Exercise training improves muscle insulin resistance but not insulin receptor signaling in obese Zucker rats

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Exercise training improves muscle insulin resistance but not insulin receptor signaling in obese Zucker rats. J Appl Physiol 92: 736–744, 2002; 10.1152/japplphysiol.00784.2001.—Exercise training improves skeletal muscle insulin sensitivity in the obese Zucker rat. The purpose of this study was to investigate whether the improvement in insulin action in response to exercise training is associated with enhanced insulin receptor signaling. Obese Zucker rats were trained for 7 wk and studied by using the hindlimb-perfusion technique 24 h, 96 h, or 7 days after their last exercise training bout. Insulin-stimulated glucose uptake (traced with 2-deoxyglucose) was significantly reduced in untrained obese Zucker rats compared with lean controls (2.2 ± 0.17 vs. 5.4 ± 0.46 μmol·g⁻¹·h⁻¹). Glucose uptake was normalized 24 h after the last exercise bout (4.9 ± 0.41 μmol·g⁻¹·h⁻¹) and remained significantly elevated above the untrained obese Zucker rats for 7 days. However, exercise training did not increase insulin receptor or insulin receptor substrate-1 (IRS-1) tyrosine phosphorylation, phosphatidylinositol 3-kinase activity associated with IRS-1 or tyrosine phosphorylated immunoprecipitates, or Akt serine phosphorylation. These results are consistent with the hypothesis that, in obese Zucker rats, adaptations occur during training that lead to improved insulin-stimulated muscle glucose uptake without affecting insulin receptor signaling through the PI3-kinase pathway.

phosphatidylinositol 3-kinase; insulin receptor substrate-1; glucose uptake; tyrosine phosphorylation

MANY DIFFERENT RODENT MODELS have been used to study the characteristics and causes of muscle insulin resistance, but probably the most popular model to date is the obese Zucker rat. Early research indicated that the muscle of the obese Zucker rat demonstrated both reduced insulin sensitivity and reduced responsiveness, suggesting that its muscle insulin resistance resulted from both receptor and postreceptor defects (12, 20, 33). These defects included a reduced insulin receptor number and a defective glucose transport system (8, 12, 14, 25). With regard to glucose transport, it was found that insulin-stimulated translocation of the glucose transporter GLUT-4 to the plasma membrane was compromised even when maximally stimulated by insulin (7, 8, 25). Thus muscle insulin resistance of the obese Zucker rat can be attributed in large part to the inability of insulin to increase the number of functional glucose transporters in the plasma membrane. This inability of insulin to translocate GLUT-4 to the plasma membrane does not appear to be due to a defect in the translocation process per se, because translocation is normal when it is stimulated by contraction (7).

More recent evidence would suggest that reduced GLUT-4 translocation is related to abnormalities in the insulin signaling cascade system involving insulin receptor substrate (IRS)-1 and phosphatidylinositol 3-kinase (PI3-kinase) (1, 22, 32).

Exercise training improves the skeletal muscle insulin resistance of the obese Zucker rat (2, 8, 11, 20, 21). This improvement is manifested as an increase in insulin responsiveness without an effect on insulin sensitivity (11, 20). The mechanism by which this occurs is not fully understood but involves an increase in GLUT-4 protein expression (2, 8). However, it is also possible that metabolic adaptations that occur in response to exercise training may involve changes in the expression and activity of proteins involved in insulin signaling. A single bout of exercise does not increase insulin receptor signaling (5, 18, 36). In contrast, in healthy, insulin-sensitive rats and humans, exercise training increases mRNA levels for the insulin receptor and IRS-1 and increases mitogen-activated protein (MAP) kinase (extracellular signal-related kinase 1) and PI3-kinase activities (19, 26, 37). Whether exercise training improves insulin receptor signaling in insulin resistant states is not known. Therefore, the purpose of this study was to determine whether the improvement in insulin-stimulated muscle glucose uptake of the obese Zucker rat after exercise training is associated with enhanced insulin receptor signaling via the IRS-1

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and PI3-kinase pathways. Secondarily, groups of rats were studied 24 h, 96 h, and 7 days after their last exercise bout to determine how long any exercise training effect would be maintained.

