Voluntary exercise training enhances glucose transport but not insulin signaling capacity in muscle of hypertensive TG(mREN2)27 rats

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HYPERTENSION IS FREQUENTLY accompanied by some degree of insulin resistance of skeletal muscle glucose transport and insulin action (11), an assessment that coexists in numerous individuals.

Our laboratory has shown previously that voluntary exercise training by male heterozygous TG(mREN2)27 rats leads to a diminution of resting systolic blood pressure (21) and to improvements in glucose tolerance and whole body insulin sensitivity, likely due to an enhancement of insulin action on skeletal muscle glucose transport activity (21). However, it remains unclear whether this exercise training-induced enhancement of insulin action on skeletal muscle glucose transport activity in the TG(mREN2)27 rat is associated with parallel increases in the protein expression or functionality of these insulin signaling factors.

In this context, the present study was designed to determine whether voluntary exercise training by male heterozygous TG(mREN2)27 rats increases insulin action on skeletal muscle glucose transport activity by inducing an upregulation of the protein expression and functionality of critical elements of the insulin signaling pathway, including tyrosine phosphorylation of IR and IRS-1, IRS-1 association with PI3-kinase, and serine phosphorylation of Akt and glycogen synthase kinase-3 (GSK-3) (35). The TG(mREN2)27 rat is, therefore, an excellent rodent model of the hypertension and insulin resistance that coexists in numerous individuals.

MATERIALS AND METHODS

Animals and voluntary exercise training. Male heterozygous TG(mREN2)27 rats were obtained from the Hypertension and Vascular Disease Center of Bowman Gray School of Medicine at Wake Forest University (Winston-Salem, NC) at ~6 wk of age. Animals were reared or overtly Type 2 diabetic individuals (reviewed recently in Ref. 11).

The TG(mREN2)27 rat overexpresses the mouse Ren-2 renin transgene (25), displays local elevations in tissue angiotensin II (ATII) levels (2, 24), and, consequently, develops severe hypertension (23, 24). The TG(mREN2)27 rat is also insulin resistant at the whole body (17, 21) and skeletal muscle levels (21). The insulin-resistant skeletal muscle of hypertensive TG(mREN2)27 rats displays numerous defects in the functionality of critical elements of the insulin signaling pathway regulating glucose transport activity, including the insulin receptor (IR), insulin receptor substrate-1 (IRS-1), phosphatidylinositol 3-kinase (PI3-kinase), Akt, and glycogen synthase kinase-3 (GSK-3) (35). The TG(mREN2)27 rat is, therefore, an excellent rodent model of the hypertension and insulin resistance that coexists in numerous individuals.

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housed in a temperature-controlled room (20–22°C) with a 12:12-h light-dark cycle (lights on from 7 AM to 7 PM) at the Central Animal Facility of the University of Arizona. All procedures were approved by The University of Arizona Animal Use and Care Committee.

All animals were housed for 6 wk in wire-mesh side cages (18 × 26 × 20 cm) with vertical stainless steel activity wheels attached (circumference of 1.13 m; Lafayette Instruments, West Lafayette, IN). Access to the wheel was blocked for the sedentary TG(mREN2)27 group by a stainless steel gate between the side cage and activity wheel, whereas the trained TG(mREN2)27 rats had unrestricted access to the wheel. Running activity was assessed daily. Body weights of both sedentary and trained TG(mREN2)27 rats were measured twice weekly.

Measurement of glucose transport activity. At the end of the 6-wk training period, at 6 AM, exercising animals were denied access to their running wheels, and chow was removed from the cages of both sedentary and trained animals. Nine hours later, animals were deeply anesthetized by using an intraperitoneal injection of pentobarbital sodium (50 mg/kg body wt). One soleus and both epitrochlearis muscles were dissected and prepared for incubation. Following dissection of these muscles, the remaining soleus, heart, and the total amount of visible fat in the abdominal cavity, including the epididymal fat pads, were removed and weighed. Each epitrochlearis was incubated intact, whereas the soleus was split into two strips (~30 mg) before incubation. Muscles were incubated initially for 1 h at 37°C in 3 ml of oxygenated (95% O2–5% CO2) Krebs-Henseleit buffer (KHB) supplemented with 8 mM glucose, 32 mM mannitol, and 0.1% BSA (radioimmunoassay grade, Sigma Chemical), in the absence or presence of a maximally effective concentration of insulin (2 μM/ml; Humulin, Eli Lilly, Indianapolis, IN). Following this initial incubation, the muscles were rinsed for 10 min at 37°C in 3 ml of KHB containing 40 mM mannitol, 0.1% BSA, and insulin, if present previously. The muscles were then transferred to 2 ml of KHB containing 1 mM 2-deoxy-[1,2-3H]glucose (2-DG) (300 μCi/ml; Sigma Chemical), 39 mM [U-14C]mannitol (0.8 mCi/mmol; ICN Radiochemicals, Irvine, CA), 0.1% BSA, and insulin, if present previously. After this final 20-min incubation at 37°C, the muscles were removed, trimmed of excess fat and connective tissue, and quickly frozen between aluminum blocks precooled in liquid-nitrogen. The frozen muscle was split into two pieces, and the mass of each piece was recorded. The specific intracellular accumulation of 2-DG was determined in one piece, as described previously (12). This method for assessing glucose transport activity in isolated muscle has been validated (8, 10).

