Enhanced muscle insulin receptor autophosphorylation with short-term aerobic exercise training

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Numerous studies have documented that regular exercise improves insulin action in humans. Evidence for the beneficial effects of a regular exercise training program comes from studies of trained athletes (27, 10), cross-sectional studies demonstrating that sedentary subjects are insulin resistant compared with physically active individuals (19, 24), and longitudinal studies documenting improved insulin action in previously sedentary subjects after an exercise training program (2, 11, 16, 18). In addition to these studies of whole body insulin action, in vivo NMR techniques in humans have directly demonstrated that, after training, insulin stimulation of glucose transport and phosphorylation in skeletal muscle is increased (29).

The improved capacity for glucose transport that occurs with training is associated with an increased muscle GLUT-4 content (16, 17). In addition, increased activation of earlier steps in the insulin-signaling pathway has been demonstrated (21). Cellular insulin action is mediated through the insulin receptor (IR). Binding of insulin to the α-subunits of this tetrameric protein results in a conformational change of the receptor that initiates the autophosphorylation of multiple tyrosine residues on the intracellular β-subunits (8, 15). This autophosphorylation is a requisite step in activating the protein tyrosine kinase activity of the β-subunits (35). IR kinase activity results in tyrosine phosphorylation of docking proteins such as the IR substrate (IRS) family of proteins. Subsequent binding of the IRS proteins to the p85 regulatory subunit of phosphatidylinositol (PI) 3-kinase leads to activation of this enzyme, a required step in the insulin-stimulated increase in glucose transport and glycogen synthesis (33, 34). Although insulin stimulation of skeletal muscle IR autophosphorylation is downregulated in certain insulin-resistant states (23, 25), it is not known whether interventions known to improve insulin action are associated with increased IR function.

Exercise training can affect skeletal muscle insulin sensitivity directly as well as through peripheral adaptations. Studies employing one-legged exercise training have demonstrated improved insulin-stimulated glucose uptake in trained, but not untrained, contralateral muscles (10). In addition to the direct effect of physical activity on muscle, insulin-stimulated glucose disposal in humans is also directly correlated with cardiovascular fitness levels (19) and inversely associated with degree of obesity (3). Because both of these latter variables can be modified by a long-term exercise training program, it can be difficult to discern the effects of increased activity alone on improvements in insulin action.

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The 7-day exercise training model has been shown to produce significant improvements in whole body insulin sensitivity in young and old subjects (7) and to enhance insulin-stimulated glucose disposal in previously sedentary young men (21). This short-term training protocol is an important tool for understanding the direct effects of physical activity on muscle, because it has no effect on either body weight or body composition and produces no improvement in maximal oxygen uptake (6, 7, 28, 32). This training protocol does produce adaptations in skeletal muscle, increasing GLUT-4 content (20) and insulin stimulation of PI 3-kinase activity (21). The present study was conducted to determine whether 7 days of exercise training would result in an increase in the capacity of muscle IR to autophosphorylate in response to insulin in a population of previously sedentary individuals.

METHODS

Materials. Microtiter plates (Maxisorb Immunoplates) were from Nunc (Copenhagen, Denmark). BSA was from Intergen (Purchase, NY). Crystalline porcine insulin was obtained from Eli Lilly (Indianapolis, IN). Biotin-conjugated anti-phosphotyrosine antibody was from UBI (Lake Placid, NY). Horseradish peroxidase (HRP)-conjugated streptavidin was from Pierce (Rockford, IL). ELAST Amplifikation kit was from Du Pont-NEN (Boston, MA). TMB (3,3',5,5'-tetrathymethylbenzidine) reagent kit was from Kirkegaard and Perry Laboratories (Gaithersburg, MD). All other reagents were from Sigma (St. Louis, MO).

Study design. Twenty subjects representing a wide range of adiposity were selected for the study. Subjects were initially screened for body composition and cardiovascular fitness level. A pretraining insulin sensitivity index (SI) was determined with an intravenous glucose tolerance test (IVGTT). Homeostasis model assessment (HOMA) measures of insulin action were calculated from fasting insulin and glucose values. Subjects then underwent seven consecutive days of supervised exercise training. A posttraining IVGTT was performed 15–17 h after the final training bout. Muscle samples (~50 mg) from the vastus lateralis muscle were obtained with the percutaneous needle biopsy technique in the fasted state before the IVGTT and before and after the exercise training for determination of IR autophosphorylation.

