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Postexercise Improvement in Insulin-Stimulated Glucose Uptake Occurs Concomitant With Greater AS160 Phosphorylation in Muscle From Normal and Insulin-Resistant Rats

METABOLISM

Earlier research on rats with normal insulin sensitivity demonstrated that acute exercise increased insulin-stimulated glucose uptake (GU) concomitant with greater phosphorylation of Akt substrate of 160 kDa (pAS160). Because mechanisms for exercise effects on GU in insulin-resistant muscle are unknown, our primary objective was to assess insulin-stimulated GU, proximal insulin signaling (insulin receptor [IR] tyrosine phosphorylation, IR substrate 1–phosphatidylinositol-3-kinase, and Akt phosphorylation and activity), and pAS160 in muscles from acutely exercised (one session) and sedentary rats fed either chow (low-fat diet [LFD]; normal insulin sensitivity) or a high-fat diet (HFD; for 2 weeks, insulin-resistant). At 3 h postexercise (3hPEX), isolated epitrochlearis muscles were used for insulin-stimulated GU and insulin signaling measurements. Although exercise did not enhance proximal signaling in either group, insulin-stimulated GU at 3hPEX exceeded respective sedentary control subjects (Sedentary) in both diet groups. Furthermore, insulin-stimulated GU for LFD-3hPEX was greater than HFD-3hPEX values. For HFD-3hPEX muscles, pAS160 exceeded HFD-Sedentary, but in muscle from LFD-3hPEX rats, pAS160 was greater still than HFD-3hPEX values. These results implicated pAS160 as a potential determinant of the exercise-induced elevation in insulin-stimulated GU for each diet group and also revealed pAS160 as a possible mediator of greater postexercise GU of insulin-stimulated muscles from the insulin-sensitive versus insulin-resistant group.

Increased insulin-stimulated glucose uptake (GU) in muscle after a single exercise session (acute exercise) is well-documented for rodents (1–6) and humans (7–10) with normal insulin sensitivity. Although the mechanisms remain incompletely understood, improved insulin sensitivity postexercise by healthy rodents and humans is not attributable to greater insulin signaling at proximal steps from insulin receptor (IR) binding (11) to Akt activation (1,5,9,12,13). These data suggest exercise's effect on insulin sensitivity may occur downstream of Akt.

The most distal insulin-regulated Akt substrate clearly linked to glucose transport (14–18), Akt substrate of 160 kDa (AS160; also known as TBC1D4), has emerged as an attractive candidate for regulating the postexercise increase

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in insulin sensitivity (1,4,5,19). Supporting this idea, several hours after acute exercise, muscle AS160 phosphorylation (pAS160) exceeds the values of unexercised control subjects in rats and humans (1,4,5,8,20). Moreover, greater pAS160 tracks with the postexercise increase in insulin-stimulated glucose transport in muscles from normal rats. Although acute exercise can improve insulin-mediated glucose disposal in insulin-resistant rats (21–26) and humans (27–29), surprisingly little is known about the mechanisms for this improvement. Studying exercise effects on individuals with normal insulin sensitivity is interesting, but a more pressing need is to learn about correcting insulin resistance, an essential defect in type 2 diabetes.

We evaluated mechanisms for improved insulin sensitivity in both normal and insulin-resistant conditions by studying the insulin-stimulated GU in muscles from rats eating standard rodent chow (low-fat diet [LFD]) or a high-fat diet (HFD). Because HFD (2 to 3 weeks) rapidly produces muscle insulin resistance (30–33), research on brief HFDs offers unique insights into the primary mechanisms for this defect. Turner et al. (33) demonstrated that muscle insulin resistance in HFD-fed mice can precede outcomes often assumed to cause insulin resistance. To focus on the primary mechanisms responsible for brief HFD-induced insulin resistance, we studied rats consuming an HFD for 2 weeks. To address physiologically relevant outcomes, GU was measured with a submaximally effective insulin dose in the range of plasma values for fed rats. To identify potential mechanisms for exercise-induced improvement in insulin sensitivity, muscles were assessed for proximal insulin-signaling steps, pAS160, and putative mediators of insulin resistance at 3 h postexercise (3hPEX). To probe possible diet-related differences in triggers for the increase in insulin sensitivity observed several hours postexercise, key metabolic and signaling outcomes were evaluated immediately postexercise.

RESEARCH DESIGN AND METHODS

Materials
Materials for SDS-PAGE and immunoblotting were from Bio-Rad (Hercules, CA). Anti-AS160, anti–glucose transporter type 4 (GLUT4), anti–IR substrate-1 (IRS-1), anti–phosphatidylinositol-3-kinase (PI3K), Akt1/protein kinase Bα Immunoprecipitation-Kinase Assay Kit, anti-Akt/pleckstrin homology domain clone SKB1 binding protein 1, Akt substrate peptide, protein G agarose beads, MILLIPLEX$_{MAP}$ Cell Signaling Buffer and Detection Kit, MILLIPLEX$_{MAP}$ Akt/mTOR Phosphoprotein Panel [including: phospho-(p)Akt^{Ser473}; IR, pIR$^{Tyr1162/1163}$; and pIRS-1^{Ser307}], MILLIPLEX$_{MAP}$ Phospho JNK/stress-activated protein kinase$^{Thr183/Tyr185}$, and Luminata Forte Western Horseradish Peroxidase Substrate were from Millipore (Billerica, MA). Anti-pAkt^{Thr308}, anti-Akt, and anti–Jun NH$_2$-terminal kinase (JNK) were from Cell Signaling Technology (Danvers, MA). pAS160^{Thr642} was from Symansis Ltd. (Auckland, New Zealand). Anti–IR-β was from Santa Cruz Biotechnology. Radioactive 2-deoxyglucose (2-DG) and mannitol were from PerkinElmer (Waltham, MA). Bicinchoninic acid protein assay and Pierce MemCode Reversible Protein Stain Kit were purchased from Thermo Fisher (Pittsburgh, PA). Insulin ELISA was from ALPCO Diagnostics (Salem, NH).

Animal Treatment
Animal care procedures were approved by the University of Michigan Committee on Use and Care of Animals. Male Wistar rats (initial body weight ~200–250 g; Harlan, Indianapolis, IN) were individually housed and provided standard rodent chow (LFD: 14% kcal fat, 58% kcal carbohydrate, and 28% kcal protein; Laboratory Diet no. 5001; PMI Nutrition International, Brentwood, MO) or HFD (60% kcal fat, 20% kcal carbohydrate, and 20% kcal protein; D12492; Research Diets, New Brunswick, NJ) and water ad libitum for 2 weeks. Rats were fasted at ~1900 on the night before the terminal experiment.

Beginning at ~0700, exercised rats swam in a barrel filled with water (35°C; ~45 cm depth; six rats per barrel, three rats per diet group) for four 30-min bouts with a 5-min rest between bouts (4). Blood was sampled from the tail vein (immediately postexercise [IPEX] and sedentary time-matched control subjects [Sedentary]) to determine plasma insulin. Some rats (IPEX and Sedentary) were then anesthetized (intraperitoneal sodium pentobarbital, 50 mg/kg weight), and epitrochlearis muscles were isolated. One muscle was used for insulin-independent GU. The contralateral muscle was frozen for glycogen, p–AMP-activated protein kinase (AMPK), and pAS160. Other exercising rats were dried and returned to their cages without food for 3 h, then anesthetized, and their epitrochlearis muscles were dissected out to measure insulin-stimulated GU. Sedentary rats from each diet group were anesthetized at times coinciding with IPEX and 3hPEX groups.