Assessment of insulin signaling protein expression and functionality. The protein expression and functionality of critical elements of the insulin signaling pathway were determined, as described previously (27, 35). Frozen pieces of muscle were homogenized in 8 volumes of ice-cold lysis buffer (50 mM HEPES, 150 mM NaCl, 20 mM sodium-pyrophosphate, 20 mM α-glycerophosphate, 10 mM NaF, 2 mM Na2VO3, 2 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM MgCl2, 1 mM CaCl2, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.5 μg/ml pepstatin, and 2 mM PMSF). A 20-min incubation on ice followed the homogenization. The samples were then centrifuged at 13,000 g for 20 min at 4°C. Protein concentration was determined by using the bicinchoninic acid method (Sigma Chemical). Proteins were separated by SDS-PAGE using 7.5% (IR, IRS-1, IRS-2) or 12% (Akt, GSK-3, and GLUT-4) polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA) and transferred to nitrocellulose membranes. The membranes were incubated with the appropriate dilution of commercially available antibodies to determine protein expression of IR-β (Santa Cruz Biotechnology, Santa Cruz, CA), IRS-1, the IRS-1 phos- phorylation subunit of PI3-kinase, Akt1/2 (all Cell Signaling Technology, Beverly, MA), GSK-3β (Upstate Biotechnology, Lake Placid, NY), and GLUT-4 (Biogenesis, Brentwood, NH). In addition, serine phosphorylation of Akt and GSK-3β was determined in muscles incubated in the absence or presence of insulin prepared as described above. Membranes were incubated with antibodies against Akt Ser673 or GSK-3β Ser21 (Cell Signaling Technology). The membranes were then incubated in secondary goat anti-rabbit antibody conjugated with horseradish peroxidase (Chemicon, Temecula, CA). Proteins were visualized on Kodak X-Omat AR film (Kodak, Rochester, NY) using an enhanced chemiluminescence detection system (Amersham Pharmacia, Piscataway, NJ). Densitometry was performed on the autoradiographs using a Bio-Rad imaging densitometer (model GS-800) with Quantity One software.

Immunoprecipitation followed by immunoblotting was performed to measure tyrosine phosphorylation of IR-β (IR/β) and IRS-1 (IRS-1/β) and of IRS-1 associated with p85 (IRS-1/p85). Muscle pieces were homogenized in 1 ml of ice-cold lysis buffer, and protein concentration was determined by using the bicinchoninic acid method. Samples were diluted to 2 mg/ml (IRS-1/β) and p85/IR and 3 mg/ml (IRS-1/p85). For assessment of IR/β, 0.5 ml of diluted homogenate was immunoprecipitated with 15 μl of agarose-conjugated anti-phosphotyrosine antibody (4G10, Upstate Biotechnology). For analysis of IRS-1/β and IRS-1/p85, 0.5 ml of diluted homogenate was immunoprecipitated with 25 μl of agarose-conjugated anti-IRS-1 antibody (Upstate Biotechnology). After an overnight incubation at 4°C, all samples were centrifuged, and the supernatant was removed. The beads were washed three times with ice-cold PBS, mixed with SDS sample buffer, and boiled for 5 min. Equal amounts of the proteins of interest were separated by SDS-PAGE on 7.5% polyacrylamide gels (Bio-Rad Laboratories) and transferred to nitrocellulose membranes for Western blotting. Immunoblotting for detection of IR/β and IRS-1/p85 was done by using antibodies against IR and p85, as described above. For analysis of IRS-1/β, the nitrocellulose membrane was incubated in anti-phosphotyrosine antibody (PY99, Santa Cruz Biotechnology). The secondary antibody incubation was performed by using secondary goat anti-mouse antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology). Protein bands were visualized and analyzed as described above.