METHODS

Experimental animals. Animal characteristics and the training protocol for this study have been previously reported (29). Three groups of obese Zucker rats (fa/fa; n = 7 each) were trained for 5 days/wk for 7 wk on a motorized treadmill beginning at 7 wk of age. Training began with the rats running at 15 m/min for 10 min on an 8% grade. The work rate was gradually increased during the next 6 wk until the rats were running for 90 min continuously at 22 m/min on an 8% grade. Thereafter, the rats continued this running protocol for the duration of training. On the last day of training, rats were required to run an additional 10 min at 26 m/min after a 5-min rest period. The training groups were studied 24 h, 96 h, or 7 days after their last exercise bout. Animals were 14 wk of age at the end of training. Eight obese female Zucker rats (fa/fa) and eight of their lean littermates (fa/?) were studied 24 h, 96 h, or 7 days after their last exercise bout. Animals were 14 wk of age, also underwent hindlimb-perfusion studies and served as nontrained controls. All rats were housed three to a cage and provided laboratory chow and water ad libitum. The temperature of the animal room was maintained at 21°C, and an artificial 12:12-h light-dark cycle was set. Food was withdrawn 12–14 h before hindlimb perfusion.

Surgical preparation and hindlimb perfusion. After an overnight fast that ended between 1000 and 1500 h, rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (6.5 mg/100 g body wt). The surgical procedure for hindlimb-perfusion of the rats and the perfusion apparatus used were similar to those described previously (29). Blood samples were collected under basal conditions for assay of plasma glucose (glucose oxidase method) and insulin (radioimmunoassay). After the completion of the surgical preparations, the left gastrocnemius muscle (and plantaris muscle for GLUT-4 determinations; see GLUT-4 protein) was removed, split into two sections, and clamp frozen. Then the right hindlimb was prepared for perfusion. The surgical technique was modified to limit perfusate flow to only the right leg by cannulating the right iliac artery and vein to the tip of the femoral artery. Once the canulas were inserted, the right hindlimb was washed out with 35 ml of Krebs-Henseleit buffer. The rat was then killed by an intracardiac injection of pentobarbital sodium. Immediately thereafter, the canulas were placed in line with the perfusion system, and the hindlimb was allowed to stabilize during a 10-min nonrecirculating washout period. The perfusion medium consisted of Krebs-Henseleit buffer (pH 7.4) containing 4.5% dialyzed bovine serum albumin, 20% washed time-expired human red blood cells, 1 mM glucose, 10 μ U/ml Humulin, and 0.2 mM pyruvate. Perfusion flow rate during the washout period was 5 ml/min. After the washout period, the arterial line was switched to a perfusate with 10 mM glucose, 6 mM glucose, 0.2 mM pyruvate, 2 mM mannitol, 0.2 μC/ml 2-deoxy-[3H]glucose (2-[3H]DG), 0.15 μCi/ml [14C]mannitol, and the same concentration of human red blood cells and BSA as used during the washout period. Perfusion was performed at 37°C and continued for a total of 22 min, at which time the right gastrocnemius muscle was excised, split, and clamp frozen. Muscle samples were stored at -80°C until analysis.

Materials. Polyclonal anti-COOH-terminal IRS-1, anti-IRS-2, anti-insulin receptor β-subunit, anti-PI3-kinase p110α and -β subunits, and monoclonal anti-phosphotyrosine (4G10) antibodies were purchased from Upstate Biotechnology, (Lake Placid, NY). An anti-phosphotyrosine (PY-20) antibody was purchased from Transduction Laboratories (Lexington, KY). An anti-Akt antibody and anti-phospho-Akt (Ser473) antibody were purchased from Cell Signaling Technology (Beverly, MA). Protein A-agarose and protein G-agarose were purchased from Sigma (St. Louis, MO). [γ-32P]ATP was purchased from NEN Life Science Products (Boston, MA). Phosphatidylinositol was purchased from Avanti Polar Lipids (Pelham, AL). GLUT-4 antibody was a kind gift of Dr. S. W. Cushman (NIH, Bethesda, MD).

Muscle processing. Muscle samples used for measuring insulin signaling were weighed while still frozen and were homogenized in ice-cold lysis buffer (1:10 wt/vol) containing 50 mM HEPES (pH 7.6), 150 mM NaCl, 20 mM sodium pyrophosphate, 20 mM β-glycerophosphate, 10 mM sodium fluoride, 2 mM sodium orthovanadate (Na3VO4), 2 mM EDTA (pH 8.0), 1% Nonidet P-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 mM MgCl2, 1 mM CaCl2, 10 μg/ml leupeptin, and 10 μg/ml aprotonin. A Polyclon homogenizer (Brinkman Instruments, Westbury, NY) set on maximum speed for 30 s was used for homogenization. Homogenates were incubated on ice for 20 min and then centrifuged at 15,000 g for 20 min at 4°C. Muscle debris was removed, and protein concentrations of crude extracts were estimated by the Lowry method (28). Supernatant was stored at -80°C until used.

