Interactions of exercise training and α-lipoic acid on insulin signaling in skeletal muscle of obese Zucker rats

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The obese Zucker (fa/fa) rat is a well-defined animal model of severe skeletal muscle insulin resistance. The defective insulin-stimulated glucose transport activity in isolated skeletal muscle from these obese animals (9, 14) can be attributed to specific defects in the insulin-signaling pathway (1) leading to impaired insulin-stimulated GLUT4 protein translocation (9, 21). For example, the insulin receptor substrate (IRS)-1 protein level and insulin-stimulated IRS-1 tyrosine phosphorylation in hind-limb muscle from obese Zucker rats is only 60 and 72%, respectively, of the level measured in muscle fromagematched lean Zucker rats (1). In addition, the amount of the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI 3-kinase) associated with the tyrosine-phosphorylated IRS-1 under insulin-stimulated condition is 29% of control, and IRS-1-associated PI 3-kinase activity in muscle immunoprecipitated from the obese animals is only 54% of the level observed in lean animals (1). Therefore, specific defects in the insulin-signaling cascade contribute to the insulin resistance of skeletal muscle glucose transport in the obese Zucker rat.

Interventions that improve insulin resistance of skeletal muscle glucose transport include diet, loss of fat mass, and pharmaceutical and nutriceutical compounds. The R-(-)-enantiomer of α-lipoic acid (R-ALA) is a nutriceutical compound that also displays antioxidant properties (23). R-ALA can modulate glucose metabolism in both insulin-sensitive (7, 10, 15) and insulin-resistant (19, 20, 29) muscle tissues. Furthermore, we have demonstrated that chronic in vivo treatment with R-ALA elicits improvements in whole body glucose tolerance and insulin sensitivity, as well as insulin action, on skeletal muscle glucose transport in insulin-resistant obese Zucker rats (25). Although the precise cellular mechanisms responsible for the stimulatory effect of R-ALA on insulin action have not been determined, we have shown that increased insulin action after R-ALA treatment in obese Zucker rats is associated with a reduction of oxidative stress (25).

Another effective intervention that improves insulin action in insulin-resistant states is exercise training (see recent review in Ref. 12). Moderate- to high-intensity exercise training increases glucose tolerance and glucose disposal in the insulin-resistant obese Zucker rat, primarily due to adaptations in the glucose transport activity (6, 17, 28) and translocation of GLUT4 protein (4, 8). We have recently demonstrated favorable metabolic adaptations in skeletal muscle of the insulin-resistant obese Zucker rat in response to treadmill training (25), including significant enhancements of insulin-stimulated glucose transport, GLUT4 protein expression, and activities of enzymes involved in glucose metabolism (25). Most importantly, we have recently shown that the combination of treatment with the antioxidant R-ALA and endurance exercise training is associated with greater improvements in insulin action on skeletal muscle glucose transport than either intervention individually (25). However, the potential cellular mechanisms, including the involvement of elements of the insulin-signaling pathway, that underlie the beneficial adaptations associated with R-ALA treatment and exercise training leading to enhanced insulin action on the insulin-resistant skeletal muscle were not elucidated in that study. Therefore, the purpose of the present study was to determine the effects of...
the R-ALA treatment and exercise training, individually and in combination, on glucose transport activity and protein expression and functionality of select insulin-signaling factors in skeletal muscle of the obese Zucker rat.

METHODS

Animals and treatments. Female obese Zucker (fat/−) and lean Zucker (Fat+) rats (Harlan, Indianapolis, IN) were received at 5–6 wk of age. The animals were housed in a temperature-controlled room (20–22 °C) at the Central Animal Facility of the University of Arizona. A reversed 12:12-h light-dark cycle (lights on 1900–0700) was maintained so that training occurred during the dark cycle, when they are most active. Animals had free access to water and chow (Teklad, Madison, WI). All procedures were approved by the University of Arizona Animal Use and Care Committee.

