Prior exercise increases phosphorylation of Akt substrate of 160 kDa (AS160) in rat skeletal muscle

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Arias EB, Kim J, Funai K, Cartee GD. Prior exercise increases phosphorylation of Akt substrate of 160 kDa (AS160) in rat skeletal muscle. Am J Physiol Endocrinol Metab 292: E1191–E1200, 2007. First published December 19, 2006; doi:10.1152/ajpendo.00602.2006.—The main purpose of this study was to determine whether the increased glucose transport (GT) found immediately postexercise (IPEX) or 4 h postexercise (4hPEx) is accompanied by increased phosphorylation of Akt substrate of 160 kDa (AS160, a protein regulator of GLUT4 translocation). Paired epiploic muscles were dissected from rats (sedentary or IPEX, 2-h swim) and used to measure protein phosphorylation and insulin-independent GT. IPEX values exceeded sedentary values for GT and phosphorylations of AS160, AMP-activated protein kinase (AMPK), and acetyl-CoA carboxylase (ACC) but not for AS160 abundance or phosphorylation of Akt serine (pSerAkt), Akt threonine (pThrAkt), or glycogen synthase kinase-3 (pGSK3). AS160 phosphorylation was significantly correlated with GT ($R = 0.801$, $P < 0.01$) and AMPK ($R = 0.655$, $P < 0.05$). Muscles from other rats were studied 4hPEx along with sedentary controls. One muscle per rat was incubated without insulin, and the contralateral muscle was incubated with insulin. 4hPEx values exceeded sedentary values for insulin-stimulated GT. The elevated AMPK and ACC found IPEX had reversed by 4hPEx. Insulin caused a significant increase in pSerAkt, pThrAkt, pGSK3, and AS160 phosphorylation with or without exercise. Exercise significantly increased AS160 phosphorylation, regardless of insulin, with unchanged AS160 abundance. Among the signaling proteins studied, insulin-stimulated GT was significantly correlated only with insulin-stimulated pThrAkt ($R = 0.720$, $P < 0.0005$). The results are consistent with a role for increased AS160 phosphorylation in the increased insulin-independent GT IPEX, and the exercise effects on AS160 phosphorylation and/or pThrAkt at 4hPEx are potentially relevant to the increased insulin-stimulated glucose transport at this time.

The increased insulin-independent glucose transport during and immediately after exercise is attributable to increased translocation of the GLUT4 glucose transporter protein to the cell surface membranes (12) with unaltered total GLUT4 abundance (8), as is the increased insulin-stimulated glucose transport at $\sim 3–4$ h postexercise (16). Although increased insulin-independent and insulin-dependent glucose transport are both achieved via redistribution of intracellular GLUT4 to the cell surface, each stimulus relies on a distinctive signaling pathway to trigger GLUT4 translocation (12, 46, 47). Compelling evidence indicates that phosphorylation of a recently identified protein, known as Akt substrate of 160 kDa (AS160), plays an important role in insulin-stimulated GLUT4 vesicle exocytosis in adipocytes (36, 48), making AS160 the most distal insulin-signaling event that has been linked to GLUT4 translocation. In light of the distinct signaling pathways for each stimulus, the recognition that AS160 in skeletal muscle becomes phosphorylated in response to either insulin or in vitro contractile activity (6) raised the surprising possibility that this protein may be a point of convergence between the insulin and exercise/contraction pathways for increasing cell surface GLUT4 and glucose transport.

AS160 phosphorylation was subsequently found also to increase immediately after in vivo exercise by mice (22) and humans (11). Akt is responsible for insulin-stimulated phosphorylation of AS160 (15). Although several studies have indicated that in vitro muscle contractions can increase Akt activation (6, 33–35), many studies that used in vivo exercise have not detected altered Akt phosphorylation or activity (27, 42, 45). However, there is considerable evidence that AMP-activated protein kinase (AMPK), which is increased during in vitro contractions or in vivo exercise, can also induce AS160 phosphorylation (6, 22). Therefore, we evaluated glucose transport and phosphorylation of AMPK, Akt, and AS160 in muscles dissected out of rats immediately after exercise.

With regard to the elevated insulin-dependent glucose transport postexercise, an appealing idea is that, several hours after exercise, one or more key steps in the insulin-signaling pathway may be amplified, allowing greater GLUT4 recruitment and thereby increased insulin-stimulated glucose transport. However, earlier studies have failed to detect effects of prior exercise on proximal insulin-signaling steps, including insulin receptor binding (4, 39), tyrosine phosphorylation of the insulin receptor (39), insulin receptor substrate-1 (IRS-1) tyrosine phosphorylation (16), and IRS-1-associated phosphatidylinositol 3-kinase (PI3-kinase) activity (14) in rat skeletal muscle stimulated by a physiological insulin concentration. Thus,
insulin signaling proximal to and including IRS-1–PI 3-kinase does not appear to be augmented by prior exercise or account for enhanced insulin sensitivity several hours after exercise.

