Contraction of insulin-resistant muscle normalizes insulin action in association with increased mitochondrial activity and fatty acid catabolism

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Submitted 5 June 2006; accepted in final form 11 October 2006

Thyfault JP, Cree MG, Zheng D, Zwetsloot JJ, Tapscott EB, Koves TR, Ilkayeva O, Wolfe RR, Muoio DM, Dohm GL. Contraction of insulin-resistant muscle normalizes insulin action in association with increased mitochondrial activity and fatty acid catabolism. Am J Physiol Cell Physiol 292: C729–C739, 2007. First published October 18, 2006; doi:10.1152/ajpcell.00311.2006.—Acute exercise can reverse muscle insulin resistance, but the mechanism(s) of action are unknown. With the use of a hindlimb perfusion model, we have found that acute contraction restores insulin-stimulated glucose uptake in muscle of obese Zucker rats to levels witnessed in lean controls. Previous reports have suggested that obesity-related insulin resistance stems from lipid oversupply and tissue accumulation of toxic lipid intermediates that impair insulin signaling. We reasoned that contraction might activate hydrolysis and oxidation of intramuscular lipids, thus alleviating “lipotoxicity” and priming the muscle for enhanced insulin action. Indeed, analysis of mitochondrial-derived acyl-carnitine esters suggested that contraction caused robust increases in β-oxidative flux and mitochondrial oxidation. As predicted, contraction decreased intramuscular triacylglycerol content; however, diacylglycerol and long chain acyl-CoAs, lipid intermediates presumed to trigger insulin resistance, were either unchanged or increased. In muscles from obese animals, insulin-stimulated tyrosine phosphorylation of the insulin receptor and insulin receptor substrate-1 remained impaired after contraction, whereas phosphorylation of the downstream signaling protein, AS160, was partially restored. These results suggest that acute exercise enables diabetic muscle to circumvent upstream defects in insulin signal transduction via mechanisms that are more tightly coupled to increased mitochondrial energy metabolism than the lowering of diacylglycerol and long chain acyl-CoA.

skeletal muscle; intramuscular lipids; signaling; exercise

INSULIN RESISTANCE, a hallmark of obesity and type 2 diabetes, is characterized by the failure of peripheral tissues to appropriately regulate glucose homeostasis in response to insulin. In skeletal muscle, impaired insulin action leads to diminished glucose uptake and contributes to systemic hyperglycemia. Research over the past decade has established a convincing connection between the development of insulin resistance and an increased storage of triacylglycerol (TAG) within skeletal muscle cells (20, 30, 34, 44). However, it has become increasingly apparent that TAG does not function as a direct mediator. Rather, increased TAG is thought to mark and/or contribute to the accumulation of other lipid moieties, such as diacylglycerol (DAG), long-chain fatty acyl CoAs (LCACoA), and ceramides, all known to participate in cellular signaling. Recent evidence suggests that intramuscular DAG and LCACoA act as ligands for serine kinases (PKC or others) that can inactivate the insulin receptor IRS-1, thus implicating these specific lipid intermediates as direct inhibitors of insulin signal transduction (20, 44). Additional support for this “lipotoxicity” model comes from studies of human and rodent obesity, in which elevated DAG and LCACoA levels are accompanied by severely impaired insulin signaling (12, 23, 24, 39). Likewise, prospective studies show that high fat feeding or lipid infusions elevate intramuscular lipids and concomitantly provoke insulin resistance (25, 32, 50). Still, despite intense investigation, the precise mechanism(s) responsible for lipid-induced insulin resistance in skeletal muscle have remained elusive.

Our laboratory uses exercise as a physiological model to study mechanisms that govern muscle insulin action. Remarkably, a single bout of acute exercise dramatically enhances insulin-stimulated glucose transport in both insulin sensitive and insensitive skeletal muscle (9, 11, 22, 41). Early work by our group (9) explored acute exercise and insulin action using hindlimb perfusions in lean and obese Zucker rats. In the muscle of lean rats, contraction followed by insulin elicited an additive effect on glucose transport (contraction plus insulin stimulated glucose transport equal to that found when adding the singular effects of insulin and contraction). Muscles from obese animals were severely insulin resistant; however, the impairment was fully overcome when insulin administration was preceded by acute contraction. In fact, only in the obese muscles did contraction and insulin act synergistically to enhance glucose transport; suggesting that contraction eliminates an inhibitory factor and primes the muscle for improved insulin action. The highly sought identity of this inhibitory factor and the mechanism(s) underlying the exercise effect are yet unknown. Like other diabetic models, the obese Zucker rats are characterized by elevated intramuscular lipids (12). We therefore hypothesized that contraction-induced mitochondrial oxidation would activate lipolysis and lipid catabolism, thereby lowering the levels of toxic intermediates (DAG and LCACoA) thought to impede insulin action.