The obese rats were randomly assigned to one of the following groups: a sedentary, vehicle-treated control group, an R-ALA-treated group, an exercise-trained group, or a combined R-ALA-treated and exercise-trained group. Animals in the obese R-ALA-treated groups received 30 mg/kg body wt of the purified R(-)-enantiomer of α-lipoic acid (Viatris, Frankfurt, Germany), dissolved in 100 mM Tris buffer (pH 7.4), by intraperitoneal injection every evening for 15 days, and were given daily treatments with R-ALA throughout the study. The contralateral soleus was removed, trimmed of fat and connective tissue, and quickly frozen in liquid nitrogen. Muscles were used to assess the protein expression and functionality of insulin-signaling factors in a tissue with normal insulin sensitivity.

Peak aerobic capacity. Peak aerobic capacity (VO2 peak) was assessed in each obese animal during a treadmill test by the method of Bedford et al. (3). No exercise was performed on the day before the VO2 peak tests. However, R-ALA was given to the obese R-ALA-treated group and the obese, combined exercise and R-ALA group on this day. Animals ran on a motorized treadmill in an air-tight Plexiglas chamber. Grade and speed of the treadmill were increased every 3 min from 0.05 vs. the R-ALA-treated group.

After this initial incubation period, the muscles were rinsed for 10 min at 37°C in 3 ml of oxygenated KHB containing 40 mM mannitol, 0.1% BSA, and insulin, if previously present. Thereafter, the muscles were transferred to 2 ml of KHB, containing 1 mM 2-deoxy-[1,2-3H]glucose (2-DOG, 300 μCi/mmol; Sigma Chemical), 39 mM [U-13C]mannitol (0.8 μCi/mmol; ICN Radiochemicals, Irvine, CA), 0.1% BSA, and insulin, if previously present. At the end of this final 20-min incubation period at 37°C, the muscles were removed, trimmed of excess fat and connective tissue, quickly frozen, weighed, and dissolved in 0.5 ml of 0.5 N NaOH. After the muscles were completely solubilized, 5 ml of scintillation cocktail were added, and the specific intracellular accumulation of 2-DOG was determined as described previously (13, 14).

Insulin-signaling factors. The contralateral soleus was removed, trimmed of fat and connective tissue, and quickly frozen in liquid nitrogen. Muscles were cut on dry ice, and pieces of the muscle were homogenized in ice-cold lysis buffer [50 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 2 mM EDTA, 10 mM NaF, 20 mM sodium pyrophosphate, 20 mM β-glycerophosphate, 1% Triton X-100, 2 mM Na3VO4, 10 μg/ml aprotinin and leupeptin, and 2 mM PMSF]. After a 20-min incubation on ice, the homogenates were centrifuged at 13,000 g for 20 min at 4°C. A portion of the lysates was analyzed for total protein concentration by use of the bicinchoninic acid (BCA) method (Sigma Chemical); the remainder was used for Western blot analysis of insulin-signaling protein expression. After denaturing by boiling with SDS-PAGE sample buffer, samples were separated on 7.5 or 12% polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA) and blotted electrophoretically onto nitrocellulose paper. Blots were incubated with commercially available antibodies against insulin receptor β-subunit (IR β), COOH-terminal IRS-1, the p85 regulatory subunit of PI 3-kinase (Upstate Biotechnology, Lake Placid, NY), and Akt1/2 (Cell Signaling Technology, Beverly, MA). For evaluation of insulin receptor tyrosine phosphorylation and Akt serine phosphorylation in soleus muscle incubated in the absence or presence of insulin, blots were incubated with antibodies against Tyr1146 on the insulin receptor and against Ser473 on Akt (Cell Signaling). For assessment of IRS-1 tyrosine phosphorylation and tyrosine-phosphorylated IRS-1 associ-

