Exercise restores insulin, but not adiponectin, response in skeletal muscle of high-fat fed rodents

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Submitted 16 April 2012; accepted in final form 4 October 2012

Since the discoveries of leptin and adiponectin (Ad), a growing body of evidence has linked these adipose tissue-derived hormones (adipokines) to changes in skeletal muscle FA oxidation, reactive lipid species accumulation, and insulin resistance (29, 32, 37, 39). In isolated skeletal muscle from both rodents and humans, these adipokines promote FA oxidation rather than storage, a purportedly protective effect (7). These adipokines stimulate AMPK in skeletal muscle (19, 36), which, in turn, phosphorylate ACC, leading to an increase in FA oxidation (32). Ad stimulation of this cascade is regulated by its surface receptors AdipoR1 and AdipoR2 (with AdipoR1 being the predominant muscle isoform) and adaptor proteins APP1 and APP2 (33). In the obese and HF-fed conditions, skeletal muscle becomes resistant to leptin and Ad, such that their ability to stimulate FA oxidation is lost or blunted (4, 25). The development of skeletal muscle resistance to leptin and Ad occurs within days of HF feeding in rats, and precedes intramuscular lipid accumulation and insulin resistance (21). Whether resistance to adipokines that promote FA oxidation is a determinant factor in the intramuscular accumulation of reactive lipid species is unknown. The importance of restoring adipokine response for the reversal of intramuscular lipid accumulation and insulin resistance is also unclear.

It is well known that endurance exercise, pharmacological interventions, and weight loss can all reverse acquired skeletal muscle and whole-body insulin resistance. Given the increasing interest in adipose tissue interaction with other insulin-sensitive tissues, the paucity of data examining the significance of adipokines in the reversal of skeletal muscle insulin resistance is surprising. Recently, our laboratory has determined that the exercise-induced decrease in muscle DAG content and restoration of insulin response in HF-fed rats occurred prior to the reversal of leptin resistance (25), suggesting that the recovery of the skeletal muscle response to these hormones is independent. To our knowledge, whether the recovery of Ad response, rather than leptin response, is an important factor in the reversal of insulin resistance has not yet been examined.

In the current study, we aimed to determine whether the recovery of skeletal muscle Ad response coincided with or preceded the recovery of insulin response with exercise or pharmacological interventions in the HF-fed rat. Rats were fed a HF diet for 4 wk to induce soleus muscle insulin and Ad resistance. This was followed by 1 or 2 wk of intervention with either treadmill exercise training, or dietary supplementation of β-guadinoproprionic acid (β-GPA), a pharmacological insulin-sensitizing agent that decreases intramuscular phosphagen stores and promotes metabolic adaptations in muscle similar to those induced by endurance exercise training (23, 27, 35). We hypothesized that exercise training or pharmacological inter-
vention would restore muscle Ad response, and this would precede or coincide with the recovery of insulin response.

METHODS

Animals and Experimental Groups

Ninety-six female Sprague-Dawley rats were randomly assigned to 1 of 8 groups (n = 12 per group; Fig. 1). Experimental procedures commenced with an initial 4-wk feeding phase, in which 12 animals were fed a low-fat diet, while 84 rodents consumed a HF diet. After this initial phase, 12 low-fat fed (CON4) and 12 HF-fed rodents (HF4) were killed as per the surgical procedures outlined below. The remaining 72 HF-fed rodents continued HF feeding for 1 or 2 wk (5 and 6 wk total) with either no additional intervention (HF5 and HF6), exercise training (HF5EX1 and HF6EX2), or pharmacological supplementation with β-GPA (HF5GPA1 and HF6GPA2). All procedures were approved by the Animal Care Committee at the University of Guelph in accordance with the Canadian Council on Animal Care guidelines.

Housing and Diets

Animals were housed in groups of 4–6 per cage at 21°C and 50% relative humidity, on a reverse 12:12-h light-dark cycle (0730 to 1930 dark). A 7-day acclimation period preceded experimental feeding, during which rodent chow and water were provided ad libitum.