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To address this hypothesis we again employed the hindlimb perfusion model using obese and lean Zucker rats. Skeletal muscle was studied under conditions of no insulin (basal), contraction, insulin, or contraction followed by insulin, in a two stage model. Using conventional lipid chemistry as well as mass spectrometry-based lipid profiling, we first investigated the effects of acute contraction on the intramuscular lipid milieu at the time of insulin administration. Therefore, stage one assessed alterations in intramuscular lipids immediately following muscle contraction. The second stage examined the impact of contraction and/or insulin on the conventional insulin signaling pathway, as well as alternative pathways known to activate glucose transport (e.g., AMPK and MAPK). Surprisingly, the salutary effects of contraction were not accompanied by a decrease in LCACoAs or DAG, and upstream signaling events remained severely impaired. Instead, our findings showed a strong association between improved insulin action, enhanced mitochondrial activity and partial recovery of downstream signaling.

MATERIALS, METHODS, AND EXPERIMENTAL PROCEDURES

Animals

Male obese Zucker rats (fa/fa) (12 wk of age) and lean littermates were obtained from Harlan (Indianapolis, IN). Animals were maintained on a 12-h light, 12-h dark cycle, provided with ad libitum standard chow (Purina rodent chow) and water.

Hindlimb Perfusions

Hindlimb perfusions and measurement of glucose transport were performed as described previously (8, 9). Hindlimbs were perfused with no insulin or with a maximal concentration of insulin (100 nM), and in all perfusions one limb was electrically stimulated to contract and the other limb served as a noncontracted control.

After an overnight fast, rats were anesthetized intraperitoneally with ketamine and xylazine (10 mg/100 g body wt). The aorta and vena cava were ligated and a hemiscorpus preparation was created. The aorta and vena cava of the hemiscorpus were quickly catheterized in succession and a prepared media (4% BSA, 100 mg/dl glucose, 33% washed bovine red blood cells, and Krebs-Henseleit buffer) was perfused at a rate of 18 ml/min and gassed with O₂/CO₂ (95:5), with chamber temperature maintained at 37°C. The first 50 ml of fluid collected from the perfusion prep was discarded while the remaining 100 ml was recirculated. After a 30-min preperfusion period, one limb was stimulated to contract for a total of 15 min, then radioactive label was added to the media at a final concentration of 20 mM sorbitol containing 0.1 µCi of [U-¹⁴C]sorbitol and 0.2 µCi of 2-deoxy [³H]glucose. Insulin was also added when appropriate at a concentration of 100 nM. For measures of glucose transport all perfusions were stopped 25 min post administration of radioactive label. For measures of skeletal muscle lipids, acylcarnitine profiling and insulin signal transduction no radioactivity was added and muscle was excised either immediately post contraction or 10 min post contraction (Table 1). Media was sampled for glucose concentration every 5 min throughout the perfusion and 50% liquid dextrose was added when needed to maintain a media glucose concentration of ~100 mg/dl.

<table>
<thead>
<tr>
<th>Timeline (30 min)</th>
<th>Metabolite Levels</th>
<th>Insulin Signaling</th>
<th>Glucose Transport</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preperfusion</td>
<td>Rest</td>
<td>Basal or insulin</td>
<td>Basal or insulin</td>
</tr>
<tr>
<td>Preperfusion</td>
<td>Contraction</td>
<td>Basal or insulin</td>
<td>Basal or insulin</td>
</tr>
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</table>

Booth lean and obese Zucker rats underwent the following procedures. Metabolite levels were measured immediately post contraction (or rest control), insulin signaling was measured 10 min post contraction (or rest control), and glucose transport rates were measured during the entire 25 min post contraction (or rest control).

Electrical Stimulation

During surgery the sciatic nerve of one limb was isolated and connected to an electrode and electrical stimulation was delivered by a Grass SD9 stimulator (West Warwick, RI) as done previously (9). For the contraction treatment, skeletal muscle was stimulated for 3 × 5-min periods separated by 1-min rest periods. Stimulation consisted of a double pulse per second frequency (duration of each pulse was 1 mS with a delay of 1 mS) delivered at 4 V.

Glucose Transport

Glucose transport was measured in tissues excised at 25 min post contraction under the following treatments: basal (resting without insulin), contraction, insulin and contraction + insulin (Con + Ins). All tissue was removed immediately after perfusion, carefully cleaned of connective tissue and fat, and freeze clamped in liquid nitrogen. Powdered tissue (50–100 mg) was added to 0.4 ml of distilled water and then dispersed via heat (40°C) and sonication, followed by the addition of 5 ml of liquid scintillation fluid, as performed previously (9). ³H/¹⁴C radioactivity (dpm) and glucose concentrations were analyzed at 5-min intervals and used to determine specific radioactivity. [¹⁴C]Sorbitol was utilized to evaluate the amount of glucose in the interstitial space, thus accumulation of intramuscular 0.2 µCi of 2-deoxy [³H]glucose was determined by subtracting extracellular 0.2 µCi of 2-deoxy [³H]glucose (determined from the [¹⁴C]sorbitol space) from total muscle 0.2 µCi of 2-deoxy [³H]glucose.