Muscle Incubations
Isolated epitrochlearis muscles were placed in vials for two incubation steps with continuous shaking and gassing (95% O$_2$/5% CO$_2$) in a heated (35°C) water bath. Muscles from the IPEX experiment were incubated for insulin-independent GU as follows: step 1 (10 min) in a vial containing 2 mL of media 1 (Krebs-Henseleit buffer with 0.1% BSA, 2 mmol/L sodium pyruvate, and 6 mmol/L mannitol); and step 2 (20 min) in a vial containing 2 mL of media 2 (Krebs-Henseleit buffer with 0.1% BSA, 1 mmol/L 2-DG [2.25 mCi/mmol ^{3}H–2-DG], and 9 mmol/L mannitol [0.022 mCi/mmol ^{14}C-mannitol]). Insulin-independent (no insulin) and insulin-dependent (100 μU/mL) GUs were measured in paired muscles from other rats at 3hPEX and Sedentary. Incubations for the 3hPEX experiment were: step 1, contralateral muscles were placed in vials (30 min) containing 2 mL of media 1 without insulin or 100 μU/mL insulin; and step 2, muscles were transferred to a vial (20 min) containing 2 mL of media 2 and the same insulin concentration as step 1. Muscles were blotted, freeze-clamped, and stored (−80°C).

Muscle Homogenization

Frozen muscles used for GU and immunoblotting were weighed and homogenized (TissueLyser II homogenizer; Qiagen Inc., Valencia, CA) in ice-cold lysis-buffer (34). After protein concentration was determined using an aliquot of supernatant, the remaining supernatant was stored ($-80°C$) until analyzed.

GU

Aliquots of supernatants were added to vials containing scintillation cocktail, ^3H and ^{14}C disintegrations per minute were measured by a scintillation counter, and 2-DG uptake was calculated (35).

Immunoblotting

Total protein concentrations for muscle lysates were determined by bicinchoninic acid assay, and equal amounts of protein for each sample were boiled (5–10 min) in SDS loading buffer, separated via SDS-PAGE, and transferred to nitrocellulose. The MemCode protein stain was used to confirm equal loading (36). Membranes were incubated with appropriate primary and secondary antibodies, and enhanced chemiluminescence of protein bands was quantified by densitometry (Alpha Innotech, San Leandro, CA) (34). Individual values were normalized to the mean value for all samples on the membrane.

IRS-1–PI3K Association

For PI3K associated with IRS-1, sample protein was combined with anti–IRS-1 and rotated overnight (4°C). The following morning, protein G agarose beads were washed (three times) with lysis buffer, resuspended in lysis buffer, and a slurry mix of protein G-agarose beads was added to the sample/antibody mix and rotated 2 h (4°C). Protein G-agarose beads were isolated (centrifugation, $4,000 \times g$; 4°C for 1 min) and washed (three times) in lysis buffer. Antigens were eluted from beads with $2\times$ SDS loading buffer and boiled before SDS-PAGE and immunoblotting with anti-PI3K.

Akt Activity Measurement

Akt activity was determined by the manufacturer's protocol (5). Sample values were normalized to the mean value for all samples per assay cohort (including all groups).

Multiplex Analysis

Bead-based multiplex analysis (MILLIPLEX$_{MAP}$ assays with the Luminex L200 instrument and xPONENT software; Luminex, Austin, TX) were performed for pAkt[Ser473], pIR[Tyr1162/1163], pIRS-1[Ser307], and pJNK/stress-activated protein kinase[Thr183/Tyr185].

Muscle Lipid Analysis

The University of Michigan Molecular Phenotyping Core performed lipid extraction and diacylglycerol (DAG) and ceramide analysis. DAG was isolated via thin-layer chromatography and analyzed by gas chromatography using a flame-ionization detector (37). Ceramides were extracted and analyzed via high-performance liquid chromatography and tandem mass spectrometry and quantified by electronspray ionization–magnetic resonance microscopy–mass spectrometry on a tandem quadrupole mass spectrometer (38).

Muscle Glycogen

Muscles were weighed and then homogenized (0.3 mol/L ice-cold perchloric acid) prior to glycogen determination (39).

Statistics

Two-way ANOVA was used to compare means among more than two groups, and Tukey post hoc analysis was used to identify the source of significant variance using SigmaPlot version 11.0 (Jandel Corporation, San Rafael, CA). Two-tailed t test analysis was used for comparing means from two groups. Data were expressed as means $\pm$ SEM. P values <0.05 were considered statistically significant.

RESULTS

Body Mass, Epididymal Mass, Epididymal/Body Mass Ratio, and Estimated Caloric Intake

After a 2-week diet intervention, HFD versus LFD values were greater ($P < 0.01$) for body mass (321 ± 5 vs. 291 ± 4 g), epididymal fat mass (2.70 ± 0.11 vs. 1.71 ± 0.06 g), epididymal/body mass ratio (8.36 ± 0.26 vs. 5.84 ± 0.14 mg/g), and estimated caloric intake (93.43 ± 2.00 vs. 85.00 ± 1.70 kcal/day).

IPEX: Plasma Insulin, Muscle 2-DG Uptake, and Glycogen

For plasma insulin (Fig. 1A), there were significant main effects of diet (HFD > LFD; $P < 0.001$) and exercise (Sedentary > IPEX; $P < 0.001$). Post hoc analysis indicated greater ($P < 0.05$) insulin for Sedentary versus IPEX within each diet group and greater ($P < 0.005$) insulin for HFD versus LFD within either Sedentary or IPEX conditions.
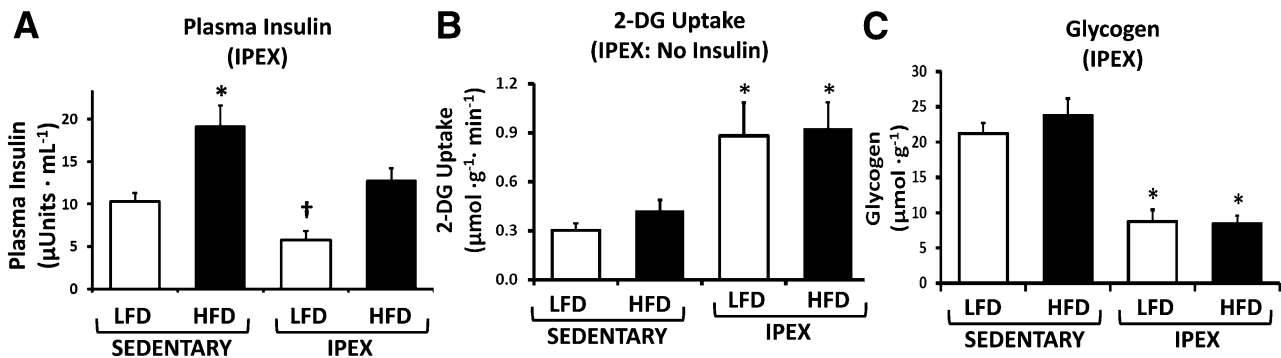
For insulin-independent 2-DG uptake by muscles (Fig. 1B), there was a significant main effect of exercise (IPEX > Sedentary; $P < 0.01$). Post hoc analysis indicated greater ($P < 0.05$) insulin-independent 2-DG uptake from muscles of IPEX versus Sedentary within each diet group. Insulin-independent 2-DG uptake did not differ between diet groups for either Sedentary or IPEX.