Low-fat diets contained 10% of kilocalories from fat (6% lard, 4% soybean oil). HF-fed groups all consumed a lard-based diet, where 60% of kilocalories were fat-derived (55% lard, 5% soybean oil). In HF5GPA1 and HF6GPA2 groups, this diet was supplemented with 1% wt/wt β-GPA (e.g., 0.2 g β-GPA into 20 g food). Food intake was monitored daily to ensure that all food was consumed.

HF5EX1 and HF6EX2 groups were exercised 5 days/wk. During the first week of exercise, the speed, duration, and inclination were gradually increased to 20 m/min, 60 min, and 10%, respectively. During the second week, the duration was incrementally increased to 120 min/session. We have previously shown this protocol to restore soleus muscle insulin response in HF-fed rats (25).

Surgical Procedures and Tissue Storage

Animals were fasted overnight (10–12 h) and anesthetized with an intraperitoneal injection of pentobarbital sodium (6 mg/100 g body mass) prior to all surgical procedures. The soleus muscle of each leg was then longitudinally sectioned into three muscle strips. Two unilateral soleus strips were immediately used for assessment of ex vivo basal (unstimulated) and insulin-stimulated (10 mU/ml Humulin R; Eli Lilly, Toronto, ON, Canada) glucose transport. The remaining muscle strip from this leg was incubated for 15 min with insulin to assess activation of signaling proteins. Two unilateral soleus strips from the opposite leg were used for assessment of basal protein content. Following removal of muscle samples, ~3 ml of blood was obtained via cardiac puncture. Blood samples were stored in ice-cold heparinized tubes and centrifuged at 5,445 g for 5 min (4°C), and plasma was stored at −80°C for further analysis.

Blood Measurements

Terminal whole blood glucose measurements were taken from all animals using a glucometer (OneTouch Ultra2; LifeScan, Milpitas, CA). Frozen plasma samples were used for measurement of insulin and Ad concentrations in duplicate. Quality controls ensured intra-assay accuracy and interassay consistency for insulin and Ad assays. Insulin measurements were made using commercially available RIA kits (cat. no. RI-13K; Millipore, Billerica, MA). Ad measurements were made using commercially available ELISA kits (cat. no. ESRADP-62K; Millipore).

Insulin Response: Basal and Insulin-Stimulated Glucose Transport

All incubation buffers contained medium 199 (9.4 g/l) and sodium bicarbonate (2.2 g/l) supplemented with 0.1% BSA. Buffers were adjusted to pH 7.0, gassed with humidified 95% O2-5% CO2, and maintained at 30°C. Upon excision, strips were placed in 2 ml of the incubation buffer plus 8 mM glucose and 32 mM mannitol to equil-
Bars represent unstimulated basal state, while black bars represent insulin-stimulated state.

HF5
HF5EX1
HF5GPA1
HF6
HF6EX1
HF6GPA2

Adiponectin Response: Basal and Adiponectin-Stimulated Fatty Acid Oxidation

All incubation buffers for FA oxidation measurements contained medium 199 (9.4 g/l), sodium bicarbonate (2.2 g/l), 4% BSA, 5 mM glucose, and 0.5 mM palmitate. This buffer was used during the 30-min equilibration period immediately following muscle excision. Subsequently, muscle strips were transferred to fresh buffer, including 30 mM d-[1-14C] mannitol (ARC-0126, American Radiolabeled Chemicals), with or without globular Ad (2.5 μg/ml) for 60 min. We have previously reported that glucose transport is linear over this period (30). Vials were gently agitated during the incubations. After incubations, muscles were blotted of excess liquid, removed of tendons, and digested in 1.0 M NaOH. This digest was used to quantify extracellular space and, subsequently, the transport of glucose into the solei, as described originally by Narahara and Özand (22) and Holloszy and Narahara (12).