Subjects. Subjects were volunteers who had not been active in an exercise program for at least the previous 2 yr. Subjects were also questioned concerning normal daily activities, and only those with relatively sedentary lifestyles were included. Other inclusion criteria were no medications that would alter insulin action or evidence of coronary artery disease, hypertension, or orthopedic injuries that would inhibit exercise training. Women were tested in the follicular phase of the menstrual cycle, based upon a recall of their previous menses.

Insulin action. An SI was determined with an IVGTT as described by Finegood et al. (12). This procedure was employed to determine the effect on the blood glucose profile of the combined endogenous secretion and exogenous insulin. Subjects were instructed to record and consume the same foods for 24 h before each IVGTT and had consumed ≥250 g carbohydrate/day for the previous 3 days. Subjects then reported to the laboratory in the morning after a 12-h fast. The pretraining IVGTT was performed in the sedentary condition; the posttraining test was performed 15–17 h after the final exercise bout. Two or three hours after the final exercise bout, subjects consumed a meal and then fasted overnight (12 h). Briefly, the IVGTT procedure consisted of obtaining four baseline samples before the intravenous injection of glucose (1.7 mmol/kg) at time 0 and insulin (150 pmol/kg) 20 min later. Twenty-five samples were obtained between 0 and 180 min and were subsequently analyzed by spectrophotometry for glucose (procedure HK 16-UV, Sigma) and by microparticle enzyme immunoassay for insulin (IMx, Abbott, Abbott Park, IL). SI was calculated with the minimal model of insulin action (MINMOD, version 3.0; R. N. Bergman, USC, Los Angeles, CA). HOMA values were calculated from glucose and insulin values from baseline plasma samples of the IVGTT. HOMA = fasting plasma insulin (μU/ml) × fasting plasma glucose (mmol/l)/22.5 (26). The HOMA values provide a determination of the effectiveness of steady-state fasting insulin levels to regulate blood glucose (26).

Cardiovascular fitness and body composition. Peak oxygen consumption (VO2 peak) was measured during incremental exercise on an electrically braked cycle ergometer (Lode, Dieren, NL) with a 5W workload. The initial workload was 5 W with an increase of 25 W every 2 min until voluntary exhaustion was achieved. For a valid test, two of the three after-criteria had to be met: 1) a respiratory exchange ratio greater than 1.0; 2) a heart rate not less than 15 beats/min below age-predicted maximal heart rate; or 3) the maintenance of oxygen consumption within 2 ml·kg-1·min-1 despite an increase in workload. Oxygen consumption was measured with open-circuit spirometry by use of a metabolic cart (model 2900, Sensor Medics, Anaheim, CA). A 12-lead electrocardiogram (EKG) recorded heart rate and EKG tracings. EKGs were monitored continuously throughout the test to look for abnormalities, and a tracing was printed during the last 15 s of each stage to calculate heart rate. The maximal exercise test was used to 1) screen for potential heart disease and 2) determine the heart rate and oxygen consumption needed to elicit the desired intensity (70–75% VO2 peak) during the 7 days of training. Body fat percentage, fat mass, and fat free mass were determined with the 7-site skinfold method (22).

Exercise training. Subjects exercised 1 h/day for 7 consecutive days on a cycle ergometer. Exercise intensity was adjusted to achieve 70–75% of VO2 peak as determined from expired air collected in Douglas bags at minute 5 and every subsequent 15 min of exercise. All subjects exercised continuously for the 1 h during the 7 days of training. To ensure weight maintenance, subjects were instructed to consume their normal diet during the exercise training period with the addition of ~450 kcal, which represented the energy expended daily upon exercise.

Preparation of muscle extracts. Soluble extracts were prepared from frozen muscle tissue to measure tissue content of IR, cytochrome c oxidase (COX), GLUT-4, membrane glyco-protein PC-1, and IR autophosphorylation capacity. Approximately 50 mg of frozen tissue were pulverized under liquid nitrogen. The resultant powder was homogenized in 0.5 ml buffer (50 mM HEPES HCl, 150 mM NaCl, 1 mM phenyl-methylsulfonyl fluoride, 2 μM leupeptin, and 2 μM pepstatin, pH 8.7) at 4°C with the use of a polytron homogenizer (Kinematica, Lucerne, Switzerland) for 10 s at a setting of 9. Triton X-100 was added to a final concentration of 1%, and the homogenates were solubilized for 60 min at 4°C. The solubilized homogenate was then centrifuged at 100,000 g for 60 min at 4°C. The supernatants were collected and stored at −70°C. Protein content of muscle extracts was determined by the Bradford method (4).