Table 1. Effects of exercise training and R-ALA treatment on body weight, VO2 peak, and maximum run time to fatigue in obese Zucker rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Initial Body Wt, g</th>
<th>Final Body Wt, g</th>
<th>Change in Body Wt/d, g/day</th>
<th>VO2 peak, ml/kg • min⁻¹</th>
<th>Maximum Run Time, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sedentary</td>
<td>172±8</td>
<td>265±6</td>
<td>6.2±0.3</td>
<td>47.2±1.1</td>
<td>10.2±0.2</td>
</tr>
<tr>
<td>R-ALA treated</td>
<td>175±13</td>
<td>266±12</td>
<td>6.0±0.2</td>
<td>49.3±1.6</td>
<td>10.1±0.2</td>
</tr>
<tr>
<td>Exercise trained</td>
<td>165±8</td>
<td>240±13</td>
<td>5.0±0.4†</td>
<td>59.4±1.2†</td>
<td>13.9±0.1†</td>
</tr>
<tr>
<td>Combined</td>
<td>161±5</td>
<td>247±5</td>
<td>5.7±0.3</td>
<td>61.2±1.0†</td>
<td>14.2±0.3†</td>
</tr>
</tbody>
</table>

Values are means ± SE for 7–8 animals/group. R-ALA, R(-)-α-lipoic acid; VO2 peak, peak aerobic capacity. *P < 0.05 vs. sedentary control group; †P < 0.05 vs. the R-ALA-treated group.
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ANOVA with a post hoc Fisher’s protected least significant difference test (StatView version 5.0, SAS Institute, Cary, NC). In some cases, values from sedentary lean Zucker rats were used as a reference control. A level of $P < 0.05$ was set for statistical significance.

**RESULTS**

**Body weights and $\dot{V}O_2_{peak}$**. The obese exercise-trained and combined-treatment groups had slightly lower (7–10%) final body weights than either the obese sedentary or the obese R-ALA-treated group (Table 1). The obese exercise-trained group displayed the lowest ($P < 0.05$) average rate of body weight gain over the experimental period compared with the obese sedentary control and the obese R-ALA-treated groups. Animals in both obese exercise-trained and obese combined-treatment groups had significantly higher peak aerobic capacities compared with the obese sedentary control group (26 and 29%, respectively) or the obese R-ALA-treated group (20 and 24%, respectively; Table 1). Moreover, exercise training alone or in combination with R-ALA treatment resulted in significantly longer maximum run times compared with those of the obese sedentary control group (36 and 39%, respectively) or the obese R-ALA-treated group (38 and 41%, respectively; Table 1). It is clear that this 2-wk exercise training regimen (8) elicits similar adaptive responses in aerobic capacity to those observed after 6 wk or more of endurance training by the obese Zucker rat (6, 25, 28, 30).

**Muscle glucose transport**. Rates of basal 2-DG uptake in both epitrochlearis and soleus muscles were not different among the obese experimental groups. In the epitrochlearis...
individual treatments with R-ALA or exercise training resulted in significant enhancement of the increase in 2-DG uptake due to insulin relative to the increases seen in the sedentary control group (40 and 41%). The greatest improvement of this parameter in the epitrochlearis was observed in the combined-intervention group (78%). In the soleus, insulin-mediated 2-DG uptake was significantly increased by R-ALA treatment (45%) and exercise training alone (67%), and to the greatest degree in the group that received R-ALA treatment and underwent exercise training in combination (124%).

GLUT4 protein and enzyme responses. The GLUT4 protein level (Fig. 2) and the activities of total hexokinase (Fig. 3) and citrate synthase (Fig. 4) enzymes were assessed in the epitrochlearis, soleus, plantaris, and myocardium. There were no increases in these parameters in any of these muscle types after R-ALA treatment. Exercise training alone or in combination with R-ALA treatment, however, caused significant increases in GLUT4 protein level in the epitrochlearis (32 and 28%, respectively), soleus (27 and 28%, respectively), and plantaris (23 and 25%, respectively).

Exercise training alone and in combination with R-ALA treatment resulted in significant enhancements of total hexokinase activity in the epitrochlearis (38 and 42%, respectively), soleus (150 and 150%), and plantaris (102 and 118%). Citrate synthase activity was increased by exercise training alone and by combination treatment in the epitrochlearis (45 and 38%), soleus (19 and 35%), and plantaris (44 and 51%). This variable was significantly enhanced (20%) in the myocardium of obese animals that received R-ALA treatment and underwent exercise training in combination.