Western Blot Analysis

Insulin signal transduction was measured in tissue excised 10 min after the addition of insulin (Insulin or Con + Ins). Contraction stimulated proteins [AMPK, ERK, P38, and AS160] were assessed in both immediate post contraction (basal and contraction) and 10-min post samples (insulin or Con + Ins). Powdered muscle was homogenized in buffer [50 mM HEPES, 50 mM Na⁺ pyrophosphate, 100 mM Na⁺ fluoride, 10 mM EDTA, 10 mM Na⁺ orthovanadate, 1% Triton X-100, and protease and phosphatase (1 and 2) inhibitor cocktails (Sigma, St. Louis, MO)] on ice. After centrifugation for 25 min at 15,000 g, supernatants were extracted and protein content was detected using a BCA protein assay (Pierce, Rockford, IL), separated by SDS-PAGE using 4–15% or 7.5% Tris-HCl gels and then transferred to PVDF membranes for probing by appropriate antibodies. The following antibodies were used: insulin receptor IRβ, IRS-1, Akt phosphoseryl473, Akt1/2, ERK phosphotyrosine204, and ERK1 purchased from Santa Cruz Biotech (Santa Cruz, CA); and phospho-serine/threo-
nine) Akt substrate, p38 MAP kinase phospho threonine^{180} tyrosine^{182}, p38 MAP kinase, AMPK-α phospho threonine^{172}, and AMPK-α purchased from Cell Signaling (Beverly, MA). Following incubation with primary antibodies, blots were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies. Horseradish peroxidase activity was assessed with ECL solution (Lumiglo and Peroxide, Cell Signaling, San Francisco, CA), and exposure to film. The image was scanned and band densitometry was assessed with Gel Pro Analyzer software (Media Cybernetics, Silver Spring, MD). Content of phospho-proteins (using phospho-specific antibodies) was calculated from the density of the band of the phospho-protein divided by the density (content) of the protein (total) using the appropriate antibody. To measure tyrosine phosphorylation of the insulin receptor (IR_\beta) and IRS-1, protein from muscle homogenate was immunoprecipitated overnight with antiphosphotyrosine agarose beads (Sigma) and homogenization buffer. Immunoprecipitates underwent western blotting as stated previously. Membranes were then probed for total IR_\beta or IRS-1 (Santa Cruz Biotech) resulting in bands detecting tyrosine phosphorylation levels of IR_\beta or IRS-1 that also account for possible protein content differences between muscle from lean and obese as has been shown by others (28).

Intramuscular Lipids

**Triacylglycerol and diacylglycerol.** Powdered muscle (~25 mg) was added to 3 ml of lipid extraction solution composed of 1:2 (vol/vol) methanol:chloroform containing 0.05 mg/ml BHT, in addition to TAG and DAG internal standards [(TAG: 1 mg Tri-C17:0/ml chloroform); DAG (0.1 mg of 1,30 dipentaedecanoyl-glycerol/ml chloroform)]. The samples were vortexed and stored for 24 h at 4°C for extraction of lipids. The samples were then centrifuged at 3,500 rpm for 30 min at 4°C. The supernatant was extracted and dried under a flow of N_2. The residue was resuspended in 50 μl of chloroform, mixed, and loaded onto TLC plates, in addition to internal standards in separate lanes. The plate was incubated in a tank containing hexane:ethyl ether:acetic acid in 70:30:1 (vol/vol) and the fluid allowed to rise to ~1 cm from the top of the plate. The plate was dried, sprayed with primuline solution, and TAG and DAG bands were visualized and marked under a UV lamp with 365 nm. Following incubation with primary antibodies, blots were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies. Horseradish peroxidase activity was assessed with ECL solution (Lumiglo and Peroxide, Cell Signaling, San Francisco, CA), and exposure to film. The image was scanned and band densitometry was assessed with Gel Pro Analyzer software (Media Cybernetics, Silver Spring, MD). Content of phospho-proteins (using phospho-specific antibodies) was calculated from the density of the band of the phospho-protein divided by the density (content) of the protein (total) using the appropriate antibody. To measure tyrosine phosphorylation of the insulin receptor (IR_\beta) and IRS-1, protein from muscle homogenate was immunoprecipitated overnight with antiphosphotyrosine agarose beads (Sigma) and homogenization buffer. Immunoprecipitates underwent western blotting as stated previously. Membranes were then probed for total IR_\beta or IRS-1 (Santa Cruz Biotech) resulting in bands detecting tyrosine phosphorylation levels of IR_\beta or IRS-1 that also account for possible protein content differences between muscle from lean and obese as has been shown by others (28).

**Statistical Analysis**

Glucose transport rates were analyzed using a 2-factor (type and treatment) ANOVA. Treatments analyzed for glucose transport included basal, contraction, insulin, and Con + Ins. Insulin signaling was analyzed using a 2-factor (type and treatment) ANOVA, with statistical analysis limited to the insulin and Con + Ins treatments. Intramuscular lipid data was also analyzed using a two-factor (type and treatment) ANOVA, with statistical analysis of the basal and contraction samples. Followup statistical measures included an independent t-test for type comparisons and Tukey’s post hoc for type or treatment conditions, where appropriate. All statistics were analyzed with the Statistical Package for the Social Sciences (version 11.0; SPSS, Chicago, IL). Data are presented as means ± SE and P values of <0.05 were considered statistically significant.