For muscle glycogen (Fig. 1C), there was a significant main effect of exercise (Sedentary > IPEX; $P < 0.01$). Post hoc analysis indicated lower ($P < 0.05$) glycogen for IPEX versus Sedentary within each diet group. Glycogen did not differ between diet groups for either Sedentary or IPEX.

IPEX: Muscle Immunoblotting

There were no significant effects of diet or exercise on total abundance of AMPK or AS160 at IPEX (Fig. 2A).

For pAMPK (Fig. 2B), there was a significant main effect of exercise (IPEX > Sedentary; $P < 0.05$). Post hoc analysis indicated greater ($P < 0.05$) pAMPK for IPEX versus Sedentary within each diet group. No diet-related differences in pAMPK were found for Sedentary or IPEX.

Figure 1—A: Plasma insulin values. *$P < 0.05$, HFD-Sedentary greater vs. all other groups; †$P < 0.05$, LFD-IPEX less vs. all other groups. B: 2-DG uptake (without insulin). *$P < 0.05$, IPEX vs. Sedentary within the same diet group. C: Glycogen. *$P < 0.05$, IPEX vs. Sedentary within the same diet group. Data were analyzed by two-way ANOVA, and Tukey post hoc analysis was performed to identify the source of significant variance. Values are means ± SEM; n = 4–17/group.

For pAS160[Thr642] (Fig. 2C), there was a significant main effect of exercise (IPEX > Sedentary; $P < 0.05$). Post hoc analysis indicated greater ($P < 0.05$) pAS160 for IPEX versus Sedentary within each diet group. There was no significant diet effect on pAS160[Thr642] in Sedentary or IPEX. For pAS160[Ser588] (Fig. 2D), there was a significant main effect of exercise (IPEX > Sedentary; $P < 0.05$), but post hoc analysis revealed no further significant differences.

3hPEX: Muscle 2-DG Uptake
For 2-DG uptake in muscles incubated without insulin (Fig. 3A), there was a significant main effect of exercise (3hPEX > Sedentary; $P < 0.05$), and post hoc analysis indicated that insulin-independent 2-DG values were greater ($P < 0.05$) for muscles from 3hPEX versus Sedentary in the LFD group.

For 2-DG uptake in muscles incubated with insulin (Fig. 3A), there were significant ($P < 0.01$) main effects of both diet (LFD > HFD) and exercise (3hPEX > Sedentary). Post hoc analysis revealed that 2-DG uptake with insulin was greater ($P < 0.05$) for muscles from LFD-3hPEX versus all other groups. In the HFD groups, post hoc analysis indicated 2-DG uptake with insulin was greater ($P < 0.05$) for muscles from 3hPEX versus Sedentary. For delta-insulin 2-DG uptake (2-DG uptake with insulin − no insulin = delta-insulin; Fig. 3B), there were significant ($P < 0.01$) main effects of diet (LFD > HFD) and exercise (Sedentary < 3hPEX). Post hoc analysis demonstrated that delta-insulin 2-DG uptake for LFD-3hPEX exceeded ($P < 0.05$) all other groups. Post hoc analysis indicated that in Sedentary groups, delta-insulin 2-DG uptake was greater ($P < 0.05$) for LFD versus HFD rats, and delta-insulin 2-DG uptake for HFD-3hPEX exceeded HFD-Sedentary ($P < 0.05$). However, delta-insulin 2-DG uptake did not significantly differ for HFD-3hPEX versus LFD-Sedentary.
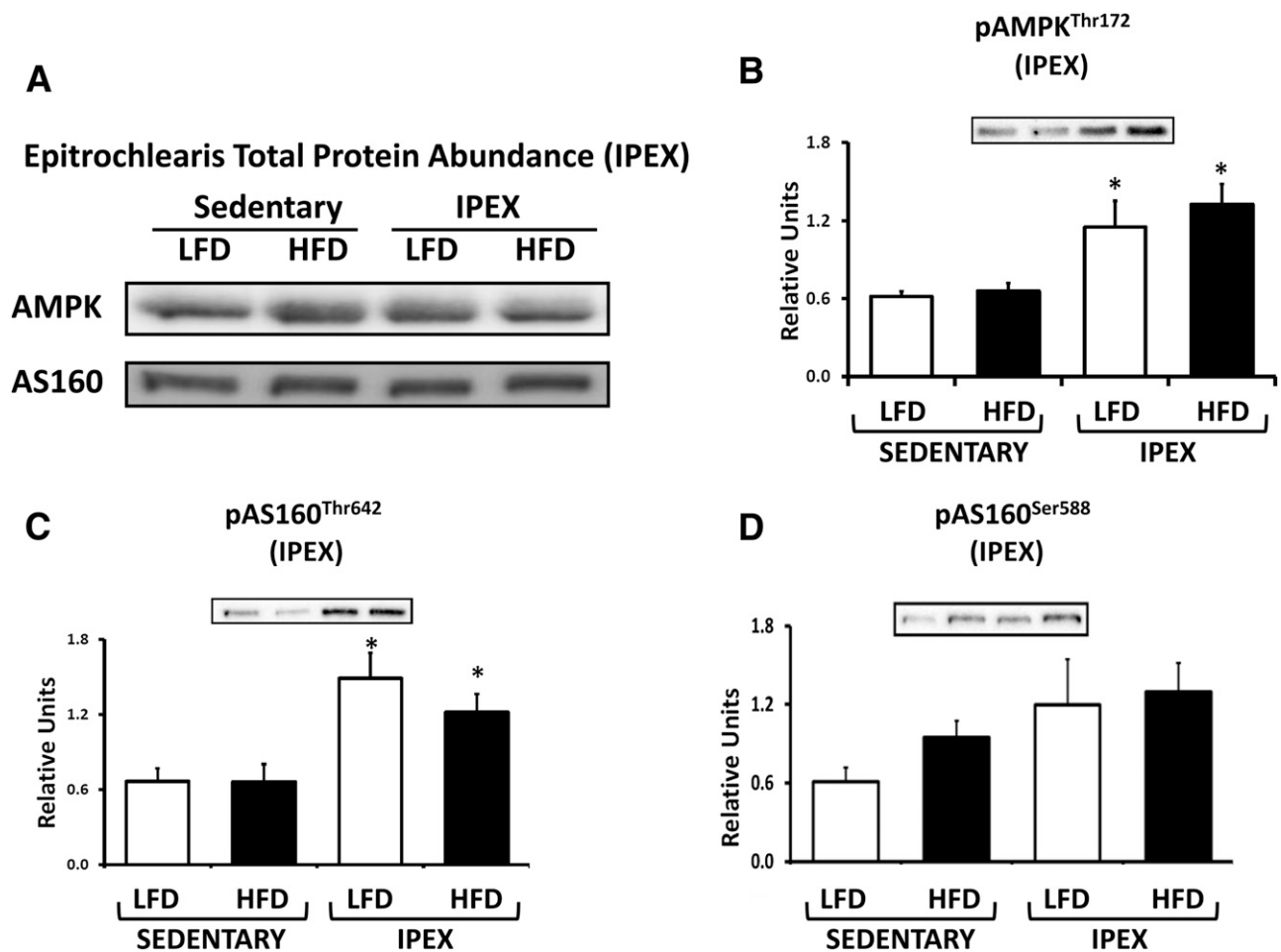
3hPEX: Muscle Immunoblotting
Neither diet nor exercise significantly altered the total protein abundance of IR, IRS-1, Akt, AS160, or GLUT4 (Fig. 4).