Intramuscular triglycerides. Triglyceride accumulation in the soleus of the obese Zucker rat was three- to fourfold higher ($P < 0.05$) than in the same tissue from insulin-sensitive lean Zucker rats (Fig. 5). Compared with the obese sedentary control group, R-ALA treatment and exercise training individually in obese animals caused significant reductions (44 and 37%, respectively) in soleus triglyceride levels. However, the combination of R-ALA treatment and exercise training did not induce a further decrease in lipid content in this muscle.

Insulin-signaling factors. Protein expression of IRβ, IRS-1, p85 regulatory subunit of PI 3-kinase (p85), and Akt1/2 after the interventions was determined in soleus muscle (Fig. 6). These parameters were expressed relative to the level obtained from soleus muscle of the age-matched insulin-sensitive lean Zucker rat. No significant differences in protein expression of IRβ, p85, and Akt1/2 were observed among experimental groups. However, the protein expression of IRS-1 in soleus muscle from the sedentary obese Zucker rat was 44% less than in the insulin-sensitive soleus muscle of lean Zucker rats. Compared with the obese sedentary group, R-ALA treatment alone or exercise training alone caused significant enhancements (30 and 31%, respectively) in IRS-1 protein expression. An additional increase in IRS-1 protein expression was observed in the soleus muscle from obese animals that received R-ALA treatment and underwent exercise training in combination (55% vs. sedentary and 19% vs. R-ALA alone or exercise trained alone; all $P < 0.05$).
Because the enhanced level of IRS-1 protein expression may play a role in the improvement of insulin action after interventions, linear regression analysis of the association between levels of IRS-1 protein expression and insulin-mediated 2-DG uptake in the soleus muscle was completed. The correlation between these two parameters was highly significant \((r = 0.597, P < 0.05)\).

The ability of insulin to affect the phosphorylation state and interactions of select insulin-signaling factors was further assessed in the incubated soleus muscle. The insulin-mediated increase in Tyr\(^{1146}\) phosphorylation of the insulin receptor was not significantly enhanced by the interventions, although there was a trend \((P = 0.073)\) for greater IR tyrosine phosphorylation in the obese exercise-trained group compared with the obese sedentary group (Fig. 7). Insulin-stimulated IRS-1 tyrosine phosphorylation in the obese soleus was only 55\% of that in the lean soleus; however, when expressed relative to equal amounts of IRS-1 protein, there was no difference among obese groups for insulin-mediated IRS-1 tyrosine phosphorylation (data not shown). The insulin-mediated increase in IRS-1 associated with p85 in soleus of the obese sedentary group was only 17\% of that measured in soleus of lean Zucker rats, and this parameter in the obese groups was significantly increased by R-ALA (317\%); both \(P < 0.05\); Fig. 8). Moreover, the greatest enhancement of insulin-mediated IRS-1-associated p85 was seen in soleus of obese animals that received the combination of interventions (435\%, \(P < 0.05\)). Whereas insulin-mediated Akt Ser\(^{473}\) phosphorylation in soleus of obese sedentary animals was only 65\% of the lean control value, the R-ALA and exercise training interventions were ineffective in enhancing insulin action on this parameter (Fig. 9).