Immunoprecipitation. For immunoprecipitation, the amounts of protein used were as follows: 250 μg for insulin receptor β-subunit, 400 μg/ml for IRS-1 and p85, and 750 μg for IRS-2. Aliquots of homogenates were incubated for 2 h on ice with specific antibodies in a final volume of 125 μl. Protein A-Sepharose beads were prepared by washing once with 0.5% Tween 20, 0.05% SDS, 0.1% BSA, and 0.02% NaN3 and twice with homogenization buffer. Protein A beads were added to the immunoprecipitation reaction, and incubation was continued for another 1.5 h at 4°C with rotation. The protein bead-antibody complexes were precipitated by brief centrifugation.

SDS polyacrylamide electrophoresis and immunoblotting. After muscle proteins were denatured by boiling for 5 min with SDS-polyacrylamide gel electrophoresis sample buffer (4% SDS and 8 M urea), proteins were separated on 7.5% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were then blocked in TBST [20 mmol/l Tris-HCl (pH 7.5), 150 mmol/l NaCl, and 0.05% Tween 20] containing nonfat dried milk for 1 h at room temperature. The membranes were incubated at 4°C overnight with the appropriate antibody at a 1:1,000 dilution in blocking buffer or TBST containing 5% bovine BSA in the case of anti-phospho-Akt and anti-Akt antibodies. After three 5-min washes in TBST, the membranes were incubated for 1 h at room temperature with anti-rabbit or anti-mouse IgG in TBST in a 1:2,000 dilution. The membranes were then washed three times in TBST and visualized on X-ray film using the enhanced chemiluminescence (ECL) detection system according to the manufacturer’s protocol (Amersham, Arlington Heights, IL). Images were digitized by scanning and band intensity was quantified by using Image Tool software.

Protein expression. To determine the expression of the insulin receptor, IRS-1, and the p85 subunit of PI3-kinase and Akt, equal amounts of protein were prepared from muscle homogenates and subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting procedures as described.
above. IRS-2 immunoprecipitatedphosphotyrosine blots were stripped using a buffer containing 0.7% β-mercaptoethanol, 7 mM SDS, and 6 mM Tris-HCl (pH 6.7) and reblotting with an anti-IRS-2 antibody to obtain IRS-2 protein.

**PI3-kinase assay.** Sample protein (250 μg) was immunoprecipitated with 16 ng/μl of anti-IRS-1 antibody, 64 ng/μl of anti-phosphotyrosine antibody (PY-20), or 32 ng/μl each of anti-p110α and anti-p110β for 2.0 h on ice. An 80-μl slurry of protein A-Sepharose was added to anti-IRS-1 and anti-phosphotyrosine immunoprecipitates for 1.5 h. Then, 80 μl of a protein A- and protein G-Sepharose slurry mixture were added to p110 immunoprecipitates for 1.5 h. Samples were centrifuged briefly at 15,000 rpm, and the immunocomplex was washed successively with the following: buffer A [PBS containing 0.5% Nonidet-40, 100 μM Na3VO4, and 1 mM dithiothreitol (DTT)], buffer B (100 mM Tris-HCl, pH 7.5, containing 500 mM LiCl2, 100 μM Na3VO4, and 1 mM DTT), and buffer C (100 mM Tris-HCl, pH 7.5, containing 100 mM NaCl, 500 μM EDTA, 100 μM Na3VO4, and 1 mM DTT). Washing was done once in buffer A and buffer B and twice in buffer C. Packed beads were suspended and incubated for 5 min in 20 μl of phosphatidylinositol dissolved (0.5 μg/ml final concentration) in 50 mM HEPES (pH 7.6) containing 1 mM EGTA and 1 mM sodium phosphate at room temperature, with occasional shaking. The kinase reaction was started by the addition of 10 μl of 50 mM HEPS (pH 7.6) containing 1 mM EGTA, 1 mM sodium phosphate, 50 mM MgCl2, 200 μM ATP, and 0.8 μCi/μl of [γ-32P]ATP (6,000 Ci/mmol; DuPont NEN, Boston, MA). After 5 min of incubation at room temperature with vigorous shaking, the reaction was terminated by the addition of 15 μl of 4 N HCl and 130 μl of MeOH-HCl3 (1:1 vol/vol). After brief centrifugation, 40 μl of the organic solvent layer were spotted onto a thin-layer chromatography plate (Silica gel 60, Whatman, Hillsboro, OR). After separation of phosphoinositides in running solvent (CHCl3-organic solvent layer were spotted onto a thin-layer chromatography plate (Silica gel 60, Whatman, Hillsboro, OR). After separation of phosphoinositides in running solvent (CHCl3-MeOH-H2O-NH4OH 40:47:11:3:2), plates were dried and exposed to X-ray film. Spots were scraped from the plates, and radioactivity was counted using a liquid scintillation counter. Kinase activity was quantified by using PI3-kinase activity in a single lot of Jurkat cell lysate as a standard.