Statistical analysis. Data are presented as means ± SE. Differences between sedentary and exercise-trained groups were analyzed by using Student’s unpaired t-test. Correlations were performed by using linear regression analysis (StaView version 5.0, SAS Institute, Cary, NC). A P value of <0.05 was considered statistically significant.

RESULTS

Effects of exercise training on body weight, muscle weights, and total abdominal fat. Peak running activity by TG (mREN2)27 rats was observed by the end of the 3rd wk of training, when these animals averaged 7.1 ± 0.8 km/day (Fig. 1). At the end of the 6-wk training period, this voluntary running activity had diminished somewhat, with the TG (mREN2)27 rats averaging 4.3 ± 0.5 km/day. This pattern of running activity in male heterozygous TG(mREN2)27 rats is similar to that reported previously by our group (21).

Initial and final body weights were not significantly different between sedentary and trained groups (Table 1), and the average weight gain over the investigation period did not differ between groups (5.2 ± 0.1 vs. 5.4 ± 0.1 g/day). The elevated cardiac mass of the TG(mREN2)27 rats (21) was not altered by the exercise training. Soleus mass in the trained animals was 19% greater (P < 0.05) than in the sedentary group. In addition, the absolute amount of total abdominal fat was 18% less (P < 0.05) in the trained group compared with the sedentary group. Similarly, total abdominal fat as a percentage of body weight was 20% lower (P < 0.05) in the trained group.

Effect of exercise training on muscle glucose transport activity. Basal 2-DG uptake in epitrochlearis and soleus muscles of TG(mREN2)27 rats was not affected by the exercise.
training (Fig. 2). However, in trained epitrochlearis, the rate of insulin-stimulated 2-DG uptake was increased by 27%, and the increase above basal due to insulin was increased by 46% (both \( P < 0.05 \)) relative to the untrained epitrochlearis. In trained soleus of the TG(mREN2)27 rats, these increases were 36 and 80%, respectively (\( P < 0.05 \)). Significant (\( P < 0.05 \)) correlations were observed between the absolute amount of total abdominal fat and the insulin-mediated increases in glucose transport activity in both the epitrochlearis (\( r = -0.705, P = 0.015 \)) and soleus (\( r = -0.749, P = 0.008 \)) muscles in the sedentary and exercise-trained TG(mREN2)27 rats (Fig. 3).

Effect of exercise training on insulin signaling factors in soleus. Because of limitations in tissue availability, a complete assessment of insulin signaling was investigated in soleus muscle only. Exercise training by the TG(mREN2)27 rats had no effect on the protein expression of IR-\( \beta \), IRS-1, the p85 regulatory subunit of PI3-kinase, Akt, and GSK-3\( \beta \) in soleus muscle (data not shown). In addition, GLUT-4 protein expression was also not enhanced in the trained soleus of the TG(mREN2)27 rat (105 ± 2 arbitrary units, \( n = 5 \)) compared with that in the soleus of the sedentary TG(mmREN2)27 rats (100 ± 5, \( n = 5 \)).

The ability of insulin to maximally activate tyrosine phosphorylation of IR-\( \beta \) and IRS-1 was similar in soleus muscle of the sedentary and trained TG(mREN2)27 rats (Fig. 4, left and center). Similarly, exercise training did not significantly modify insulin action on IRS-1-associated p85 in the soleus, a surrogate measure of PI3-kinase activation (Fig. 4, right).

Insulin action on distal elements of the insulin signaling pathway was determined in soleus muscle from sedentary and exercise-trained TG(mREN2)27 rats (Fig. 5). Whereas insulin-stimulated Ser\(^{473} \) phosphorylation of Akt was not different between sedentary and trained groups (Fig. 5, left), insulin-stimulated Ser\(^{9} \) phosphorylation of GSK-3\( \beta \) was 22% greater (\( P < 0.05 \)) in the trained soleus of TG(mREN2)27 rats (Fig. 5, right).