**RESULTS**

**Animals**

The obese Zucker rats were significantly heavier than their lean littermates (437.9 ± 8.8 g vs. 307.8 ± 3.9 g; P < 0.001). For all experiments, n = 6–8 animals were examined.

**Glucose Transport**

Glucose transport was measured in whole gastrocnemius muscle to ensure replication of earlier findings following treatments: basal (resting without insulin), contraction, insulin, and Con + Ins. Results were similar to previous findings (9) with the muscle of the obese rats showing markedly lower insulin stimulation of glucose transport (P = 0.026) (Fig. 1). Contraction-stimulated glucose transport tended to be higher in lean than obese muscle, but this difference did not reach statistical significance (P = 0.115). When muscles were first contracted
and then exposed to insulin, glucose transport in the obese group was restored to levels that were equal to those achieved by the lean group ($P = 0.927$).

**Intramuscular Lipids and Glycogen**

To determine whether improvements in insulin action corresponded with changes in intramuscular lipids we measured TAG, DAG, and LCACoAs in rested and contracted muscles that were harvested at the same time at which insulin was administered to a parallel set of animals (Table 1). Muscle glycogen was also measured to assess reliance upon endogenous carbohydrate sources. Basal TAG content was 766% higher in muscle from obese compared with lean rats ($P < 0.001$) (Fig. 2A) ($P = 0.001$). In the obese rats, contraction lowered muscle TAG content (72%; $P = 0.022$) to levels that were closer to (but still higher than) those measured in lean group. Contraction decreased TAG in muscles from the lean animals, but the 40% decline was not significant ($P = 0.38$). Contraction also decreased muscle glycogen levels in both lean (33%; $P = 0.04$) and obese (45%; $P = 0.015$) rats compared with the basal condition. Thus the energy requirements of contraction were met via the activation of both lipolysis and glycogenolysis.

Total intramuscular DAG content tended to be increased (32%) in the obese compared with lean ($P = 0.1$) (Fig. 2B). In the lean animals, contraction increased DAG levels ($P = 0.05$), whereas in their obese counterparts they remained unchanged. The finding that basal DAG content in the obese rats did not parallel the high TAG levels implied a low lipolytic rate, and/or that the DAG products of lipolysis were hydrolyzed in a rapid manner. Likewise, in the obese state, DAG did not accumulate with contraction, despite the marked depletion of TAG.

Total intramuscular LCACoA levels were 50% higher in obese than in lean rats ($P = 0.02$) (Fig. 2C), consistent with their predicted roles in insulin resistance. In contrast to our hypothesis, muscle contraction increased total LCACoA levels by 2-fold in the lean ($P = 0.008$) and ~30% in the obese ($P = 0.03$); thus the exercise-induced improvements in insulin action were associated with an increase rather than a decrease in these lipid metabolites. The rise in LCACoA is consistent with the notion that exercise liberates fatty acids from storage (TAG) to satisfy increasing energy demands. These results were surprising considering previous studies that have reported a strong negative relationship between insulin sensitivity and LCACoA levels (10, 13).

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**Fig. 1.** Rates of glucose transport in whole gastrocnemius from lean (open bars) and obese (solid bars) animals were measured following basal [resting without insulin (Ins)], contraction, insulin, or contraction followed by insulin (Con + Ins) stimulation. Contraction increased glucose transport in both lean and obese (*$P < 0.05$). Insulin increased glucose transport in lean mice; however, obese showed no insulin stimulation of glucose transport and was decreased compared with lean (†$P < 0.05$). Con + Ins restored insulin stimulated glucose transport in the obese. Values are means ± SE.

**Fig. 2.** Endogenous substrate levels were measured in whole gastrocnemius muscle at rest or following contraction. **A:** muscle of obese rats demonstrated markedly elevated intramuscular triglyceride levels. †Contraction presumably activates lipolysis, thus lowering triglycerides in obese; however, levels are still higher than those found in lean (note *). Contraction did not significantly lower triglyceride content in lean. **B:** under basal conditions, diacylglycerol content tended to be elevated in the obese compared with the lean state ($P = 0.10$). Contraction increased ($P = 0.05$) diacylglycerol content in muscles from lean but not obese rats. **C:** muscle of obese rats had increased long-chain acyl CoA levels compared with lean (†). Contraction significantly increased fatty acyl CoA levels in lean and obese. **D:** contraction lowered muscle glycogen levels in both the lean and obese rats. Significance was set at *$P < 0.05$; values are means ± SE.

