor signaling,[34] while IRS-1 is dominant in muscle tissue.[35]

In conclusion, our study shows that physical exercise ameliorates the insulin signaling response in diabetes-prone *Psammomys obesus* and inhibits PEPCK activity. These effects of exercise may play an important role in the prevention of type 2 diabetes mellitus. Further studies are required, however, to fully characterize the gluconeogenic and glycogenolytic process in this context and to directly measure the hepatic glucose output in the different groups. Other factors that may influence these mechanisms, such as corticosteroids and nutrients, should also be evaluated.

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