Table 1. Body mass and blood data

<table>
<thead>
<tr>
<th></th>
<th>Initial Body Mass, g</th>
<th>Final Body Mass, g</th>
<th>Blood Glucose, mM</th>
<th>Plasma Insulin, ng/ml</th>
<th>Plasma Ad, μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON4</td>
<td>153.1 ± 1.5</td>
<td>254.8 ± 9.0</td>
<td>8.9 ± 0.3</td>
<td>5.2 ± 0.7</td>
<td>9.6 ± 0.4</td>
</tr>
<tr>
<td>HF</td>
<td>153.0 ± 2.1</td>
<td>299.9 ± 10.5†</td>
<td>9.8 ± 0.4</td>
<td>6.2 ± 0.5</td>
<td>8.3 ± 0.4</td>
</tr>
<tr>
<td>HF5EX1</td>
<td>152.8 ± 2.9</td>
<td>305.8 ± 6.1†</td>
<td>9.4 ± 0.5</td>
<td>8.1 ± 0.6†</td>
<td>8.0 ± 0.5</td>
</tr>
<tr>
<td>HF5GPA1</td>
<td>155.0 ± 1.0</td>
<td>275.6 ± 5.1*</td>
<td>8.7 ± 0.3</td>
<td>4.3 ± 0.6</td>
<td>7.0 ± 0.4†</td>
</tr>
<tr>
<td>HF6</td>
<td>151.3 ± 1.9</td>
<td>330.3 ± 6.4†</td>
<td>8.8 ± 0.2</td>
<td>5.2 ± 0.6</td>
<td>8.3 ± 0.7</td>
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<tr>
<td>HF6GPA2</td>
<td>146.8 ± 2.3</td>
<td>260.2 ± 6.6*</td>
<td>7.9 ± 0.3</td>
<td>2.3 ± 0.5*†</td>
<td>5.5 ± 0.5*†</td>
</tr>
</tbody>
</table>

Data shown as mean ± SE. *Significant difference (P < 0.05) compared to the corresponding HF group. †Significant difference (P < 0.05) when compared to CON4.

Western Blot Analyses

Proteins measured in unstimulated strips include total GLUT4, total and phosphorylated (Thr172) AMPK, total and phosphorylated (Ser79) ACC, APP1, APP2, AdipoR1, and AdipoR2 (GLUT4, Millipore CBL243; total AMPK, Cell Signaling 2603; phosphorylated AMPK, Cell Signaling 2535; total ACC, Cell Signaling 07–303; phosphorylated ACC, Cell Signaling 3661; APP1, Cell Signaling 3858S; APP2, Abcam ab95156; AdipoR1, Abcam ab70362; AdipoR2, Abcam ab77612). Total and Thr308 phosphorylated Akt content were measured after a 10-min insulin stimulus (total Akt, Upstate 07–372; phosphorylated Akt, Abcam ab66134).

Soleus muscle was prepared using a whole-cell lysate procedure. Briefly, muscles were homogenized in ice-cold buffer and centrifuged for 15 min at 1,500 g, and the supernatant was collected. Electrophoresis was conducted using 8% acrylamide gels at 160 V for 1–1.5 h, and transferred onto polyvinylidene fluoride membranes at 100 V for 1–2.5 h, dependent on size of the protein to be investigated. The membrane was then soaked in an antibody-free blocking buffer for 1 h to reduce nonspecific binding, followed by overnight incubation in the primary antibody. The membrane was washed of excess antibody and incubated with a horseradish-peroxidase-linked secondary antibody for 1 h. The membrane was once again washed, and proteins of interest were detected via chemiluminescence and quantified via densitometry. Blotting for α-tubulin and nonspecific Ponceau staining of the membrane confirmed equal protein loading.

Muscle Metabolites

Phosphagens. To determine the extent of high-energy phosphagen perturbation with β-GPA supplementation at the time of partial
restoration of insulin sensitivity, soleus muscle ATP, and PCr content was compared between HF5 and HF5GPA1 animals (n = 6 or 7). ATP and PCr contents were quantified in freeze-dried soleus muscles. Muscle metabolites were extracted from solei using 0.5 M perchloric acid, and neutralized using 2.2 M KHCO3. An NADPH-dependent absorptive assay (2) was then used to determine intramuscular ATP and PCr content in triplicate.

Glycogen. Muscle glycogen content was measured in the same samples as used for phosphagen determination. Muscles were digested with 0.1 M NaOH and brought to pH of 5.0 using 0.1 M HCl and 0.2 M citric acid; glycogen was broken down by amyloglucosidase. Free glucose was then assayed in quadruplicate (2).