**Determination of 2-[3H]DG uptake.** Muscle samples used to determine glucose uptake were weighed, homogenized in 1 ml of 10% TCA at 4°C, and centrifuged in a microcentrifuge for 10 min. Duplicate 0.3-ml samples of the supernatant were transferred to 20-ml scintillation vials containing the muscle homogenates. Samples were deproteinized in 10% TCA and treated the same as the muscle homogenates. Samples were counted for radioactivity in an LS-350 liquid scintillation spectrophotometer (Beckman, Fullerton, CA). Efficiency and channel cross-over were determined by counting 3H and 14C standards of known activities. The accumulation of intracellular 2-[3H]DG, which is indicative of muscle glucose uptake, was calculated by subtracting the concentration of 2-[3H]DG in the extracellular space from the total muscle 2-[3H]DG concentration. The 2-[3H]DG in the extracellular space was quantified by measuring the concentration of [14C]mannitol in the muscle homogenate.

**GLUT-4 protein.** Plantaris muscle samples were homogenized (1:15) in HES buffer (pH 7.4) with a Polytron (Brinkman Instruments). Sample homogenates and standards were diluted 1:1 with Laemmli sample buffer (27). Muscle homogenates containing 75 μg of protein were then subjected to SDS-polyacrylamide gel electrophoresis run under reducing conditions on a 12.5% resolving gel. Two GLUT-4 standards from the rat heart, containing 15 and 30 μg of protein, were loaded in parallel with the muscle samples. Protein determinations were performed on each homogenate via the method of Bradford (6). Resolved proteins were transferred to a polyvinylidene fluoride membrane (BioRad, Hercules, CA) as described previously (2). GLUT-4 antiserum diluted 1:1,000 was used for immunoblotting. GLUT-4 protein was visualized on Hyperfilm (Eastman Kodak, Rochester, NY) using the ECL Western blot detection kit (Amersham) according to the manufacturer's instructions.

**Citrate synthase activity.** To verify that there was a training response of the gastrocnemius muscle, citrate synthase activity was assayed by homogenizing weighed muscle samples in HES buffer [20 mM HEPES, 1 mM EDTA, and 250 mM sucrose, pH 7.4, 1:20 wt/vol] on ice with three consecutive 15-s bursts of a Virtishear homogenizer (Virtishear, Gardner, NY) set at its highest speed. A 300-μl aliquot was further diluted 1.2 in HES buffer and 1:10 in 0.1 M Tris and 0.4% Triton X-100, pH 8.1. Citrate synthase activity was determined spectrophotometrically according to Srere (34).

**Statistical analysis.** All data are expressed as means ± SE. Statistical differences among groups were determined by using two-way repeated-measures ANOVA and Fisher’s post hoc tests, with P < 0.05 considered to be statistically significant. Paired t-tests were used to determine statistical differences between basal and insulin-stimulated values within groups, with P < 0.05 considered to be statistically significant.

**RESULTS**

**Animal characteristics.** The characteristics of these animals have been reported previously (29) and are summarized in Table 1. Untrained obese Zucker rats were significantly heavier than their lean littermates (373.1 ± 4.8 vs. 200.1 ± 4.5 g; P < 0.001), but exercise training did not decrease body weight significantly in the obese Zucker rats. Citrate synthase activity in