**DISCUSSION**

The TG(mREN2)27 rat is a rodent model of hypertension of monogenetic origin (25) and displays marked insulin resistance of whole body glucose disposal (17, 21) and skeletal muscle glucose transport (21). This muscle insulin resistance is associated with defects in the functionality of critical elements of the insulin signaling pathway, including reduced tyrosine phosphorylation of IR and IRS-1, diminished IRS-1-associated PI3-kinase, and reduced serine phosphorylation of Akt and GSK-3\( \beta \) (35). Moreover, the insulin resistance at the whole body and skeletal muscle levels in the TG(mREN2)27 rat can be overcome with voluntary exercise training (21). A primary finding of the present investigation is that this exercise training-induced enhancement of insulin action on skeletal muscle glucose transport (Fig. 2) occurs without an upregulation of the protein expression or functionality of the insulin signaling factors IR, IRS-1, PI3-kinase, and Akt (Figs. 4 and 5), and without any increase in the protein expression of GLUT-4. The only adaptive response to the voluntary exercise training observed in the insulin signaling cascade was a small (22%) but statistically significant increase in insulin-stimulated Ser\(^{9} \) phosphorylation of GSK-3\( \beta \) (Fig. 5). The functional meaningfulness of this small increase in GSK-3\( \beta \) phosphorylation (and therefore a small decrease in GSK-3 activity) in the trained soleus remains unclear, but it could facilitate a greater capacity for glycogenesis, as GSK-3 normally inhibits GS activity through serine phosphorylation (6, 36). It should be noted that, because of the relatively short period (9 h) after the trained animals were denied access to the running wheels, there may have been some influence of the last bout of exercise on insulin-stimulated skeletal muscle glucose transport activity in these TG(mREN2)27 rats.

Previous studies of exercise training and its effects on insulin action and insulin signaling in normal and insulin-resistant skeletal muscle have provided some contradictory information. Insulin action on whole body glucose disposal or on skeletal muscle glucose transport activity is typically enhanced following exercise training (reviewed in Ref. 11). With regard to muscle insulin signaling, endurance exercise training of animals with normal insulin signaling leads to an enhancement of specific steps in the insulin signaling cascade, including increased mRNA and protein expression and functionality of the IR, IRS-1, PI3-kinase, and MAP kinase (ERK1) (3, 19, 20). Several studies have addressed whether insulin signaling is altered by exercise training in the obese Zucker rat, a

**Table 1. Body weights, muscle weights, and total abdominal fat in sedentary and exercise-trained TG(mREN2)27 rats**

<table>
<thead>
<tr>
<th>Group</th>
<th>Initial BW, g</th>
<th>Final BW, g</th>
<th>Heart Weight, g/100 g BW</th>
<th>Soleus Weight, g/100 g BW</th>
<th>Total Abdominal Fat, g</th>
<th>%BW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sedentary TG(mREN2)27</td>
<td>126±4</td>
<td>344±8</td>
<td>369±26</td>
<td>39.3±1.3</td>
<td>11.3±0.4</td>
<td>3.28±0.01</td>
</tr>
<tr>
<td>Trained TG(mREN2)27</td>
<td>128±3</td>
<td>354±4</td>
<td>395±14</td>
<td>46.8±1.3*</td>
<td>9.3±0.2*</td>
<td>2.64±0.01*</td>
</tr>
</tbody>
</table>

Values are ± SE for 5–6 animals per group. BW, body weight. *\( P < 0.05 \) vs. sedentary TG(mREN2)27 group.

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prediabetic model of insulin resistance. Hevener et al. (15) demonstrated that 3 wk of treadmill training by obese Zucker rats leads to increases in IR and IRS-1 tyrosine phosphorylation, whereas Saengsirisuwan et al. (31) detected increases in IR tyrosine phosphorylation, IRS-1 protein expression, and IRS-1-associated p85, but not in Akt serine phosphorylation, in skeletal muscle following 2 wk of treadmill training by these animals. In contrast, Christ et al. (4), using a 7-wk treadmill training regimen, found no upregulation of any of these elements of the insulin signaling pathway in muscle of obese Zucker rats.

The influence of exercise training on skeletal muscle insulin signaling has recently been investigated in middle-aged, insulin-resistant human subjects with prediabetes or overt Type 2 diabetes (5). Eight weeks of aerobic exercise training increased whole body glucose disposal (assessed using a hyperinsulinemic, euglycemic glucose clamp) in both groups of insulin-resistant subjects. However, the exercise training did not enhance insulin action on IRS-1-associated PI3-kinase activity in muscle biopsies. Only the protein expression of Akt was increased by training in muscle of the Type 2 diabetic subjects. It is evident that further investigations are warranted to more clearly delineate how endurance exercise training impacts the protein expression and functionality of the insulin signaling pathway for activation of muscle glucose transport in insulin-resistant animal models and humans.