Statistical Analyses

For comparison of basal and stimulated glucose transport and palmitate oxidation values between and within groups, a two-way ANOVA test was conducted (treatment \times condition). When treatment, condition, and interaction effects were observed, differences in means were elucidated using a Tukey post hoc test.

To compare proteins measured via Western blots and blood analyses, one-way ANOVA procedures were undertaken to determine overall group effects of treatment. When overall group effects were seen, group means were compared using a Tukey post hoc test. Statistical significance was accepted at P < 0.05.

RESULTS

Body Mass and Blood Measurements

There were no significant differences in initial body mass between groups (Table 1). All HF-fed groups gained mass compared with CON4, except for \( \beta \)-GPA-supplemented groups (HF5GPA1 and HF6GPA2). There was no significant effect of treatment on blood glucose (Table 1). Plasma insulin levels in HF5EX1 were signif-
significantly higher than CON4 (P = 0.036), although no difference was observed between CON4 and HF4EX2. Supplementation of the HF diet with β-GPA lowered plasma insulin levels in HF6GPA2 compared with CON4 (P = 0.035) and HF6 (P = 0.041).

High-fat feeding and exercise had no significant effect on plasma Ad levels. β-GPA supplementation with the HF diet lowered plasma Ad levels, with HF5GPA2 showing a significant reduction compared with CON4 (P < 0.001) and HF6 (P < 0.01). Plasma Ad was significantly reduced in HF5GPA1 compared with CON4 (P = 0.022).

Basal and Insulin-Stimulated Glucose Transport

There was no treatment effect on basal soleus muscle glucose transport rates between groups (Fig. 2). Within groups, glucose transport was increased by insulin in soleus from CON4 animals (+83%, P < 0.05), but insulin treatment did not significantly increase glucose transport in HF4 and HF5 (+11%, P = 0.289 and 15%, P = 0.498, respectively). Insulin significantly increased glucose transport following interventions with exercise (HF4EX1 +27%, P = 0.032; HF5EX2 +48%, P < 0.001) and β-GPA (HF5GPA1 +48%, P < 0.001; HF6GPA2 +66%, P < 0.001). Unexpectedly, glucose transport was significantly increased in response to insulin in HF6 (+30%; P = 0.037).

Insulin-Signaling Proteins

In insulin-stimulated strips, there was no treatment effect on total Akt content (Fig. 3A). Phosphorylated Akt (pAkt) content was significantly decreased in HF4, HF5, and HF6 compared with CON4, corresponding to the observed decrease in insulin-stimulated glucose transport (Fig. 3B). With the exception of the HF4EX1 group, insulin-stimulated pAkt content was not decreased in the exercise and β-GPA-supplemented rats. No effect of treatment was seen on total muscle GLUT-4 content (Fig. 3C).

Basal and Adiponectin-Stimulated Fatty Acid Oxidation

Basal FA oxidation rates were unaffected by treatment (Fig. 4). Ad increased FA oxidation in the CON4 group (+34%, P < 0.001), but this stimulation was lost in all HF-fed groups. Neither exercise nor β-GPA restored this stimulation in HF-fed rats.

Adiponectin-Signaling Proteins

No significant differences were found between groups in any of the proteins involved in fat oxidation or Ad signaling (total and Ser79 pACC, total and Thr172 pAMPK, APPL1, APPL2, AdipoR1, AdipoR2; Figs. 5 and 6), as measured in the unstimulated, fasted state. The absence of any treatment effect on AMPK and ACC phosphorylation was confirmed in a small number of animals in the fed state, as an increase in FA metabolism during fasting could potentially mask any treatment effects (9). Because of tissue limitations of the soleus, these phosphorylated protein measurements were not assessed in the Ad-stimulated state.