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Insulin Signaling

Activation of the classic insulin signaling cascade was severely diminished in muscle from obese Zucker rats compared with lean controls (Fig. 3A). The impairments were observed in insulin-stimulated tyrosine phosphorylation of the insulin receptor (IR) (Fig. 3B; *P* = 0.043) and IRS-1 (Fig. 3C; *P* < 0.0001), as well as serine phosphorylation of Akt serine^473^ (Fig. 3D; *P* = 0.001), and AS160 (Fig. 3E; *P* = 0.022). Contraction alone did not stimulate tyrosine phosphorylation of IR or IRS-1, or serine phosphorylation of Akt (data not shown); but did increase phosphorylation of AS160 in both the lean and obese groups (Fig. 3E; *P* = 0.05). Contraction did not potentiate insulin-stimulated phosphorylation of IR, IRS-1, or Akt in the obese group (Fig. 4, B–D). However, the combination of insulin and contraction synergistically enhanced phosphorylation of AS160 in both groups (obese; *P* = 0.03, and lean; *P* = 0.001). Interestingly, insulin-mediated phosphorylation of AS160 was substantially restored in the obese group when hormone administration was preceded by contraction.

MAPK and AMPK pathway(s). Consistent with other reports, contraction caused robust increases in the phosphorylation state of ERK, p38MAPK (Fig. 4), as well as AMPK (Fig. 5). Notably, these responses were similar between skeletal muscle of lean and obese animals; although total AMPK protein content was lower in obese compared with lean muscles (*P* = 0.015). Insulin alone tended to activate ERK, but only in lean rats and the effect was not significant (*P* = 0.066), whereas it did not affect p38MAPK or AMPK. Insulin appeared to diminish the contraction-induced phosphorylation of ERK and p38 in both the lean and obese groups. Similarly, insulin administration reduced AMPK phosphorylation in the contracted muscles of obese, but not lean, rats. However, it is important to note that the insulin-treated muscles were excised 10 min after those in the basal and contracted (no insulin) groups, thus the differences between the insulin and Con + Ins conditions could be an artifact of time. Nonetheless, the phosphorylation state of AMPK in muscles of the contracted/obese group returned to near baseline levels in response to time and/or insulin admin-
Fig. 4. MAPK signaling. ERK1 tyrosine \textsuperscript{204} phosphorylation was compared between basal and contraction or between insulin and Con+Ins in gastrocnemius of lean (open bars) and obese (solid bars) Zucker rats. A: contraction had a significant effect upon ERK activation in both lean and obese. B: lean animals showed a significant increase in ERK activation from insulin to Con+Ins while the obese Con+Ins response was blunted (note †). P38 threonine\textsuperscript{180} / tyrosine\textsuperscript{182} phosphorylation was also assessed under the same conditions. C: lean and obese both showed an increase in P38 phosphorylation in response to contraction, however. D: no effect was found from the insulin to the Con+Ins stimulus. *Significance was set at $P < 0.05$; values are means ± SE.

Fig. 5. AMPK-α threonine\textsuperscript{172} phosphorylation was compared between basal and con or between insulin and Con+Ins in gastrocnemius of lean (open bars) and obese (solid bars) Zucker rats. A: contraction increased phosphorylation of AMPK in both lean and obese. B: in response to Con+Ins, both groups had increased AMPK phosphorylation, however, the obese response was blunted compared with lean (note †). *Significance was set at $P < 0.05$. Values are means ± SE.
Acylcarnitine Profiling of Mitochondrial Activity

In skeletal muscle, acylcarnitine esters (Table 2 and Fig. 6) represent by-products of mitochondrial substrate catabolism that are often used to assess pathophysiological changes in metabolic flux (6, 29, 33, 48). In the context of our experiment, the fatty acylcarnitines (most even chain species) were derived only from endogenous sources because lipids were not added to the perfusion medium. Contraction tended to decrease the major long-chain acylcarnitine species (C16 and C18:1) in the lean animals; whereas short- and medium-chain acylcarnitines (C2, C4, C6, C8, and C10) levels increased in both groups (Fig. 6). These results suggest that contraction promoted flux through the β-oxidative pathway. Further support for this notion is offered by the higher ratio of short chain to long chain acylcarnitines that was measured in the contracted muscles (Table 2). The most abundant carnitine ester, acetylcarnitine (C2), reflects intramitochondrial production and/or accumulation of excess acetyl-CoA. Production of the C2 metabolite depends on the balance between acetyl-CoA supply via substrate catabolism and acetyl-CoA entry into the TCA cycle. Our finding that exercise lowered the ratio of acetylcarnitine to short-chain acylcarnitines (C2/SC) implies that the contraction-induced generation of acetyl units was accompanied by an increase in TCA cycle activity (Table 2). The basal ratio of short- to long-chain acylcarnitines tended to be higher in muscles from the Zucker rats compared with lean controls (Table 2), suggesting that the high lipid environment of obesity favors increased β-oxidative activity but without an accompanying increase in TCA cycle activity.

**DISCUSSION**

The present study sought to determine the mechanism whereby acute exercise restores insulin action in obesity-associated insulin resistant muscle. Previous reports have advanced the concept that muscle insulin resistance develops secondary to accumulation of lipid intermediates. Whereas muscle TAG content is considered a strong marker of the pathology, DAG and LCACoA have emerged as more likely “lipotoxic” inhibitors of insulin signaling (20, 44). We therefore predicted that contraction might lower the initially high levels of these metabolites in muscles of obese/insulin resistance rats, subsequently priming the tissue for enhanced insulin action. Our studies demonstrated a dramatic contraction-induced depletion of TAG in muscles from obese rats; however, the DAG and LCACoA species were either unchanged or increased, and transduction via the classic insulin signaling cascade was only partially restored. Whereas these results did not concur with our predictions, profiling of mitochondrial-derived acylcarnitines revealed robust contraction-induced increases in lipid catabolism and mitochondrial fatty acid flux. These findings support emerging evidence that links the insulin sensitizing effects of exercise to enhanced mitochondrial activity (3, 14, 17, 29, 35, 38). Moreover, these putative changes in mitochondrial energy metabolism and/or other contraction-induced events appear to circumvent upstream impairments in insulin signaling.