<table>
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<tr>
<th>Time Since Last Training Bout</th>
<th>Lean Zucker Rats</th>
<th>Obese Zucker Rats</th>
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<tbody>
<tr>
<td>24 h</td>
<td>200.1 ± 4.5</td>
<td>373.1 ± 4.8*</td>
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<td>356.1 ± 3.2*</td>
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<td>348.5 ± 8.2*</td>
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<tr>
<td>96 h</td>
<td>200.1 ± 4.5</td>
<td>373.1 ± 4.8*</td>
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<td>373.9 ± 4.4*</td>
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<td>59.7 ± 2.6*c</td>
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<td>7 days</td>
<td>200.1 ± 4.5</td>
<td>373.1 ± 4.8*</td>
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<td>75 ± 5</td>
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<td></td>
<td>19 ± 4</td>
<td>248 ± 24*b</td>
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<td></td>
<td>197 ± 21</td>
<td>248 ± 20*b</td>
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<tr>
<td></td>
<td></td>
<td>224 ± 20*b</td>
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<tr>
<td>24 h</td>
<td>22 ± 0.46</td>
<td>2.2 ± 0.17*b</td>
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<td>4.9 ± 0.41*c,d</td>
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<td></td>
<td></td>
<td>3.9 ± 0.44*c,e</td>
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<tr>
<td>96 h</td>
<td>22 ± 0.46</td>
<td>2.2 ± 0.17*b</td>
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<td></td>
<td>4.9 ± 0.41*c,d</td>
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Values are means ± SE. *P < 0.05 was considered statistically significant. **P < 0.05 vs. lean Zucker rats. ***P < 0.001 vs. lean Zucker rats. *P < 0.05 vs. obese Zucker rats. **P < 0.001 vs. obese Zucker rats. *P < 0.05 vs. 96 h. **P < 0.05 vs. 7 days.
untrained obese Zucker rats was greater than in untrained lean Zucker rats, and training increased citrate synthase activity significantly. Seven days after the last exercise bout, citrate synthase activity was still increased compared with the untrained value (P < 0.05). Fasting plasma glucose and insulin concentrations also are given in Table 1. Plasma glucose concentrations did not differ among the groups. Plasma insulin concentrations were increased in the untrained obese Zucker rats compared with lean controls (P < 0.001) and were not significantly decreased by exercise training.

**Muscle glucose uptake.** Insulin-stimulated hindlimb uptake of 2-[3H]DG was significantly reduced in the untrained obese Zucker rats compared with lean Zucker rat controls (Table 1). In obese Zucker rats, 7 wk of training significantly increased insulin stimulation of hindlimb glucose uptake to a value that was not different from that of the lean control rats 24 h after the last exercise bout. By 96 h, insulin-stimulated glucose uptake was still significantly less in obese Zucker rats than the lean control rats (P < 0.05), but glucose uptake was still significantly greater than in the untrained obese Zucker rats (P < 0.05). This effect was still observed 7 days after the end of training (Table 1).

**Insulin receptor.** Portions of gastrocnemius muscle were excised and snap-frozen under basal conditions and during perfusion with insulin (10 mU/ml). Muscle samples were homogenized, and Western blot analysis was used to measure insulin receptor expression and tyrosine phosphorylation. Figure 1A shows representative Western blots of insulin receptor tyrosine phosphorylation and insulin receptor protein expression under both basal and insulin-stimulated conditions. There was no difference in expression of the insulin receptor among any of the study groups. There was no significant difference in basal values for insulin receptor tyrosine phosphorylation, and training did not affect these values (Fig. 1A). Insulin increased insulin receptor tyrosine phosphorylation in all groups of rats, although to a significantly greater degree in untrained lean Zucker rats than in untrained obese Zucker rats. Seven weeks of training had no significant effect on insulin-stimulation of insulin receptor tyrosine phosphorylation in the obese Zucker rats. Therefore, the insulin-stimulated increment in tyrosine phosphorylation of the insulin receptor was significantly decreased in all groups of obese Zucker rats (Fig. 1B).

**IRS-1 and p85.** Figure 2A shows representative Western blots of IRS-1 tyrosine phosphorylation and protein expression from homogenized gastrocnemius muscle samples under both basal and insulin-stimulated conditions. There was no difference in IRS-1 expression among any of the study groups. Basal values of IRS-1 tyrosine phosphorylation were not significantly different among the groups of rats and were not affected by training (Fig. 2A). Like the insulin receptor, insulin increased IRS-1 tyrosine phosphorylation in all groups, but it did so to a significantly greater degree in untrained lean Zucker rats (Fig. 2A). Seven weeks of training had no significant effect on insulin stimulation of IRS-1 tyrosine phosphorylation in the trained obese Zucker rats. Figure 2B shows that the insulin-stimulated increment in IRS-1 tyrosine phosphorylation was significantly decreased in all groups of obese Zucker rats compared with untrained lean Zucker rats.

IRS-1 tyrosine phosphorylation in response to insulin stimulation increases IRS-1 association with p85, the regulatory subunit of PI3-kinase. To determine p85 association with IRS-1, IRS-1-immunoprecipitated proteins were resolved by using SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and immunoblotted with either an anti-phosphotyrosine antibody or anti-insulin receptor antibody as described in METHODS. Data (means ± SE) are presented as insulin-stimulated increase over basal levels. Units are arbitrary density units based on scanning and digitizing the scans. Basal values of insulin receptor tyrosine phosphorylation were not significantly different among the groups, and they averaged 0.02 ± 0.02 arbitrary units. P < 0.05 was considered statistically significant. *P < 0.05 vs. lean Zucker rats, †P < 0.01 vs. lean levels, ‡P < 0.05 vs. basal levels.
was intermediate between lean and obese untrained Zucker rats. Figure 3A (top) demonstrates that, whereas the increment of p85 association with IRS-1 24 h postexercise (lanes 5–6) was not significantly different from lean untrained Zucker rats (lane 1–2), the response was not normalized in response to training. At 96 h and 7 days after exercise training, the insulin-stimulated increment of p85 association with IRS-1 was significantly decreased compared with lean untrained controls. The expression of p85 was not significantly different among any of the study groups (Fig. 3A).