Neither diet nor exercise significantly altered phosphorylation of the IR with or without insulin (pIR[Tyr1162/1163]; Fig. 5A) or delta-insulin pIR[Tyr1162/1163] (Fig. 5B). There were also no significant diet or exercise effects on IRS-1–PI3K association with or without insulin (Fig. 5C) or delta-insulin IRS-1–PI3K (Fig. 5D).

Neither diet nor exercise significantly altered pAkt[Thr308] for muscles with or without insulin (Fig. 6A). For delta-insulin pAkt[Thr308] (Fig. 6B), there was a significant main effect of diet (LFD > HFD; $P < 0.05$), but no further significance was revealed by post hoc analysis. There were no significant effects of either diet or exercise on pAkt[Ser473] with or without insulin (Fig. 6C) or delta-insulin pAkt[Ser473] (Fig. 6D). Akt activity was not significantly different regardless of diet or exercise with or without insulin or for delta-insulin (Fig. 6E and F).

For pAS160[Thr642] (Fig. 7A), there was a significant main effect of exercise in muscles incubated without insulin (3hPEX > Sedentary; $P < 0.05$); post hoc analysis indicated that for LFD rats, 3hPEX exceeded Sedentary values ($P < 0.05$). For pAS160[Thr642] in muscles with insulin, there was a significant main effect of diet (LFD > HFD; $P < 0.01$); post hoc analysis revealed that for 3hPEX rats, LFD exceeded HFD values. For delta-insulin pAS160[Thr642] (Fig. 7B), there was a significant main effect of diet (LFD > HFD; $P < 0.05$), and post hoc analysis indicated that for 3hPEX groups, LFD values exceeded HFD values ($P < 0.05$).

For pAS160[Ser588] (Fig. 7C) in muscles incubated without insulin, there was a significant main effect of diet (LFD > HFD; $P < 0.05$), and post hoc analysis indicated that within Sedentary groups, the LFD values exceeded HFD values ($P < 0.05$). For pAS160[Ser588] muscles incubated with insulin, there were significant main effects of both diet (LFD > HFD; $P < 0.01$) and exercise (3hPEX > Sedentary; $P < 0.01$). Post hoc analysis of muscles incubated with insulin indicated that pAS160[Ser588] for the LFD-3hPEX group exceeded all other groups ($P < 0.05$), pAS160[Ser588] for the HFD-Sedentary group was lower

A

Epitrochlearis Total Protein Abundance (IPEX)

B

pAMPK^{Thr172}
(IPEX)

C

pAS160^{Thr642}
(IPEX)

D

pAS160^{Ser588}
(IPEX)

Figure 2—*A*: Total protein abundance for AMPK and AS160 in epitrochlearis muscles of IPEX and time-matched Sedentary rats. There were no significant differences for total abundance of either protein. *B*: AMPK^{Thr172} phosphorylation. *C*: AS160^{Thr642} phosphorylation. *D*: AS160^{Ser588} phosphorylation in epitrochlearis muscles of IPEX and Sedentary rats. Data were analyzed by two-way ANOVA, and Tukey post hoc analysis was performed to identify the source of significant variance. Values are means $\pm$ SEM; n = 4–6 per group. *P < 0.05, IPEX vs. Sedentary within the same diet group.

than all other groups (P < 0.05), and pAS160^{Ser588} from LFD-Sedentary and HFD-3hPEX were not significantly different. For delta-insulin pAS160^{Ser588} (Fig. 7*D*), there were significant main effects of both diet (LFD > HFD; P < 0.01) and exercise (3hPEX > Sedentary; P < 0.01). Post hoc analysis indicated that values from the LFD 3hPEX group exceeded all other groups (P < 0.05).

For pIRS-1^{Ser1101} (Supplementary Fig. 1), muscles incubated without insulin had a significant main effect of exercise (3hPEX > Sedentary; P < 0.01). Post hoc analysis revealed HFD-3hPEX exceeded HFD-Sedentary values (P < 0.05). For muscles incubated with insulin, there was a significant main effect of diet on pIRS-1^{Ser1101} (LFD > HFD; P < 0.05), but post hoc analysis revealed no further significant differences.

For p–IRS-1^{Ser307} (Supplementary Fig. 1), there were no significant differences in muscles incubated without insulin, but there was a significant main effect of exercise (3hPEX > Sedentary; P < 0.05) in muscles incubated with

insulin. Post hoc analysis revealed no further significant differences.

Neither diet nor exercise altered JNK abundance or pJNK regardless of insulin concentration (Supplementary Fig. 1).

Lipid Metabolites

A significant interaction between diet and exercise was found for muscle DAG 20:4 (Table 1); post hoc analysis indicated that for HFD rats, 3hPEX exceeded Sedentary (P < 0.05).

Neither diet nor exercise significantly altered the level of any ceramide species (Table 1).