The “lipotoxicity” model of insulin resistance has been supported by studies in which high-fat diets or lipid infusion caused accumulation of intramuscular lipid metabolites, activation of serine kinases, blunted insulin signaling transduction, and impaired glucose metabolism in skeletal muscle (7, 25, 50). In contrast, exercise has long been known to confer therapeutic effects on insulin sensitivity (19, 22), although the underlying mechanisms are incompletely understood (21). Herein, we show that contraction led to a robust reduction of intramuscular TAG (Fig. 2A), although levels in the muscle of obese rats remained elevated compared with the lean basal group. However, following contraction, DAG levels were increased in lean muscle and unchanged in the obese. Muscle LCACoA levels were higher in obese rats and increased with contraction in both groups (Fig. 2C), suggesting that the lipolytic provision of fatty acids exceeded rates of β-oxidation. Interestingly, contraction enhanced insulin sensitivity in the face of elevated LCACoA levels. Although these findings were unexpected, others have likewise found that insulin sensitivity does not always track with reciprocal changes in intramuscular lipid levels. For example, endurance trained athletes are highly insulin sensitive while having elevated intramuscular TAG content (16). Furthermore, insulin-resistant human subjects who underwent 12 wk of exercise training improved insulin sensitivity despite elevated intramuscular TAG content (38). Chronic exercise training has been shown to improve insulin action in humans with type II diabetes, and although intramuscular TAG was reduced, LCACoA levels were unchanged (4). Similarly, obese/overweight human subjects who were exercise trained demonstrated improved insulin sensitivity but showed no changes in muscle TAG or LCACoA levels (14). Finally, a genetic rescue of insulin sensitivity in rats fed a

**Table 2. Muscle acylcarnitine ratios**

<table>
<thead>
<tr>
<th>Flux ratio</th>
<th>Lean Basal mean ± SE</th>
<th>Lean Contraction mean ± SE</th>
<th>P Value</th>
<th>Obese Basal mean ± SE</th>
<th>Obese Contraction mean ± SE</th>
<th>P Value</th>
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<tbody>
<tr>
<td>C4/LC</td>
<td>1.57±0.10</td>
<td>9.28±2.71</td>
<td>0.03</td>
<td>4.27±1.79</td>
<td>9.04±3.12</td>
<td>0.26</td>
</tr>
<tr>
<td>C6/LC</td>
<td>1.04±0.10</td>
<td>9.13±2.11</td>
<td>0.01</td>
<td>2.57±0.73</td>
<td>14.38±5.60</td>
<td>0.07</td>
</tr>
<tr>
<td>C8/LC</td>
<td>0.80±0.25</td>
<td>13.13±4.14</td>
<td>0.04</td>
<td>2.07±0.97</td>
<td>13.64±6.04</td>
<td>0.07</td>
</tr>
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<table>
<thead>
<tr>
<th>Entry into TCA</th>
<th>Lean Basal mean ± SE</th>
<th>Lean Contraction mean ± SE</th>
<th>P Value</th>
<th>Obese Basal mean ± SE</th>
<th>Obese Contraction mean ± SE</th>
<th>P Value</th>
</tr>
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<tbody>
<tr>
<td>C2/SC</td>
<td>9.40±1.94</td>
<td>3.41±0.29</td>
<td>0.02</td>
<td>11.09±3.60</td>
<td>3.12±0.46</td>
<td>0.04</td>
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Values are means ± SE. Muscle contraction increased the ratio of short- (C4, C6, C8) to long-chain acylcarnitines, implying accelerated β-oxidative flux in gastrocnemius of lean and obese Zucker rats. In addition, contraction lowered the acetyl-carnitine (C2) to short-chain acylcarnitine (SC) ratio, suggesting rapid entry of acetyl-CoA (the end product of β-oxidation) into the TCA cycle. P values compare within-group treatment effect (basal vs. contraction).
high-fat diet corresponded with increased muscle TAG and no change in LCACoA levels (1). Taken together, the foregoing findings could imply that intramuscular LCACoA do not play a direct role in mediating insulin resistance, or alternatively, that changes in total LCACoA do not always reflect levels in the specific cellular compartment that influences insulin signaling. Thus, there may be distinct LCACoA pools stored in the cytosol or bound to the sarclemma that provide ligands for the insulin inhibitory serine kinases, whereas other LCACoA pools, such as those liberated by contraction, might be channeled directly to the mitochondria to provide oxidative substrate. This paradigm could explain why a twofold increase in LCACoA levels had no effect on insulin signaling in muscle of lean animals. Although several reports have shown a strong positive correlation between LCACoA levels and insulin resistance (10, 13), emerging evidence from human cross sectional and exercise training studies indicates that muscle oxidative capacity (as measured by β-hydroxacycl CoA dehydrogenase and citrate synthase activity), systemic fatty acid oxidative capacity and whole body aerobic capacity are strong predictors of insulin sensitivity, and in some cases better indicators than intramuscular lipid levels (3, 14, 17, 38). These results again implicate muscle mitochondrial function as a major determinant of insulin action.