IRS-2 and p85. Although IRS-2 expression is less than IRS-1 in skeletal muscle (13), it is another substrate for the insulin receptor and may play a role in mediating insulin’s response to exercise training. We investigated IRS-2 tyrosine phosphorylation and expression in muscle samples from untrained lean and obese Zucker rats as well as muscle samples from obese Zucker rats 24 h that had undergone 7 wk of exercise training. Muscle samples (750 μg protein) were immunoprecipitated with IRS-2 antibody, and proteins were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with an anti-phosphotyrosine antibody. The blot was then stripped and reprobed with an anti-IRS-2 antibody as described in METHODS. IRS-2 protein and associated tyrosine phosphorylation were undetectable in obese Zucker rats, either untrained or trained, and could not be quantified. IRS-2 protein appeared to be more abundant in lean Zucker rats than in obese rats (data not shown). IRS-2 protein did not appear to be affected by training in obese Zucker rats.

PI3-kinase. Figure 4A shows a representative PhosphorImage of IRS-1-associated PI3-kinase activity. Insulin significantly stimulated IRS-1-associated PI3-kinase activity in lean untrained Zucker rats compared with untrained obese rats. There was no significant effect of training on IRS-1-associated PI3-kinase activity. Because PI3-kinase can associate with proteins other than IRS-1 in an insulin-responsive manner, association of PI3-kinase activity with tyrosine-phosphorylated proteins was also assayed (Table 2). The pattern was not different from that of the association of PI3-kinase activity with IRS-1, with the obese Zucker rats.
rats displaying insulin resistance and a lack of response of this measurement to training. In addition to measuring PI3-kinase activity associated with IRS-1 or other tyrosine phosphorylated protein, we also measured total PI3-kinase activity, using p110 immunoprecipitates (Fig. 5). Total PI3-kinase activity was significantly reduced in obese compared with lean Zucker rats and was not effected by training.

Akt. To determine whether signaling downstream of PI3-kinase was affected by training, we measured Akt serine phosphorylation and protein expression in the lean and obese untrained Zucker rats as well as 24 h after the last training bout in obese Zucker rats that had undergone 7 wk of exercise training. Basal values of Akt phosphorylation were not significantly different from 0 for any group. Compared with a value of 1 for lean Zucker rats analyzed on the same immunoblot, untrained obese Zucker rats had a value of 0.60 ± 0.08 (P < 0.001). Twenty-four hours after the last training bout, Akt phosphorylation was 0.69 ± 0.08 (P < 0.02 vs. lean Zucker rats). Akt phosphorylation did not differ significantly between trained and untrained obese Zucker rats. Similarly, trained (0.82 ± 0.03) and untrained (0.86 ± 0.03) obese Zucker rats had significantly decreased Akt protein expression compared with a value of 1 for lean Zucker rats (P < 0.002). The decrease in Akt phosphorylation could be accounted for by the decreased Akt protein expression, because there

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Table 2. Basal and insulin-stimulated PI3-kinase activity associated with tyrosine phosphorylated immunoprecipitates

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<th>Time Since Last Training Bout</th>
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<tr>
<td></td>
<td>Basal</td>
<td>Insulin</td>
<td>24 h</td>
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<tr>
<td>Basal</td>
<td>0.52 ± 0.11</td>
<td>2.95 ± 0.77</td>
<td>0.50 ± 0.13</td>
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<tr>
<td>Insulin</td>
<td>1.13 ± 0.24*</td>
<td>1.33 ± 0.24</td>
<td>0.53 ± 0.12</td>
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Values are means ± SE (in ng/µg protein relative to rat liver control). PI3-kinase, phosphatidylinositol 3-kinase. P < 0.05 was considered statistically significant. *P < 0.01 vs. lean Zucker rats.
were no significant differences among the groups when Akt phosphorylation was expressed relative to Akt protein.

GLUT-4 protein. To determine whether training increased GLUT-4 protein concentration, protein expression of GLUT-4 was determined by immunoblot analysis. There was no significant difference in GLUT-4 protein between untrained lean and obese Zucker rats. A significant increase in muscle GLUT-4 protein concentration was observed 24 h after the last training bout in the obese Zucker rats (Table 1). GLUT-4 protein concentration had started to decline by 96 h after training, but it was still significantly elevated above the untrained obese and lean Zucker rats. By 7 days after the last training bout, the GLUT-4 protein expression was no longer significantly elevated, but it still was 13% greater than that of the untrained obese rats.