IR ELISA. IR content of muscle extracts was determined by specific ELISA as described previously (13). Briefly, mi-
crotter 96-well plates were coated with 2 μg/ml of a monoclonal antibody to the IR α-subunit (MA-20) for 1 h at 4°C. After the plate was washed and blocked, solubilized cellular extract containing 10 μg protein of each sample was added to each well for triplicate determination and allowed to bind overnight at 4°C. Readout of bound IR was accomplished with the sequential addition of biotinylated monoclonal anti-IR antibody CT-1, HRP peroxidase-conjugated streptavidin (Pierce), the ELAST ELISA Amplification System (NEN Research Products) for signal enhancement, and the TMB peroxidase substrate system (Kirkegaard and Perry) for color development. The absorption at 450 nm of each well was measured in a microtiter plate reader (Du Pont-NEN). IR content for each sample was calculated as an average of triplicate values.

**IR autophosphorylation ELISA.** The autophosphorylation capacity of muscle IR was determined in soluble extracts by use of an ELISA specific for IR tyrosine phosphorylation as described previously (36). In this assay, solubilized cellular extract containing 10 pg of IR was added to each well of a 96-well microtiter plate coated with 2 μg/ml MA-20 and allowed to bind overnight. Immuno captured IR was then incubated in 50 mM HEPES, 150 mM NaCl, 10 mM MgCl2, 2 mM MnCl2, 0.1% Triton X-100, 0.05% BSA, 10 μM ATP, pH 7.6, ±0.1–100 nM insulin for 1 h at 22°C. The tyrosine phosphorylation state was then determined by incubation with a biotinylated anti-phosphotyrosine antibody (UBI, Lake Placid, NY), followed by an identical procedure for color development, as employed in the IR content ELISA.

**PC-1 content ELISA.** Tissue content of membrane glycoprotein PC-1 was determined by specific ELISA as described previously (13). The protocol was similar to that described above for the IR ELISA with the capture antibody to PC-1 provided by Dr. I. Yamashina (Kyoto University, Kyoto, Japan). In addition, 2 μg of muscle extract protein were added to each well, and the standard curve was constructed by multiple dilutions of 0–1.5 ng of purified PC-1. The secondary antibody was a biotinylated anti-PC-1 monoclonal antibody (13).

**Determination of COX content.** Muscle COX content was determined by SDS-PAGE, with 30 μg of soluble muscle protein loaded onto an 8–16% polyacrylamide minigel. The proteins were electrophoretically transferred to a nitrocellulose membrane at 30 V for 1.6 h. After transfer, the membranes were incubated for 30 min in SuperBlock blocking buffer (Pierce). The membranes were then incubated at 4°C with a monoclonal antibody raised against subunit 1 of human COX (Molecular Probes, Eugene, OR), 0.5 μg/ml in PBS with 0.05% Tween-20 (PBST) containing 5% powdered milk. After an overnight incubation, the membranes were washed with PBST. Next, membranes were incubated with HRP-conjugated sheep anti-mouse IgG (Amersham), diluted 1:3,000 in PBST with 5% milk for 1.5 h at 22°C. Membranes were washed again with PBST. COX protein was visualized by enhanced chemiluminescence (Pierce).

**Statistics.** Preliminary data analysis was performed to confirm that there was no effect of body composition on the training response. Analysis comparing individuals with body mass index (BMI) values >28 with those <28 was accomplished with a 2 (group)-by-2 (treatment, before and after exercise training) repeated-measures ANOVA. There was no influence of body composition on insulin action or any other variables; data from all subjects were then pooled into one group and compared before and after training with repeated-measures ANOVA (treatment, before vs. after exercise training). The effect of training on IR autophosphorylation was also compared before and after training (treatment) at the various insulin concentrations (0, 0.1, 1, 10, 100 nM) with a 2 (treatment)-by-5 (insulin concentration) repeated-measures ANOVA. Contrast comparisons were used to determine the specific difference when a significant interaction was obtained. All analyses were performed with significance at the P < 0.05 level.

**RESULTS**

Subject characteristics. Clinical and anthropometric data on the subjects before exercise training are shown in Table 1. The subjects represented a wide range of body composition. Peak aerobic capacity (VO2peak) also varied widely among individuals, although all were within accepted levels for sedentary individuals (17.0–35.5 ml·min⁻¹·kg⁻¹). In addition to indicating that the subjects were sedentary individuals, VO2peak values provided heart rate and oxygen consumption data for the training protocol. Exercise training intensity was set at 74.2 ± 0.6%. VO2peak. There was no significant effect of 7 days of exercise training on body mass or any measure of adiposity (data not shown).