**DISCUSSION**

We have previously demonstrated that 6 wk of endurance exercise training and simultaneous treatment with the metabolic antioxidant R-ALA result in an additive interaction in enhancing maximal insulin-mediated glucose transport activity in insulin-resistant skeletal muscle of the obese Zucker rat (25). In the present investigation, we have shown that this interactive effect between exercise training and R-ALA treatment on insulin-mediated glucose transport activity can be demonstrated after just 2 wk of treatment. Moreover, we have made the novel observation that this additive interaction between exercise training and R-ALA treatment on glucose transport activity was associated with specific alterations in the insulin-signaling pathway in insulin-resistant skeletal muscle. For example, compared with the increases seen in the individual treatment groups, the greatest enhancement in IRS-1 protein phosphorylation in the obese soleus was only 55\% of that in the lean soleus; however, when expressed relative to equal amounts of IRS-1 protein, there was no difference among obese groups for insulin-mediated IRS-1 tyrosine phosphorylation (data not shown). The insulin-mediated increase in IRS-1 associated with p85 in soleus of the obese sedentary group was only 17\% of that measured in soleus of lean Zucker rats, and this parameter in the obese groups was significantly increased by R-ALA (317\%); both \(P < 0.05\); Fig. 8). Moreover, the greatest enhancement of insulin-mediated IRS-1-associated p85 was seen in soleus of obese animals that received the combination of interventions (435\%, \(P < 0.05\)). Whereas insulin-mediated Akt Ser\(^{473}\) phosphorylation in soleus of obese sedentary animals was only 65\% of the lean control value, the R-ALA and exercise training interventions were ineffective in enhancing insulin action on this parameter (Fig. 9).

**Fig. 5.** Effects of chronic treatment with R-ALA, EXER, or COMBO on triglyceride level in soleus muscle of obese Zucker rats. Muscle from lean Zucker rats was used as a reference control. Values are means ± SE for 5–7 animals per group. *\(P < 0.05\) vs. lean group; †\(P < 0.05\) vs. obese SED group.

**Fig. 6.** Effects of chronic treatment with R-ALA, EXER, or COMBO on protein expression of insulin-signaling factors in soleus muscle of obese Zucker rats. Muscle from lean Zucker rats was used as a reference control. Values are means ± SE for 7–8 animals per group. *\(P < 0.05\) vs. SED group; †\(P < 0.05\) vs. R-ALA group; ‡\(P < 0.05\) vs. all other obese groups.
expression was observed in skeletal muscle from the obese animals that received R-ALA treatment and underwent exercise training in combination (Fig. 6). Importantly, these increases in IRS-1 protein expression in the various groups were significantly correlated with the enhancement of insulin action on glucose transport activity, underscoring the physiological importance of this adaptive response. In addition, the insulin-stimulated association of tyrosine-phosphorylated IRS-1 with the p85 regulatory subunit of PI 3-kinase was enhanced to the greatest degree in skeletal muscle from the combined-treatment group compared with the other groups (Fig. 8). Therefore, the IRS-1/PI 3-kinase pathway appears to be a critical site of regulation by exercise training and R-ALA treatment.

The 2-wk exercise training protocol employed in the present study was effective in enhancing whole body aerobic capacity (Table 1), maximal run time (Table 1), and insulin-stimulated glucose transport activity in skeletal muscle (Fig. 1). We have shown in the present (Figs. 2–4) and previous (25) investigations that the exercise training-induced improvements in insulin action are associated with enhanced GLUT4 protein expression and capacities for glucose phosphorylation (hexokinase) and glucose oxidation (citrate synthase). In addition, we have demonstrated in the present investigation that this 2-wk exercise training regimen was effective in significantly enhancing the protein expression of IRS-1 (Fig. 6) in the soleus muscle of the obese Zucker rat, which was markedly decreased compared with the level seen in the soleus muscle of the insulin-sensitive lean Zucker rat. These findings support the previous findings of Hevener et al. (16), who showed that IRS-1 protein expression in red quadriceps was substantially less in the obese Zucker rat than in the lean Zucker rat and could be increased with 3 wk of endurance exercise training. In contrast, Christ et al. (5), using the gastrocnemius muscle, could not demonstrate either a defect in IRS-1 protein expression in obese vs. lean Zucker rat or any enhancement of IRS-1 protein expression after 7 wk of exercise training by obese Zucker rats. It should be noted that the gastrocnemius muscle consists of a mixture of types I and II fibers (2), whereas the soleus and red quadriceps are made up of predominantly type I fibers (2), and the differences in muscles used and their fiber type compositions may account for the discrepant responses to exercise training for IRS-1 protein expression.