In the present study, we found that contraction restored insulin-stimulated glucose uptake in obese rats without rescuing the activation of IR, IRS-1, or Akt (Fig. 3). Thus the elevated lipids found in the obese state may be imposing a persistent impingement on the insulin signaling cascade. Notably, contraction permitted a marked improvement in insulin-mediated phosphorylation of the downstream signaling molecule, AS160. Recent work has shown that AS160 (RabGAP protein) is a direct substrate of Akt. Akt phosphorylates and inhibits AS160 GAP, allowing Rab to be in an active GTP form, a necessary step for Glut4 translocation and subsequent glucose transport (31, 51). Contraction and insulin induced AS160 phosphorylation independently, and the combined treatments acted synergistically (5). Prior contraction of obese muscles enabled insulin to phosphorylate AS160 to levels found in the lean, insulin-stimulated (minus contraction) condition. It is yet unclear if this partial rescue of AS160 accounted for full recovery of glucose uptake. Perhaps the combination of contraction and insulin potentiated AS160 phosphorylation to a threshold level that in turn permitted maximal Glut4 translocation and glucose transport to occur in the obese state. Although questions still remain, AS160 appears to function at the crossroads of insulin- and contraction-induced glucose uptake, and may play a key role in mediating the insulin-sensitizing actions of acute exercise.

Finally, we also explored alternative signaling pathways that are thought to contribute to contraction-induced changes in glucose uptake, including AMPK and MAPK (P38 and ERK). AMPK is a critical energy sensing enzyme that is activated by muscle contraction and may play a role in contraction activation of glucose transport (26). In the present study, AMPK phosphorylation was increased by contraction in both the lean and obese animals, but it was not activated by insulin. In addition to promoting glucose uptake, AMPK activation increases fatty acid oxidation by inactivating acetyl CoA carboxylase and thus synthesis of its product, malonyl CoA, which is a potent inhibitor of β-oxidation (18). Therefore, AMPK activation is an integral signal for enhanced mitochondrial oxidation in muscle during exercise and was likely a key component of the results shown here. The parallel MAPK pathways of ERK and P38 signaling are known to be activated by both contraction and insulin (49). In particular, P38 activation is thought to participate in both the contraction and insulin stimulation of glucose transport in skeletal muscle (42, 45). We were most interested in determining whether the combined treatment (Con + Ins) might confer a synergistic effect on MAPK signaling. Only the lean animals had an increase in ERK phosphorylation from Ins to Con + Ins, whereas there was no effect on P38 phosphorylation in either group. Thus, consistent with an earlier report (15), contraction induced improvements in insulin action do not appear to be mediated through the MAPK pathway.

To gain additional insight into contraction-induced changes in fuel metabolism we analyzed muscle acylcarnitines. The enzymes that produce these metabolites are localized primarily
in the mitochondrial compartment and are absent from the cytosol (40). As such, relative changes in the acylcarnitine profile are largely reflective of adjustments and/or perturbations in mitochondrial energy metabolism. Most of the even chain species are produced as byproducts of incomplete fatty acid catabolism, which occurs when rates of \( \beta \)-oxidation exceed TCA cycle activity, and/or when complete oxidation is limited by low activity of a specific catabolic enzyme. Accordingly, these profiles are widely used as a diagnostic tool to detect inborn errors in metabolism (6), including deficiencies in the carnitine palmitoyltransferase (CPT1) system (48), medium-chain acyl-CoA dehydrogenase (33) and long-chain acyl-CoA dehydrogenase (46). CPT1 deficiency or inhibition results in decreased levels of most even chain acylcarnitines, including acetyl-carnitine. In general, genetic defects involving one of the early steps in \( \beta \)-oxidation are characterized by accumulation of long-chain acylcarnitines and decreased levels of medium- and short-chain species. In comparison, inactivation of the medium- or short-chain acyl-CoA dehydrogenases leads to elevated levels of acylcarnitine species coinciding with the acyl-CoA substrate of the affected enzyme, with accompanying reductions in the downstream product(s). The flux ratios presented in Table 2 quantify relative changes in one metabolite compared with another that is produced by an upstream or downstream step in the same pathway. This calculation is commonly used as a crude surrogate for more precise flux analyses performed with stable isotope tracers (33, 48). For instance, in myocytes from patients with CPTII deficiency, palmitoyl-carnitine increases 7-fold compared with controls, whereas C2 and C12 levels decrease markedly because the palmitoyl-carnitine increases 7-fold compared with controls, instance, in myocytes from patients with CPTII deficiency, analyses performed with stable isotope tracers (33, 48). For example, several studies (27, 36, 37) have shown that exercise training enabled coordinated regulation of \( \beta \)-oxidative flux, TCA cycle activity, and total mitochondrial oxidation, which coincided with improved glucose tolerance despite continued feeding of the high fat regimen (29). Further analysis of oxidative capacity in isolated mitochondria showed that exercise training enhances mitochondrial capacity to completely oxidize a heavy load of fatty acid. Thus, improved insulin action appeared to be closely related to exercise-induced adaptations in mitochondrial performance. In previous reports, we observed a strong association between muscle acylcarnitine accumulation and insulin resistance (1, 29), raising the possibility that one or more of these metabolites might function as novel diabetogenic mediators. However, in the present study, contraction restored insulin action while also increasing acylcarnitine levels. These results imply that the acylcarnitines per se do not directly interfere with insulin-stimulated glucose uptake, but may instead reflect changes in mitochondrial energy flux that bear on insulin desensitization. More rigorous testing of this hypothesis is now warranted.