DISCUSSION

A considerable amount of earlier research has evaluated the improved insulin sensitivity in normal individuals, but strikingly few studies have investigated potential mechanisms for greater insulin sensitivity after acute exercise
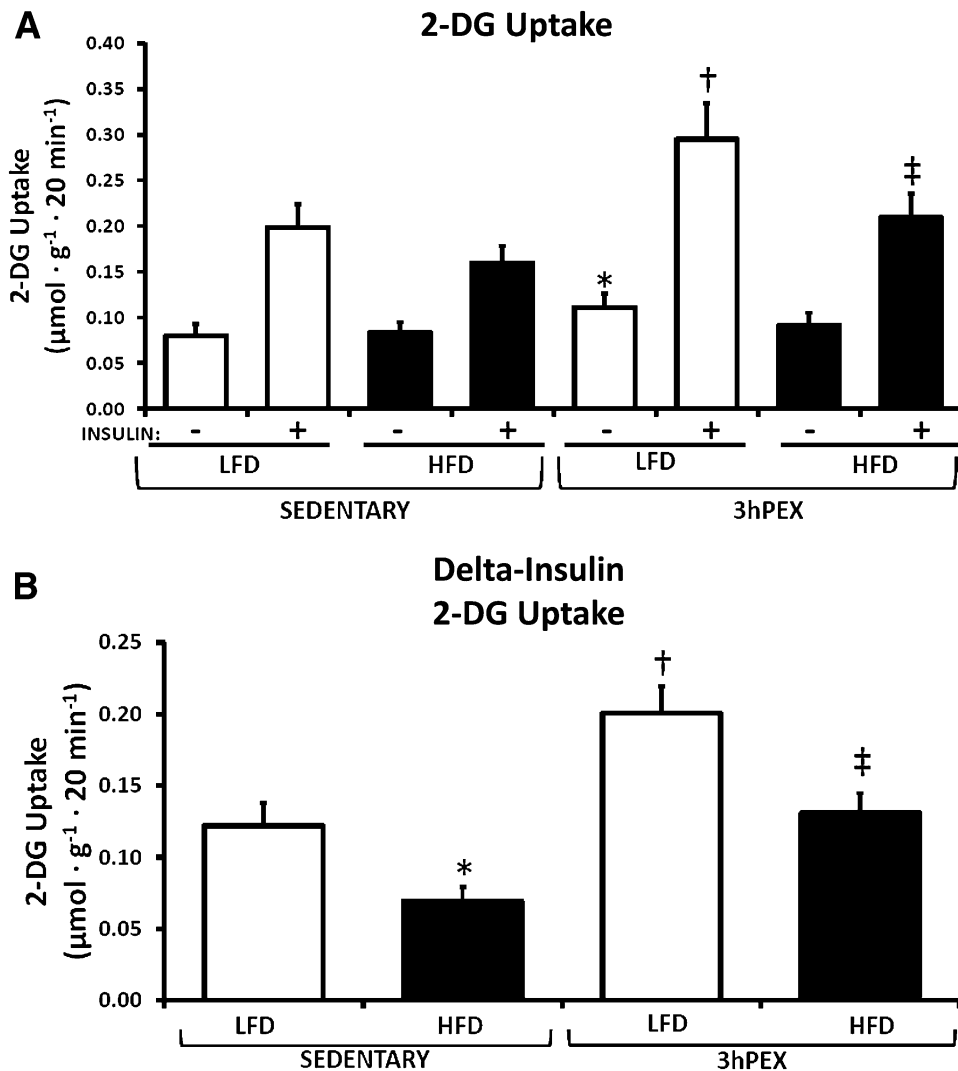
Figure 3—*A*: 2-DG uptake measured in paired epitrochlearis muscles without or with insulin 3hPEX. **P* < 0.05, LFD-Sedentary vs. LFD-3hPEX without insulin; †*P* < 0.05, LFD-3hPEX group with insulin vs. all other groups with insulin; ‡*P* < 0.05, GU with insulin of HFD-Sedentary vs. HFD-3hPEX rats. *B*: 2-DG uptake delta-insulin values (delta = value with insulin − value without insulin from paired muscles). **P* < 0.05, HFD-Sedentary vs. all other delta-insulin groups; †*P* < 0.05, LFD-3hPEX vs. all other delta-insulin groups; ‡*P* < 0.05, HFD-Sedentary vs. HFD-3hPEX. Data were analyzed by two-way ANOVA within each insulin level (minus or plus insulin) or for delta values, and Tukey post hoc analysis was performed to identify the source of significant variance. Values are means ± SEM; *n* = 17–22/group.

by directly comparing normal and insulin-resistant conditions. Therefore, the current study focused on the processes by which acute exercise improves insulin-stimulated GU in both insulin-sensitive and insulin-resistant rat skeletal muscles. The most important, novel results included: *1*) each of the key metabolic and signaling outcomes determined for muscles IPEX (insulin-independent GU, glycogen, pAMPK^{Thr172}, pAS160^{Thr642}, and pAS160^{Ser588}) was indistinguishable in LFD versus HFD rats; *2*) skeletal muscles from HFD-Sedentary versus LFD-Sedentary rats exhibited modest but significant insulin resistance for GU accompanied by modest but significant deficits in delta-insulin pAkt^{Thr308} and pAS160^{Ser588}; *3*) skeletal muscles from LFD-3hPEX rats versus LFD-Sedentary rats were characterized by substantially elevated

insulin-stimulated GU concomitant with significantly increased phosphorylation of AS160 (pAS160^{Thr642}, pAS160^{Ser588}, and delta-insulin pAS160^{Ser588}) in the absence of significant changes in proximal insulin signaling steps; *4*) muscles from HFD-3hPEX rats had insulin-stimulated GU values that exceeded the HFD-Sedentary group and were not different from LFD-Sedentary group, but were significantly less than the LFD-3hPEX group; *5*) the improvement in insulin-stimulated GU of muscles from HFD-3hPEX rats was accompanied by elimination of the diet-induced deficit in pAS160^{Ser588}, but HFD-3hPEX rats did not attain values as great as LFD-3hPEX rats for pAS160^{Ser588}, pAS160^{Thr642}, delta-insulin pAS160^{Ser588}, or delta-insulin pAS160^{Thr642}; and *6*) the improved insulin-stimulated GU by muscles from both LFD and HFD rats at 3hPEX was

Epitrochlearis Total Protein Abundance
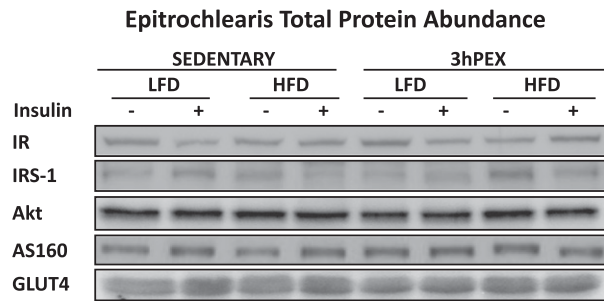
Figure 4—Total protein abundance for IR, IRS-1, Akt, AS160, and GLUT4. Data were analyzed by two-way ANOVA within each insulin level (minus or plus insulin). There were no significant differences for the total abundance of any of these proteins.

not accompanied by significant reductions in a number of the proposed mediators of muscle insulin resistance (DAG and ceramide species, pIRS-1[Ser307], pIRS-1[Ser1101], and pJNK[Thr183/Tyr185]). The results for both normal and insulin-resistant rats demonstrate that the level of muscle insulin-stimulated GU consistently tracks with the extent of postexercise effects on elevated pAS160.

Supporting previous findings for rats with normal insulin sensitivity (4,5,20), the improved insulin-mediated GU by muscles from LFD-3hPEX versus LFD-Sedentary rats occurred with increased pAS160[Thr642] and pAS160[Ser588] despite unaltered proximal insulin signaling (IR phosphorylation, IRS-1–PI3K association, and pAkt or Akt activity).

These results are also consistent with earlier research (9,10) demonstrating greater insulin-stimulated glucose disposal after acute exercise by humans without improved proximal insulin signaling (including pAkt). Treebak et al. (8) also found greater pAS160 in muscles from healthy humans after one exercise session. The current results demonstrate that acute exercise by healthy rats induces greater pAS160 on Ser[588] and Thr[642], the phosphomotifs most important for regulating insulin-stimulated glucose transport (17). Thus, enhanced pAS160 is a leading candidate for the mechanism accounting for this exercise benefit in healthy individuals.

Previous research indicated that chronic exercise (10–12 weeks) by obese (40) or older (41) humans caused greater insulin sensitivity and pAS160, but these studies did not assess AS160's role in acute exercise's benefits. Perseghin et al. (28) reported glucose disposal was elevated after acute or chronic (6 weeks) exercise versus unexercised values, but glucose disposal after chronic exercise was only modestly greater than achieved after acute exercise. Clearly, elucidating mechanisms for acute exercise's effects is essential for fully understanding exercise's health benefits.