Insulin action. Insulin action in the subjects was determined via several measurements. The IVGTT minimal model was employed to determine the effect of combined exogenous and endogenous insulin on the blood glucose profile of the subjects. The calculated SI values demonstrated a wide range in the subject population before training (0.7–13.0 min·μU⁻¹·ml⁻¹). Complete SI data (pre- and posttraining) were obtained from 16 subjects. In these subjects, 7 days of exercise training produced a significant increase in SI (6.2 ± 0.9 vs. 8.2 ± 1.1 min·μU⁻¹·ml⁻¹, pre- vs. posttraining, respectively, P < 0.05). HOMA values varied widely among these 16 individuals, exhibiting a greater than sixfold range (0.47–3.17). Pretraining HOMA values were significantly correlated with SI values (r = -0.71, P < 0.005). In addition, HOMA values were significantly correlated with both BMI and percent body fat (r = 0.44 and 0.48, respectively, P < 0.05). Unlike the increase in SI in response to training, the decline in HOMA values (indicating an improvement in insulin action) after 7 days of exercise training did not reach statistical significance (1.83 ± 0.18 vs. 1.58 ± 0.15, pre- vs. posttraining, respectively, P = 0.20). However, training-induced improvements in the two measures of insulin action were

<table>
<thead>
<tr>
<th>Variable</th>
<th>Range</th>
<th>Mean ± SE</th>
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<tbody>
<tr>
<td>Age, yr</td>
<td>20–37</td>
<td>29 ± 1</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>19.8–38.1</td>
<td>28.0 ± 1.1</td>
</tr>
<tr>
<td>% Body fat</td>
<td>13.7–33.6</td>
<td>24.4 ± 1.4</td>
</tr>
<tr>
<td>Fasting insulin, μU/ml</td>
<td>2.6–13.8</td>
<td>8.1 ± 0.7</td>
</tr>
<tr>
<td>Fasting glucose, mg/dl</td>
<td>72.3–118.5</td>
<td>88.0 ± 2.4</td>
</tr>
<tr>
<td>HOMA</td>
<td>0.47–3.17</td>
<td>1.83 ± 0.18</td>
</tr>
<tr>
<td>SI, min·μU⁻¹·ml⁻¹</td>
<td>0.7–13.0</td>
<td>6.2 ± 0.9</td>
</tr>
<tr>
<td>VO2peak, ml·min⁻¹·kg⁻¹</td>
<td>17.0–35.5</td>
<td>28.3 ± 1.1</td>
</tr>
</tbody>
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BMI, body mass index; HOMA, homeostasis model assessment; SI, insulin sensitivity index; VO2peak, peak O2 uptake.
significantly correlated with each other across all subjects ($r = -0.62$, $P < 0.01$).

**COX.** In muscle biopsies obtained before the training program, we observed a significant correlation between the content of the mitochondrial marker enzyme COX and subject $V_{O2}$ peak ($r = 0.65$, $P < 0.05$). The COX content of biopsies after training was 21% higher than pretraining values, ($P < 0.05$) (Fig. 1).

**IR content.** There was no significant effect of the training program on muscle IR content as determined by ELISA (7.4 ± 0.8 vs. 7.0 ± 0.8 ng IR/mg protein, pre- vs. posttraining, respectively, $n = 14$). IR content values were then employed to calculate the volume of lysate required to add 10 pg of IR into the autophosphorylation ELISA.

**IR autophosphorylation.** IR autophosphorylation was determined across a range of insulin concentrations (0–100 nM). IRs displayed half-maximal activation at an insulin concentration of ~5 nM (Fig. 2). Exercise training resulted in a significant increase in IR autophosphorylation at 1, 10, and 100 nM insulin (Fig. 2). The increase in IR maximal autophosphorylation was significantly correlated with the increase in muscle oxidative capacity, as determined by biopsy content of COX protein ($r = 0.543$, $P = 0.05$). After training, maximal IR autophosphorylation was significantly inversely correlated with subject BMI ($r = -0.61$, $P < 0.05$), a relationship which was not observed in pretraining samples.

**PC-1 content.** Seven days of exercise training resulted in a slight increase in muscle content of PC-1 (14.5 ± 1.0 vs. 17.0 ± 1.1 ng/mg, pre- vs. posttraining, respectively, $P < 0.05$, $n = 14$).

**DISCUSSION**

In the present study we observed significant increases in insulin sensitivity in a mixed population after 7 days of exercise training. $S_I$ values increased by ~33% with no change in body mass or body composition. Furthermore, improvements in $S_I$ values were not related to any measure of body weight or composition. This finding is in agreement with previous reports of 7-day training employing an identical protocol in an aged population (7) and is consistent with 7-day training studies in which insulin action was quantified by hyperinsulinemic glucose clamp (21) or estimated via oral glucose tolerance test in elderly (6) and diabetic subjects (32).