Muscle Phosphagen and Glycogen Content in Soleus Muscle

The effects of β-GPA administration on muscle high-energy phosphagens and glycogen were examined in HF-fed rats. Because β-GPA was only administered to high-fat fed animals, muscle metabolites were not compared between HF and control-fed animals. Soleus muscle PCr content was decreased in rats supplemented for 1 wk with β-GPA compared with HF-fed rats only (32.9 ± 3.27 vs. 51.5 ± 2.7 nmol/g dry wt, P < 0.001; Table 2). There was no significant reduction in ATP content following 1 wk of β-GPA supplementation (Table 2). Glycogen content was also unaffected by β-GPA supplementation (Table 2). Metabolite values reported in the HF-fed rats were similar to those that we normally observe in control-fed rats (18).

DISCUSSION

Numerous studies have attempted to determine whether exercise training can alter circulating levels of Ad (and leptin), presumably as part of a mechanism to improve insulin response. Surprisingly, the effects of chronic exercise on skeletal muscle responsiveness to these adipokines has not been examined. Since the development of Ad resistance in muscle is rapid and precedes insulin resistance, it is reasonable to postulate...
that a rapid improvement in Ad response (as an insulin-sensitizing adipokine) precedes the improvement in insulin response. Our present data indicate that the exercise-induced restoration of insulin response (glucose transport) in muscle of HF-fed rats is not associated with an improvement in the ability of Ad to stimulate FA oxidation. These findings were confirmed when a pharmacological (β-GPA) approach was used to restore insulin response. In fact, while both exercise and β-GPA were able to rapidly restore insulin-stimulated glucose transport in muscle after 1 and 2 wk of treatment, neither was able to restore Ad response during this period. These observations lead us to the conclusion that the ability of Ad to stimulate FA oxidation is not an essential requirement for the improvement of skeletal muscle insulin-stimulated glucose transport by exercise.

**Insulin, but not Adiponectin Response, Is Restored With Exercise and β-GPA Supplementation**

Similar to our previous findings (25), insulin-stimulated glucose transport in skeletal muscle of HF-fed rats was partially restored after only 1 wk of endurance exercise training, as well as after 2 wk. Supplementation with β-GPA similarly improved insulin-stimulated glucose transport. High-fat feeding caused depression of insulin-stimulated phosphorylation of Akt (Thr308), corresponding to the observed impairment of insulin-stimulated glucose transport. Our laboratory (21, 30) and others (17) have previously shown similar findings. Interestingly, 1 wk of exercise restored insulin-mediated stimulation of glucose transport, despite a sustained reduction in Akt Thr308 phosphorylation. However, it must be recognized that this captures only one aspect of insulin signaling, in that Akt Ser phosphorylation was not assessed, or other downstream proteins (e.g., AS160). A dissociation of phosphorylation of insulin-signaling proteins, such as Akt, and insulin-stimulated glucose uptake has also been previously demonstrated (14).

In contrast to the restoration of insulin response, neither exercise training nor β-GPA supplementation resulted in the recovery of soleus muscle Ad response. In the present study, muscles were sampled for incubation to determine insulin and Ad responsiveness ~48 h after the last exercise bout. A
postexercise delay of ~48 h is commonly used to ensure that chronic adaptations to exercise are observed rather than the residual effects of the last acute exercise bout. For example, there is a residual contraction-induced increase in GLUT-4 content and insulin response in skeletal muscle immediately following exercise (24), which is fully reversed within 40 h (13); accordingly, we did not observe any increase in total GLUT-4 content 48 h after the last exercise bout. We cannot rule out the possibility that any effect exercise may have had on Ad response may have been lost within the 48 h intervening the last exercise bout and assessment of FA oxidation. However, it is important to view this possibility in the context of recent findings; namely, that β-GPA and fish oil supplementation (31) do not restore Ad response within 2 wk, well after the observed recovery of insulin response. Together, these studies indicate that at the time of detecting the recovery of muscle insulin response in muscle, there is no evidence of Ad’s ability to stimulate FA oxidation. Thus, the two responses appear to be independent.