The conventional approach to study HFD-induced insulin resistance, using long-term HFD that produces marked obesity, fails to reveal primary mechanisms for insulin resistance caused by brief HFD. In this context, Turner et al. (33) provided valuable new information by reporting 3 weeks of HFD by mice caused muscle insulin
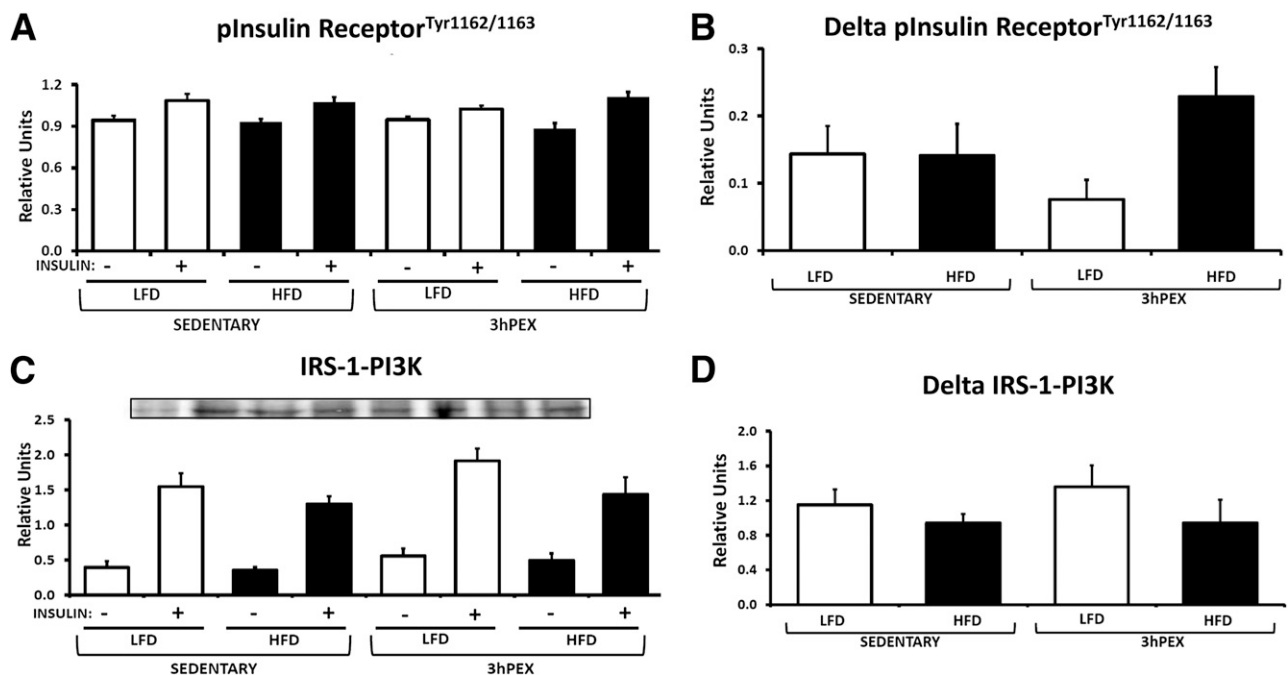
Figure 5—A: IR[Tyr1162/1163] phosphorylation measured by multiplex analysis (with this bead-based method, there is no representative blot to display) in paired epitrochlearis muscles at 3hPEX. B: Delta IR[Tyr1162/1163] phosphorylation (delta = value with insulin − value without insulin from paired muscles). C: IRS-1–PI3K association measured in paired epitrochlearis muscles at 3hPEX. D: Delta IRS-1–PI3K association. Data were analyzed by two-way ANOVA within each insulin level (minus or plus insulin) or for delta values. Values are means ± SEM; n = 8–12/group.
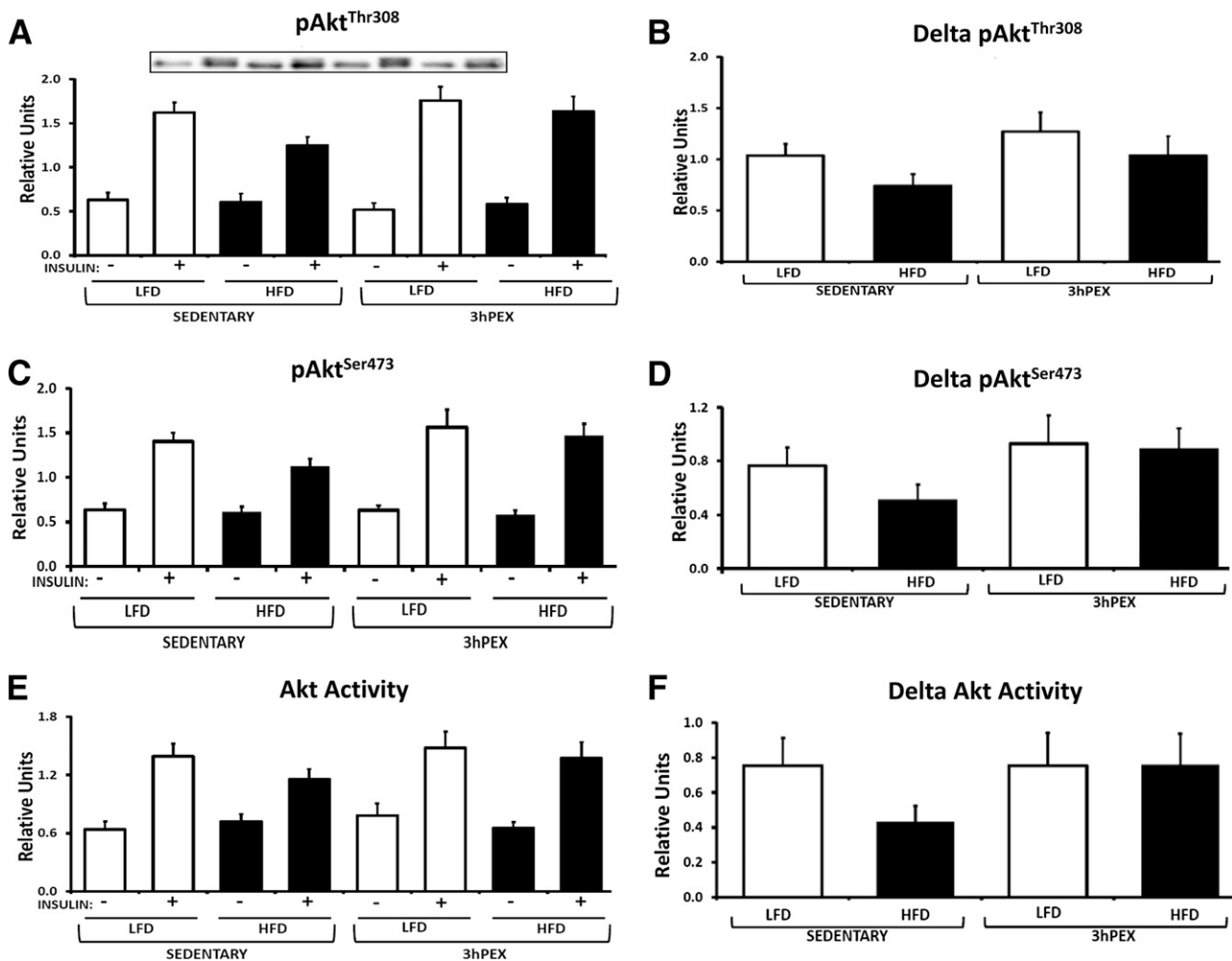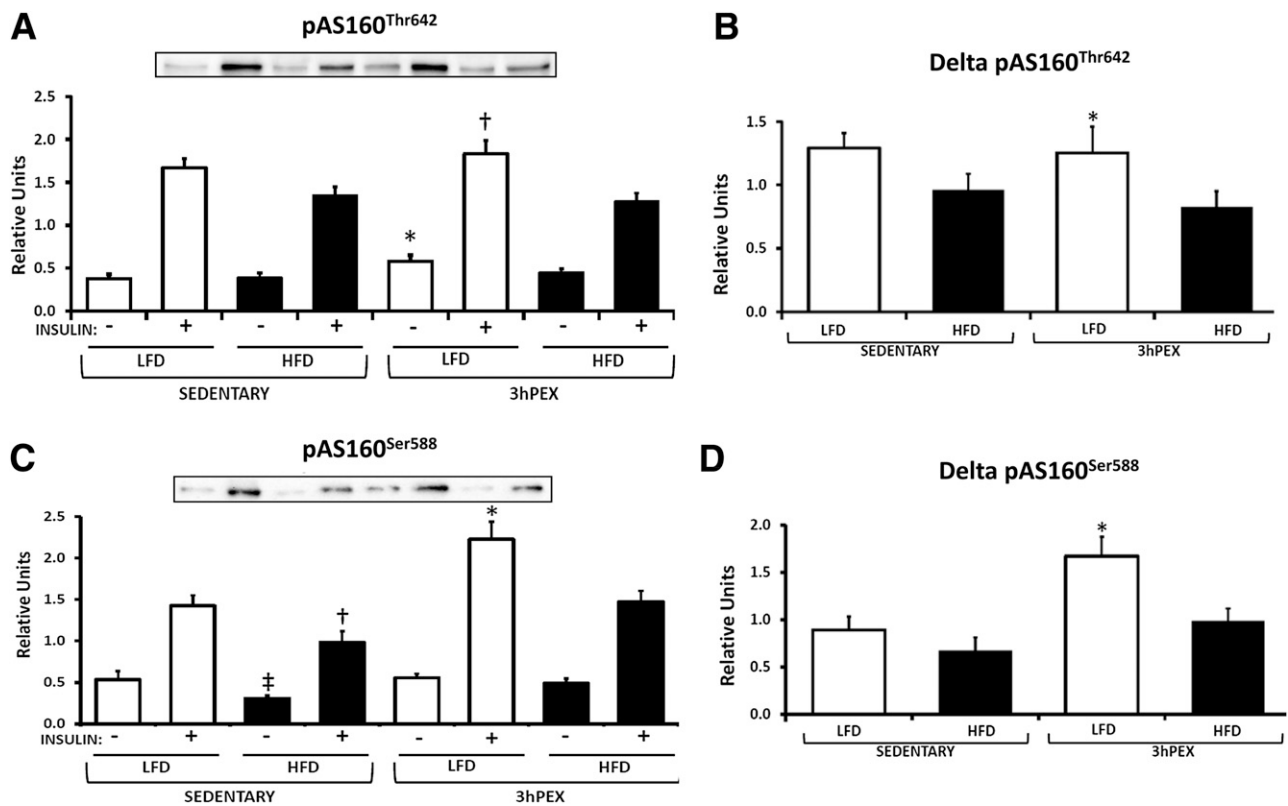
Figure 6—*A*: Akt[Thr308] phosphorylation measured in paired epitrochlearis muscles at 3hPEX. *B*: Delta Akt[Thr308] phosphorylation (delta = value with insulin − value without insulin from paired muscles). *C*: Akt[Ser473] phosphorylation measured by multiplex analysis (with this bead-based method, there is no representative blot to display) in paired epitrochlearis muscles at 3hPEX. *D*: Delta Akt[Ser473] phosphorylation. *E*: Akt activity measured in paired epitrochlearis muscles at 3hPEX. *F*: Delta Akt activity. Data were analyzed by two-way ANOVA within each insulin level (minus or plus insulin) or for delta values, and Tukey post hoc analysis was performed to identify the source of significant variance. Values are means ± SEM; *n* = 19–21/group.