DISCUSSION

The obese Zucker rat is a well-characterized model of insulin resistance that results from its hyperphagic behavior due to a leptin receptor mutation (30). It also has been used as a model for the response of insulin resistance to exercise training (8, 11, 20, 21). Previous studies have characterized the insulin receptor signaling system in skeletal muscle of the obese Zucker rat and found extreme insulin resistance consistent with the decrease in insulin-stimulated glucose uptake into skeletal muscle (1, 25, 33). Exercise is known to increase insulin responsiveness of skeletal muscle (11, 20). Therefore, our purpose was to determine whether the improvement in insulin-stimulated muscle glucose uptake that is associated with exercise training in the obese Zucker rat is accompanied by an improvement in insulin receptor signaling.

In agreement with previous reports, the present data confirm that insulin stimulation of the proximal steps in insulin receptor signaling events is reduced in muscle of obese Zucker rats (1, 22). Insulin stimulation of tyrosine phosphorylation of the insulin receptor β-subunit and IRS-1 as well as association of the p85 regulatory subunit of PI3-kinase with IRS-1 were all reduced. Consistent with the latter was the reduction in stimulation of association of PI3-kinase activity with IRS-1 or in anti-phosphotyrosine immunoprecipitates. Whereas the stimulation of insulin receptor tyrosine phosphorylation was significantly decreased in the obese Zucker rats, subsequent steps in the signaling cascade, IRS-1 tyrosine phosphorylation, and PI3-kinase activity were more severely affected. This suggests that there may be multiple abnormalities responsible for decreased insulin receptor signaling in this animal model of insulin resistance. For instance, an abnormality in insulin receptor autophosphorylation may be accompanied by impairment in the ability of IRS-1 to become tyrosine phosphorylated.

Seven weeks of training improved insulin-stimulated hindlimb glucose uptake to near-normal values in the obese Zucker rats, consistent with previous reports (20, 21). Despite this increase in the response of skeletal muscle to insulin, the animals were still insulin resistant on a systemic level, as indicated by their elevated basal plasma insulin concentrations. This basal hyperinsulinemia could be a reflection of insulin resistance in the liver that was not ameliorated by the exercise training. On the other hand, it is also possible that the skeletal muscle response to a submaximally stimulating insulin concentration was impaired. The maximally effective insulin concentration used in the hindlimp perfusions (10 mU/ml) did not allow determination of insulin action at lower, more physiological insulin concentrations. Regardless of the use of the supraphysiological insulin concentration, insulin receptor signaling was still significantly blunted in the obese Zucker rats after training, even though insulin-stimulated glucose uptake was increased to levels near those observed in the lean Zucker rats. Although insulin stimulation of the association of PI3-kinase with IRS-1 was not improved by training, it would still be possible for PI3-kinase activity to be increased by association with other tyrosine phosphorylated proteins. However, exercise training did not improve total (p110 immunoprecipitable) PI3-kinase activity, nor did it increase PI3-kinase activity in anti-phosphotyrosine immunoprecipitates. In addition, IRS-2 protein expression was extremely low in obese Zucker rats and was not increased by training. Therefore, it is unlikely that the increase in insulin-stimulated hindlimb glucose uptake is dependent on training-induced changes in PI3-kinase activity.

These results conflict with those from Chibalin et al. (10), who showed that 1 or 5 days of exercise training of Wistar rats increased insulin-stimulated insulin receptor and IRS-1 tyrosine phosphorylation as well as IRS-1-associated PI3-kinase activity. Insulin receptor protein expression was also increased in response to exercise in the Wistar rats in their study, whereas no change in protein expression of any proteins involved in the early steps of insulin signaling was observed in the present study. Several experimental differences, including animal model (Wistar vs. Zucker rats), training method (swimming vs. treadmill), and duration of training (1 and 5 days vs. 7 wk), could account for these discrepancies. Therefore, in the case of the obese Zucker rat, extended exercise training is not associated with improvements in insulin signaling in skeletal muscle, similar to acute exercise (5, 18, 36).