Previous studies have likewise described a potential link between enhanced \( \beta \)-oxidative flux and protection of insulin action. A recent report evaluated the impact of accelerated \( \beta \)-oxidative flux on glucose metabolism in L6 myocytes that overexpressed CPT1, an enzyme regarded as the rate limiting step in mitochondrial fatty acid oxidation (35). This genetic manipulation resulted in higher rates of insulin-stimulated glucose transport and glycogen synthesis, and partial protection against palmitate-induced insulin resistance. Interestingly, the CPT1-induced improvements in insulin action occurred despite increased levels of TAG, DAG, and LCACoA compared with the control cells. The authors speculated that enhanced \( \beta \)-oxidative flux plays a role in modifying and/or enhancing insulin action, regardless of cellular lipid levels.

Persistent changes in mitochondrial energy status may also contribute to the insulin sensitizing effects of chronic exercise. In a recent study, mice fed a high-fat diet gained weight and became glucose intolerant in association with high rates of incomplete \( \beta \)-oxidation and intramuscular accumulation of most fatty acylcarnitine species. The acylcarnitine profile of the diet-induced obese mice suggested that increased \( \beta \)-oxidative flux, resulting from lipid oversupply, exceeded the capacity of the TCA cycle to accommodate incoming short chain acyl-CoAs. Interestingly, exercise training enabled coordinated regulation of \( \beta \)-oxidative flux, TCA cycle activity, and total mitochondrial oxidation, which coincided with improved glucose tolerance despite continued feeding of the high fat regimen (29). Further analysis of oxidative capacity in isolated mitochondria showed that exercise training enhances mitochondrial capacity to completely oxidize a heavy load of fatty acid. Thus, improved insulin action appeared to be closely related to exercise-induced adaptations in mitochondrial performance. In previous reports, we observed a strong association between muscle acylcarnitine accumulation and insulin resistance (1, 29), raising the possibility that one or more of these metabolites might function as novel diabetogenic mediators. However, in the present study, contraction restored insulin action while also increasing acylcarnitine levels. These results imply that the acylcarnitines per se do not directly interfere with insulin-stimulated glucose uptake, but may instead reflect changes in mitochondrial energy flux that bear on insulin desensitization. More rigorous testing of this hypothesis is now warranted.

The apparent association between mitochondrial events and insulin action could explain the paradoxical observation that exercise trained humans have elevated intramuscular lipids while remaining highly insulin sensitive (16). Our proposed model also fits with previous work linking the etiology of obesity and diabetes to mitochondrial dysfunction (27, 36, 43). For example, several studies (27, 36, 37) have shown that humans with peripheral insulin resistance, type II diabetes, and
obesity exhibit reduced mitochondrial oxidative capacity in skeletal muscle. Notably, insulin resistance in obese Zucker rats has also been associated with impaired mitochondrial function (2). The results presented herein suggest that these obesity-related mitochondrial lesions might be corrected by acute exercise.

In summary, the present study highlights the powerful impact of acute exercise on insulin action, particularly in the context of obesity. We tested the hypothesis that contraction might restore lipid homeostasis and alleviate LCACoAs/DAG-mediated opposition of insulin signaling in muscles of obese Zucker rats. Our findings deviated from this paradigm as increased insulin-stimulated glucose uptake occurred despite elevated intramuscular LCACoAs and depressed insulin signaling. Instead, the data prompt speculation that insulin action may be modulated by changes in mitochondrial energy flux. These results reveal new insight into the mechanism(s) by which acute exercise promotes insulin sensitivity, and are certain to encourage future studies to determine the precise metabolic signals that link accelerated mitochondrial oxidation to enhanced insulin action. Given the current worldwide epidemic of obesity and diabetes, our findings place even greater emphasis on the importance of routine physical activity as a means to combat peripheral insulin resistance.

ACKNOWLEDGMENTS

The authors thank Erin Coburn and Robert Fish for their help with data collection.

GRANTS

This work was funded by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-46121 (to G. L. Dohm) and DK-56112, and the American Diabetes Association (to D. M. Muoiio).

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