resistance for GU despite unaltered pAkt[Ser473]. To assess primary mechanisms for insulin resistance, we performed more extensive Akt analysis with a 2-week HFD by rats and found muscle insulin resistance for GU was accompanied by reduced delta-insulin pAkt[Thr308] without significant changes in pAkt[Ser473], pAkt[Thr308], Akt activity, or delta-insulin for pAkt[Ser473] or Akt activity. The current study was apparently the first to evaluate muscle pAS160 with brief HFD, revealing lower pAS160[Ser588] as potentially contributing to the insulin resistance. The uncoupling of multiple markers of Akt activation from pAS160 was reminiscent of the observations by Tonks et al. (42), who noted "little correspondence between insulin-dependent phosphorylation of Akt substrates and Akt itself across the different groups" that included lean, obese, and diabetic humans. Thus, there can be discordance between Akt activity and pAS160 with either

insulin resistance or improved insulin sensitivity postexercise (1,4,5,8,20). The explanation may conceivably include the roles of colocalization of Akt with AS160 and/or AS160 dephosphorylation by phosphatases.

For insulin-resistant rats (21–26) and humans (27–29,43), one exercise session has been shown to elevate insulin-stimulated GU. However, surprisingly few studies have addressed the mechanisms for this improvement. Previous research suggested that acute exercise can correct some defects in proximal insulin signaling of insulin-resistant muscle stimulated with a supraphysiologic insulin dose (25,26,44). In contrast, the current results indicate that exercise did not induce increases in proximal signaling with a physiologic insulin dose in muscles from brief HFD-3hPEX versus HFD-Sedentary rats. Differences between the current and previous studies may relate to the different insulin doses used. The current

Figure 7—*A*: AS160^{Thr642} phosphorylation measured in paired epitrochlearis muscles at 3hPEX. *$P < 0.05$, LFD-Sedentary vs. LFD-3hPEX with no insulin; †$P < 0.05$, LFD-3hPEX vs. HFD-3hPEX with insulin. *B*: Delta AS160^{Thr642} phosphorylation (delta = value with insulin − value without insulin from paired muscles). *$P < 0.05$, LFD-3hPEX vs. HFD-3hPEX with no insulin. *C*: AS160^{Ser588} phosphorylation measured in paired epitrochlearis muscles 3hPEX. *$P < 0.05$, LFD-3hPEX with insulin is greater than all other groups with insulin; †$P < 0.05$, HFD-Sedentary with insulin is less than all other groups with insulin; ‡$P < 0.05$, LFD vs. HFD within Sedentary. *D*: Delta AS160^{Ser588} phosphorylation. *$P < 0.05$, for LFD-3hPEX vs. all other groups. Data were analyzed by two-way ANOVA within each insulin level (minus or plus insulin) or for delta values, and Tukey post hoc analysis was performed to identify the source of significant variance. Values are means ± SEM; $n = 13–16$ per group.

study also provided the first information about the effect of acute exercise on insulin-stimulated pAS160 in insulin-resistant rat skeletal muscle using a physiologic insulin dose. The insulin resistance for GU in HFD-Sedentary versus LFD-Sedentary rats was accompanied by reduced pAS160^{Ser588}. In HFD-3hPEX versus HFD-Sedentary rats, pAS160^{Ser588} with insulin was significantly increased, and these values were restored to levels similar to LFD-Sedentary rats. Elimination of the diet-related decrement in pAS160^{Ser588} of insulin-stimulated muscles from HFD-3hPEX versus LFD-Sedentary rats occurred with elimination of the diet-related decrease in insulin-stimulated GU in HFD-3hPEX rats.