Although improvements in insulin action have been demonstrated in training studies, the underlying mechanisms have not been elucidated. In the present study we demonstrated that 7 days of exercise training significantly improved skeletal muscle IR autophosphorylation capacity. These findings are consistent with previous reports of improved insulin stimulation of muscle PI 3-kinase activity after an identical training protocol, although the contribution of the upstream insulin signaling pathway was not examined in that study. Similar results were also obtained in a recent study of swim-trained rats (5). In that exercise model, five consecutive days of swim-training resulted in a significant increase in insulin stimulation of muscle IR autophosphorylation. Insulin stimulation of the activity of downstream, second messenger enzymes PI 3-kinase and Akt was similarly increased with training (5).

Although a relationship between impaired muscle IR tyrosine kinase activity and insulin resistance has been well documented in humans (23, 36), only two...
previous studies have examined the role of the IR in exercise training of humans. Neither Dela et al. (9) nor Bak et al. (1) documented a significant effect of exercise on IR tyrosine kinase activity. Several key methodological differences between these studies and ours could explain the discrepant results. The study by Dela et al. involved measuring IR function in trained vs. untrained muscle after one-legged ergometer training, rather than repeating biopsies before and after training. Both studies employed partially purified receptors in a wheat germ preparation in 32P substrate labeling assays. In contrast, the present study employed immunopurified IR in an ELISA technique that measures IR autophosphorylation with increased sensitivity and precision. In addition, in our study, IR autophosphorylation was examined across a full range of insulin concentrations rather than at one concentration, increasing the capacity to determine changes in the insulin response of IR.

Moreover, the relationship between improvements in autophosphorylation capacity and increased content of COX protein suggests that the improved IR function after exercise training might be related to the increased oxidative capacity of muscle. Other genes involved in carbohydrate metabolism (hexokinase, GLUT-4) appear to be jointly upregulated with mitochondrial enzymes in response to increased contractile activity (31). Thus it is possible that some gene(s) involved in regulating IR function are under similar coordinate regulation. The two previous studies that did not find an effect of exercise training on IR function employed subjects with significantly higher aerobic capacities than in the present study. Thus it is possible that improvements in IR function with training might result from low muscle oxidative capacity secondary to physical inactivity and may not develop as readily in previously physically active subjects.

In a previous study of a cross section of Pima Indians (36), we reported a significant inverse correlation between obesity and IR autophosphorylation capacity. Although a similar relationship was not observed in biopsies from untrained individuals in the present study, we observed a significant inverse correlation after exercise training between BMI and IR autophosphorylation capacity. Thus it is possible that the adverse effect of increasing obesity on IR function can be obscured by an additional negative impact of physical inactivity. By increasing and normalizing the physical activity of all subjects, we were able to observe again a direct relationship between increasing adiposity and impaired IR function.

The mechanisms of improved IR autophosphorylation capacity with training are unknown. Several factors have been postulated to downregulate IR function in cases of insulin resistance. Muscle content of the IR inhibitor, membrane glycoprotein PC-1, is inversely associated with whole body insulin action, muscle glucose transport, and IR tyrosine kinase activity in several models of human insulin resistance (14, 37). In the present study, however, short-term exercise training resulted in a slight increase in muscle PC-1 content concurrent with improved IR autophosphorylation. Thus it is unlikely that PC-1 downregulation plays a significant role in the beneficial effects of exercise on insulin action. Whether muscle IR function is improved with training secondarily to some other effector molecule(s) (such as protein tyrosine phosphatase-1B or protein kinase C) is unknown.

The recent study on swim-trained rats (5) demonstrated increased insulin-stimulated IR autophosphorylation after 5 days, but not after 1 day, of exercise. Thus, although the question of the time course of increased IR signaling capacity in human muscle in response to exercise training (and the inevitable decay of the training effect) remains to be determined, we believe that, in studying muscle biopsies obtained 17 h after the last of seven consecutive exercise sessions, we are observing a cumulative response to the program and not solely the effects of the final session.

In conclusion, the results of the present study suggest that improved IR autophosphorylation capacity is an early adaptation to exercise training in humans and may play a role in the enhanced insulin sensitivity resulting from training. Furthermore, the data suggest that improvements in IR function with exercise are related to the enhanced mitochondrial capacity. The exact mechanisms whereby IR signaling capacity are improved with exercise remain unknown.

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