To examine more distal steps in signaling through the PI3-kinase pathway, we determined the ability of insulin to induce serine phosphorylation (Ser473) of Akt in untrained and trained Zucker rats. We found that insulin-stimulated Akt serine phosphorylation was decreased in untrained obese Zucker rats compared with lean Zucker controls and that 7 wk of exercise training did not improve Akt phosphorylation. Unlike more proximal steps in insulin signaling, the decrease in Akt phosphorylation in the obese Zucker rats could be accounted for by decreased Akt protein expression. This finding confirms earlier results (24). Exercise training also had no effect on Akt protein expression. Thus the
GLUT-4 protein expression and another involving alternate signaling pathways, may explain the increase in insulin-stimulated muscle glucose uptake in the absence of an improvement in insulin receptor signaling through the PI3-kinase pathway. With regard to GLUT-4, an increase in protein expression could have been responsible in part for the improvement in insulin-stimulated muscle glucose uptake (2, 8, 15, 17, 31). Training increases the expression of GLUT-4, and there is a direct relationship between the increase in GLUT-4 protein concentration and the increase in insulin-stimulated glucose transport (2, 8). In this regard, we observed that, after 7 wk of exercise training, the pattern of change in GLUT-4 protein expression was similar to that of insulin-stimulated glucose uptake. The mechanism by which GLUT-4 overexpression reduces muscle insulin resistance is unclear, particularly when this condition results from a defect in GLUT-4 translocation.

Using a membrane isolation-Western blotting procedure to localize GLUT-4 in muscle of obese Zucker rats, Brozinick et al. (7) found that there was an increase in the GLUT-4 associated with the plasma membrane fraction, but not with the microsomal membrane fraction, after exercise training. Muscle glucose uptake during hindlimb perfusion in the absence of insulin was unaffected by training, but in the presence of a maximally stimulating concentration of insulin there was a significant improvement. In agreement with Brozinick et al., Etgen et al. (16), using the isolated epididymal fat pad preparation, found no difference in basal glucose transport between untrained and trained obese Zucker rats. Nor was cell surface GLUT-4 protein concentration [as determined by 2-N-4-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis(D-mannose-4-yloxy)-2-propylamine binding] different. However, after insulin stimulation, glucose transport was significantly improved in trained compared with untrained muscle, and cell surface GLUT-4 protein concentration was closely associated with transport. These results suggest exercise training does not correct the defect in GLUT-4 translocation but compensates for this defect by increasing the GLUT-4 concentration at a site distal to the defect (8). The results of the present study support this hypothesis in that training did not amend the defects in the insulin signaling pathway proposed to control GLUT-4 translocation but increased GLUT-4 protein expression. It would seem then that insulin activates a minimum of two cellular processes controlling glucose transport. One would be the movement of GLUT-4 vesicles from their primary storage site to the plasma membrane. Another would be the insertion of GLUT-4 into the plasma membrane or its activation once inserted.

Seven days after the last training bout, GLUT-4 expression still was increased by 13%, although it was no longer statistically significantly greater than that in untrained animals. On the other hand, glucose uptake after 7 days of detraining was increased by over 60%. Therefore it would seem likely that the increase in GLUT-4 protein does not by itself entirely explain the increase in glucose uptake. These considerations lead to the second possibility that training-induced changes may have occurred within an alternate insulin receptor signaling pathway. One candidate pathway that has been implicated in GLUT-4 translocation in adipocytes involves proteins such as e-Cbl, CAP, and TC10 (3, 4, 9). There is no evidence to date of the importance of this pathway in skeletal muscle. Another possibility is the involvement of p38 MAP kinase. Sweeney et al. (35) recently found that blocking insulin-activation of p38 MAP kinase with SB-203580 prevented the activation of GLUT-4 in L6 muscle cells but did not prevent its translocation or its insertion into the plasma membrane. SB-203580 had no effect on insulin-activated IRS-1, PI3 kinase, or Akt isoenzymes, which would be consistent with the present findings. It was suggested that insulin causes both the translocation and activation of GLUT-4 and that two different and independent enzymatic pathways control these processes.

In summary, 7 wk of exercise training improved insulin-stimulated skeletal muscle glucose uptake in obese Zucker rats. This improvement was associated with an increase in GLUT-4 protein expression, but it was not accompanied by any changes in the response of the insulin receptor or IRS-1 tyrosine phosphorylation, the association of p85 protein and PI3-kinase activity with IRS-1 or tyrosine phosphorylated proteins, or Akt Ser473 phosphorylation. These findings gain significance in light of the importance of the PI3-kinase signaling cascade in mediating insulin’s effects on glucose uptake and metabolism. Thus the results further support the hypothesis that exercise training does not ameliorate the defect in insulin signaling through the PI3-kinase pathway in the obese Zucker rat but that it modifies the muscle in a manner that compensates for this defect. This modification may involve the increase in muscle GLUT-4 protein concentration and redistribution, and it can be speculated that insulin activation of an alternative signaling pathway might be required for this compensation to occur.

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