Two earlier studies also assessed acute exercise effects on pAS160 in insulin-resistant individuals, but they used very different approaches to produce insulin resistance. Diabetic (induced by streptozotocin treatment combined with 20-week HFD) rats were sedentary or exercised, and isolated muscles were stimulated with a supraphysiologic insulin concentration (45). Exercise did not significantly alter pAS160^{Thr642} in diabetic rats, but they did not evaluate exercised healthy rats, nondiabetic insulin-resistant

rats, muscles stimulated with a submaximal insulin dose, or pAS160^{Ser588}. Pehmøller et al. (46) assessed insulin-stimulated GU and pAS160 in muscles from healthy humans undergoing two trials: *1*) saline-infused control; and *2*) insulin resistance induced by 7-h intralipid infusion. Lipid-induced insulin resistance led to lower pAS160^{Ser341} and a nonsignificant trend for lower pAS160^{Ser588} with unaltered IRS-1–PI3K, pAkt, or pS160^{Thr642}. Subjects also performed one-legged exercise. Exercised versus nonexercised legs had greater GU, pAS160^{Ser588}, and pAS160^{Thr642} in both trials without exercise effects on IRS-1–PI3K or pAkt. Lipid infusion provides exceptional experimental control, but this control is achieved by eliminating the usual physiologic process of eating complex food. Accordingly, the current study was the first to use a dietary intervention to implicate greater muscle pAS160 as part of the mechanism by which acute exercise improves the action of a physiologic insulin dose on insulin-resistant muscle.

A single exercise session by either normal or insulin-resistant rats led to greater GU for each group, but prior exercise did not equalize the insulin-stimulated GU between

Table 1—DAG and ceramide species in epitrochlearis muscles

	DAG (nmol · mg⁻¹)										Ceramide (μg · g⁻¹)				
	14:0	16:0	16:1	18:0	18:1 (n-7)	18:1 (n-9)	18:2	20:0	20:1	20:4	16:0	18:0	18:1	20:0	24:0
LFD-Sedentary	1.41 ± 0.30	69.17 ± 10.87	1.34 ± 0.15	59.25 ± 12.43	3.44 ± 0.59	31.09 ± 3.47	15.61 ± 2.83	3.46 ± 0.66	0.69 ± 0.17	3.35 ± 0.96	7.74 ± 1.89	76.89 ± 7.24	1.77 ± 0.51	3.83 ± 0.55	9.88 ± 0.81
HFD-Sedentary	1.42 ± 0.31	70.46 ± 14.99	1.57 ± 0.46	57.77 ± 10.67	3.13 ± 0.71	33.78 ± 7.96	14.63 ± 1.56	3.89 ± 0.62	0.80 ± 0.36	2.32 ± 0.34	6.74 ± 0.72	88.50 ± 4.71	1.91 ± 0.19	5.12 ± 0.35	11.79 ± 1.96
LFD-3hPEX	1.37 ± 0.25	59.40 ± 12.70	1.18 ± 0.29	48.68 ± 8.34	2.92 ± 0.51	27.43 ± 4.74	12.85 ± 2.02	4.56 ± 1.07	0.83 ± 0.23	2.59 ± 0.44	7.59 ± 0.93	81.51 ± 8.86	1.52 ± 0.30	4.15 ± 0.61	10.74 ± 0.76
HFD-3hPEX	1.17 ± 0.17	56.84 ± 8.92	1.19 ± 0.29	47.11 ± 7.20	4.18 ± 0.96	45.45 ± 11.96	21.85 ± 7.11	3.68 ± 0.69	1.24 ± 0.26	4.22 ± 0.78*	5.68 ± 0.68	74.20 ± 6.00	1.17 ± 0.33	4.20 ± 0.48	9.56 ± 1.75

Data are mean ± SEM; n = 5–9 per group. Data for DAG and ceramide species in epitrochlearis muscles from rats that were either fed LFD or HFD and either Sedentary or 3hPEX. Analysis by two-way ANOVA within each insulin level (minus or plus insulin) and Tukey post hoc analysis were performed to identify the source of significant variance. *$P < 0.05$, HFD-Sedentary vs. HFD-3hPEX.

the diet groups. Examination of earlier studies reveals similar results (22,23,27,28), but these previous publications failed to identify mechanisms for this outcome. Results of the current study identified a plausible mechanism for this outcome by revealing that diet-related differences in insulin-stimulated GU postexercise were accompanied by greater pAS160[Ser588] and pAS160[Thr642] in muscles from LFD versus HFD rats independent of significant postexercise differences in proximal signaling. It was particularly striking that the diet-related differences in postexercise values for pAS160 and insulin-stimulated GU were evident after brief HFD that caused only 10% greater body mass, did not alter key metabolic outcomes immediately postexercise that are potential triggers for subsequently increased insulin-stimulated GU (insulin-independent GU, pAMPK, and glycogen), and had little effect on proximal insulin signaling.

Because a widely held view is that accumulation of lipid species causes insulin resistance, we tested the idea that prior exercise might improve insulin sensitivity by reducing levels of lipid metabolites. However, there were no significant exercise-induced reductions in muscle concentrations of DAG or ceramide species in either diet group. Earlier research linked DAG-associated insulin resistance to JNK activation and greater serine phosphorylation of IRS-1 (47,48) and demonstrated that ceramides can promote Akt dephosphorylation (49,50). Therefore, the lack of diet-related changes in DAG and ceramide species with unaltered proximal insulin signaling is consistent with the observation that, regardless of diet, exercise did not lower muscle levels of pJNK[Thr183/Tyr185], p–IRS-1[Ser307], or p–IRS-1[Ser1101]. Although elevations in these putative mediators of insulin resistance were not essential for the reduced GU with brief HFD, they may participate in insulin resistance with extreme obesity or long-term HFD.

The novel results of the current study implicate greater pAS160 for exercise benefits on insulin sensitivity in individuals with either normal or subnormal insulin sensitivity. Furthermore, the failure of the HFD group to attain insulin-stimulated GU values equal to the LFD group postexercise was accompanied with a deficit in exercise enhancement of pAS160. The consistent relationship between pAS160 and postexercise insulin sensitivity along with pAS160's crucial role for insulin-stimulated glucose transport supports our working hypothesis that enhanced pAS160 is important for elevated insulin-stimulated GU postexercise in both normal and insulin-resistant muscle. We further hypothesize that the difference between groups for insulin-mediated GU postexercise was secondary, at least in part, to a lesser exercise effect on pAS160 in muscles from insulin-resistant rats. The current study provides the foundation for future research focused on: 1) identifying the specific processes leading to greater pAS160 postexercise; 2) elucidating the explanation for the lack of the full exercise effect on pAS160 in insulin-resistant individuals; and 3) determining if greater

pAS160 is an essential cause for greater insulin-stimulated GU after exercise.

Author Contributions. C.M.C. performed the experiments, analyzed the data, designed the experiments, discussed the manuscript, developed the hypothesis, and wrote the manuscript. E.B.A. and N.S. performed the experiments, analyzed the data, and discussed the manuscript. G.D.C. designed the experiments, coordinated and directed the project, developed the hypothesis, discussed the manuscript, and wrote the manuscript. G.D.C. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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