Changes in Adiponectin Signaling

None of the interventions had a significant effect on total APPL1 protein content, which propagates Ad signaling from the AdipoR1 receptor to downstream signaling proteins (33). The inhibitory adaptor protein APPL2 can alter this process by blocking the interaction between AdipoR1 and APPL1 (33).
We have previously shown that AdipoR1 content is not altered with HF feeding (21, 25). Therefore, it is possible that the impaired Ad response that we observed as a consequence of HF feeding was a result of an altered association of APPL1 and/or APPL2 with AdipoR1. The elucidation of these interactions could not be addressed in the current study. However, we did not observe any changes in total protein levels of AdipoR1, AdipoR2, or APPL2. Finally, there were no differences in phosphorylation of AMPK and ACC between groups. This is consistent with prior studies from this laboratory demonstrating that phosphorylation of these proteins in the fasted, unstimulated state is unaffected by HF feeding (21, 25).

A limitation of the current study is that the soleus was not of sufficient size to provide additional strips to assess the phosphorylation of signaling proteins, such as ACC under Ad-stimulated conditions. However, these assessments are secondary to our demonstration that the restoration of insulin-stimulated glucose transport and response of FA oxidation to Ad are independent of each other. Interestingly, and somewhat unexpectedly, total circulating Ad concentrations decreased with exercise and β-GPA treatment. Whether this indicates that other tissues, other than muscle, have become sensitive to Ad warrants further investigation.

**Metabolic Effects of β-GPA Administration**

In the current study, dietary supplementation of 1% wt/wt β-GPA led to the restoration of soleus muscle insulin response, but not Ad response; these effects mirrored those of endurance exercise training. To our knowledge, this is one of the first studies to determine metabolic effects of β-GPA supplementation of this brief duration, as most studies with chronic supplementation extend between 4 and 10 wk (26, 27, 38). Prolonged supplementation typically decreases intramuscular PCr and ATP by ~90% and 50%, respectively (35), leading to increased phosphorylation of AMPK (34), increased GLUT-4 expression, and mitochondrial biogenesis (23, 41). In the current study, 1 wk of β-GPA supplementation induced a 46% decrease in intramuscular PCr content, without corresponding changes in ATP, AMPK phosphorylation, or GLUT-4 content. Nonetheless, this small perturbation of intramuscular phosphagen content corresponded with partially restored insulin, but not Ad response in soleus muscle. Interestingly, we show metabolic effects of β-GPA supplementation without observable changes in AMPK, or its downstream effects on GLUT-4.

An important consequence of β-GPA supplementation is the reduced body mass of β-GPA-supplemented groups compared with exercise-trained and HF-fed groups. To avoid this would have necessitated food restriction in the exercise-trained groups. Since caloric restriction has been used for decades as an insulin-sensitizing therapy (8, 10), this would have confounded the metabolic effects of endurance exercise training. Regardless of whether the β-GPA-induced alterations in skeletal muscle insulin response are attributable primarily to changes in intramuscular energy charge or loss of body mass/fat, the current study shows that two different treatment paradigms produced a rapid restoration of skeletal muscle insulin response without recovery of Ad response.

**Perspectives and Significance**

This study has demonstrated that insulin response can be restored independently of Ad response. The dissociation of insulin response (glucose transport) and Ad response (FA oxidation) in muscle is evidenced by the observations that the development of their resistance occur on different time scales, and that insulin response can be restored while Ad response remains impaired. Ad resistance is induced in muscle with the administration of HF diets and prior to any demonstration of insulin resistance (21); conversely, interventions such as exercise and β-GPA administration (present study), as well as fish oil supplementation (31) all restore muscle insulin response prior to any observed improvement in Ad response. Furthermore, we have recently demonstrated that an exercise training protocol similar to that employed in the present study is capable of restoring muscle leptin response, but not until after insulin response has recovered (25). It should be recognized that the responses to insulin, adiponectin, and leptin in these studies are assessed in soleus muscle ex vivo, and in a whole-body context. In spite of the seemingly universal acceptance that Ad, as well as leptin, are important insulin-sensitizing adipokines, it is intriguing and paradoxical that such different paradigms (exercise, pharmacological, dietary fish oil) can all reverse the insulin resistance (in terms of glucose transport) induced by diets high in saturated fat, but without the requirement of the restoration of Ad or leptin response (at least in terms of stimulating FA oxidation). These data challenge the necessity and role of Ad as a mediator of muscle insulin response in skeletal muscle.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**


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MUSCLE INSULIN AND ADIPONECTIN RESPONSE ARE INDEPENDENT


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