It should be noted that, in contrast to exercise training by the insulin-resistant TG(mREN2)27 rat, which enhanced muscle glucose transport but caused no increase in muscle GLUT-4 protein (present study and Ref. 21), exercise training by insulin-resistant obese Zucker rats induces substantial increases in muscle GLUT-4 protein expression (summarized in Ref. 11). One possibility, which needs further experimental testing, is that the local elevations in ATII in the TG(mREN2)27 rat may be inhibiting the normal adaptive response to exercise training of GLUT-4 biosynthesis (reviewed in Ref. 13). It is clear, however, that, at least in the TG(mREN2)27 rat, an adaptive

Fig. 2. Effect of voluntary exercise training on in vitro rates of 2-deoxy-[1,2-3H]glucose (2-DG) uptake in epitrochlearis and soleus muscles of TG(mREN2)27 rats. 2-DG was assessed in the absence (–) or presence (+) of insulin (2 mU/ml). The increase above basal due to insulin (Δ) for 2-DG uptake is also presented. Values are means ± SE for 5–6 animals per group. *P < 0.05 vs. sedentary TG(mREN2)27 group.

Fig. 3. Correlations between total abdominal fat and glucose transport activity in epitrochlearis (A) and soleus muscles (B) of sedentary and exercise-trained TG(mREN2)27 rats. Data are taken from Table 1 and Fig. 2 and were analyzed by linear regression.
response of GLUT-4 biosynthesis is not an obligatory step for the development of enhanced insulin action following exercise training, and other factors must be responsible for the training-induced enhancement of insulin action.

Interestingly, ATII, which is elevated in tissues of the TG(mREN2)27 rat (2, 24), has been associated with the development of insulin resistance of skeletal muscle glucose transport activity (26, 30). Acute or chronic infusion of ATII induces skeletal muscle insulin resistance that is independent of ATII’s well-known vasoconstrictive influences (26, 30). In addition, treatment of insulin-resistant obese Zucker rats (14), fructose-fed rats (16), or spontaneously hypertensive rats (33) with AT1-selective ATII-receptor antagonists enhances both whole body insulin sensitivity and insulin action on skeletal muscle glucose transport. Importantly, AT1-selective ATII-receptor antagonism also improves insulin sensitivity in the TG(REN2)27 rat (34). The present results indicate that exercise training by the TG(mREN2)27 rat can overcome the effects of ATII to reduce insulin action on skeletal muscle glucose transport activity.

In summary, voluntary exercise training by the hypertensive male heterozygous TG(mREN2)27 rat enhances insulin action on skeletal muscle glucose transport, but without an upregulation of the protein expression or functionality of the insulin signaling factors IR, IRS-1, PI3-kinase, and Akt, and without any increase in the protein expression of GLUT-4. Only insulin stimulation of GSK-3β Ser9 phosphorylation is increased (22%) by this exercise training regimen. The exercise training-induced increase in insulin-stimulated muscle glucose transport in the TG(mREN2)27 rat is correlated with a diminution of total abdominal fat mass. The underlying mechanisms responsible for the beneficial effects of voluntary exercise training in the TG(mREN2)27 rat remain to be elucidated.

![Graph](https://jap.physiology.org/content/99/3/361/F4)

**Fig. 4.** Tyrosine phosphorylation of insulin receptor (IR) (**left**), tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) (**center**), and IRS-1 associated with the p85 subunit of phosphatidylinositol 3-kinase (PI3-kinase) (**right**) in soleus muscles of sedentary and exercise-trained TG(mREN2)27 rats. Muscles were incubated in the absence (−) and in the presence (+) of insulin (2 μU/ml). Representative bands from the autoradiograph are displayed at the top of each panel. ND, not detectable. Values are means ± SE for 4–6 animals per group.

![Graph](https://jap.physiology.org/content/99/3/361/F5)

**Fig. 5.** Akt serine phosphorylation (**left**) and glycogen synthase kinase-3 (GSK-3) serine phosphorylation (**right**) in soleus muscles of sedentary and exercise-trained TG(mREN2)27 rats. Ser473 phosphorylation of Akt1/2 and Ser9 phosphorylation of GSK-3β were assessed in the absence (−) and in the presence (+) of insulin (2 μU/ml). Representative bands from the autoradiograph are displayed at the top of each panel. Values are means ± SE for 5–6 animals per group. *P < 0.05 vs. sedentary TG(mREN2)27 group in the presence of insulin.
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