Lipoic acid is a water-soluble biological antioxidant that also displays antihyperglycemic effects (11). When administered acutely in vivo, lipoic acid decreases plasma glucose in hyperglycemic streptozotocin-diabetic rats (20). Previous findings from our research group (29) demonstrated that the beneficial metabolic effects of lipoic acid on glucose metabolism in the insulin-resistant state are stereospecific to the R(-)-enantiomer (R-ALA). In addition, we have shown that chronic administration of R-ALA improves glucose tolerance and skeletal muscle glucose transport in the obese Zucker rat (25). Unlike exercise training, chronic R-ALA treatment has no effect on GLUT4 protein expression, hexokinase activity, or citrate synthase activity in muscle (Ref. 25; and in this study Figs. 2–4).

In the present study, we have shown that 2-wk treatment of obese Zucker rats with R-ALA is associated with a substantial, although not statistically significant, enhancement of insulin-stimulated insulin receptor tyrosine phosphorylation (Fig. 7), significant increases in IRS-1 protein expression (Fig. 6), and
insulin-mediated phosphotyrosine IRS-1 associated with the p85 subunit of PI 3-kinase (Fig. 8). However, the defect in insulin action on serine phosphorylation of Akt in soleus muscle of the obese Zucker rat was not affected by the antioxidant treatment (Fig. 9). Taken together, these results indicate that the improvements in insulin action on skeletal muscle glucose transport activity in R-ALA-treated obese Zucker rats are likely related to improvements in insulin stimulation of the IRS-1/PI 3-kinase pathway, but not Akt. The molecular mechanisms for these improvements in IRS-1 protein expression and IRS-1-p85 interaction remain to be investigated.

Increasing evidence in humans and animal models indicates that accumulation of muscle triglyceride is associated with decreased insulin action on glucose disposal by skeletal muscle (18, 22, 24), possibly related to locally elevated free fatty acids impairing insulin signaling at the level of IRS-1 and PI 3-kinase (32). Previous findings (25, 26) indicate that the concentration of circulating free fatty acid of the insulin-resistant obese Zucker rat is about two- to threefold higher than the value seen in the insulin-sensitive lean Zucker rat. In addition, we have shown in the present study that the triglyceride concentration in the insulin-resistant soleus muscle of the obese Zucker rat is also threefold higher than that in the soleus muscle of the lean Zucker rat (Fig. 5). Moreover, we have demonstrated that the triglyceride concentration in the obese soleus can be significantly reduced by exercise training or by R-ALA treatment (Fig. 5). These observations provide further support for the concept that elevated tissue triglyceride accumulation and systemic free fatty acids can negatively modulate skeletal muscle insulin action in the obese Zucker rat. However, because there was no further decrease in muscle triglyceride in the combined-treatment group, it is clear that the additional improvement in insulin action on glucose transport activity in this group was due to factors other than the triglyceride concentration.

Because of the multifactorial etiology of insulin resistance of skeletal muscle glucose transport and metabolism, it is unlikely that a single intervention in isolation will be adequate to bring about normalization of insulin action. The present investigation is one of several using obese Zucker rats demonstrating that exercise training combined with a pharmaceutical or nutriceutical intervention, including the antioxidant R-ALA, in beneficially modulating the molecular defects in insulin action observed in skeletal muscle of the obese Zucker rat.

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GRANTS

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15. Henriksen EJ, Jacob S, Streeper RS, Folt DL, Hokama JY, and Trischler HJ. Stimulation by α-lipoic acid of glucose transport activity of 3-kinase and with a decrease in triglyceride concentration. Most importantly, the combination of exercise training and antioxidant treatment in the obese Zucker rat resulted in the greatest enhancement of muscle insulin-mediated glucose transport activity, likely due to further enhancements of IRS-1 protein expression and insulin-mediated IRS-1-associated p85. These results further underscore the utility of a combination therapy involving endurance exercise training and a pharmaceutical intervention, including the antioxidant R-ALA, in beneficially modulating the molecular defects in insulin action observed in skeletal muscle of the obese Zucker rat.