It seems possible that a change in post-PI 3-kinase insulin signaling may induce the subsequent increase in insulin sensitivity after exercise. Akt (also known as protein kinase B) is downstream of PI 3-kinase and important for insulin-stimulated glucose transport (2, 44). Akt activity is increased by phosphorylation on two sites (Ser473 and Thr308). Phosphorylation on Thr308 is necessary and sufficient for partial Akt activation, but full Akt activation requires phosphorylation on both sites (1). Fisher et al. (14) found that prior exercise (2-h swim) did not enhance pSerAkt in submaximal insulin-stimulated rat epitrochlearis muscle determined ∼3.5 h postexercise. However, the effect of acute exercise on Akt Thr308 phosphorylation (pThrAkt) in rat skeletal muscles stimulated by insulin has not been previously reported. Accordingly, we also evaluated the effect of exercise performed ∼4 h earlier on glucose transport, pSerAkt, pThrAkt, and AS160 phosphorylation in skeletal muscle with a physiological insulin concentration.

We hypothesized that the increased insulin-independent glucose transport in rat skeletal muscle immediately postexercise would be accompanied by elevated AMPK and AS160 phosphorylation with unaltered Akt phosphorylation. We also hypothesized that insulin-stimulated glucose transport in skeletal muscle ∼4 h after exercise would be accompanied by elevated insulin-stimulated pThrAkt and AS160 phosphorylation without increased pSerAkt or AMPK phosphorylation (pAMPK).

METHODS

Materials. Human recombinant insulin was obtained from Eli Lilly (Indianapolis, IN). Reagents and apparatus for SDS-PAGE and immunoblotting were from Bio-Rad (Richmond, CA). Protein concentration was measured using the bicinchoninic acid method (Pierce Biotechnology, Rockford, IL). Enhanced chemiluminescence (ECL; Amersham Pharmacia Biotechnology, Piscataway, NJ) or SuperSignal (Pierce Biotechnology, Rockford, IL). Enhanced chemiluminescence (ECL; Amersham Pharmacia Biotechnology, Piscataway, NJ). Immunoblotting technology (Beverly, MA). PAS recognizes Akt phosphorylation motif peptide sequences (RXRXXpT/S). Immunoblotting for total AS160 abundance and AS160 immunoprecipitation was performed using an antibody from Upstate USA (Charlottesville, VA; no. 07-741). 3-O-methyl-[3H]glucose ([3H]3-MG) was from New England Nuclear Life Science Products (Boston, MA), and [3H]mannitol was from Amersham Pharmacia Biotechnology (Piscataway, NJ). Other reagents were from Sigma-Aldrich (St. Louis, MO).

Animal treatment. Procedures for animal care were approved by the University of Michigan Committee on Use and Care of Animals. Male Wistar rats (~150–200 g; Harlan, Indianapolis, IN) were provided with rodent chow (Lab Diet; PMI Nutritional International, Brentwood, MO), and water ad libitum until 1700 the night before experiment and did not have access to food thereafter. Five to six rats swam in a barrel filled to a depth of ~80 cm with water at ∼35°C for four 30-min bouts with a 5-min rest period between each 30-min bout. Other rats served as sedentary controls with each experiment.

In vitro incubation. Rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body wt) either immediately postexercise (IPEX) or 3–4 h postexercise (4hPEX) along with time-matched sedentary rats. For the IPEX experiment, following anesthetization, one epitrochlearis muscle from each rat was rapidly dissected out, trimmed, freeze-clamped using aluminum clamps cooled to the temperature of liquid N2, and stored at ~−80°C until analyzed. After dissection, the contralateral muscle in the IPEX study was incubated for 10 min at 30°C in flasks containing Krebs-Henseleit buffer (KHB) supplemented with 0.1% bovine serum albumin (BSA), 6 mM sodium pyruvate, and 2 mM mannitol (solution 1). For all incubation steps, flasks were continuously gassed from above with 95% O2–5% CO2 and shaken in a heated water bath.

For the 4hPEX experiment, rats were dried following the final exercise bout and returned to their cage for 3–4 h before being anesthetized. After dissection, both muscles from each animal were incubated for 30 min at 35°C in flasks containing KHB supplemented with BSA, 8 mM glucose, and 2 mM mannitol (solution 2). One muscle from each rat was incubated in solution 2 without insulin, and the contralateral muscle was incubated in solution 2 with 50 μU/ml insulin. After this initial incubation, the muscles were incubated for 10 min at 30°C in flasks containing solution 1 with the same insulin concentration as in the prior incubation step. Some muscles, which were used for immunoblotting but not measurement of 3-MG transport, were incubated identically to that described above except that radiolabeled 3-MG and mannitol were not included during the final incubation step. After incubation with 3-MG for 15 min, the muscles were rapidly blotted on filter paper dampened with incubation media, trimmed, freeze-clamped, and stored at ~−80°C until processed as described below.

Muscle preparation. Muscles were prepared as previously described (3). Briefly, frozen muscles were weighed and then homogenized in ice-cold buffer (20 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1% Igepal, 2 mM NiCl2·6H2O, 10 mM NaF, 2 mM EDTA, 2 mM EGTA, 2.5 mM sodium pyrophosphate, 20 mM β-glycerophosphate, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Muscle homogenates were solubilized by end-over-end rotation for 1 h (4°C) and then centrifuged at 12,000 g for 15 min (4°C). Aliquots of the supernatant from some muscles were used for scintillation counting, and 3-MG transport was determined as previously described (7). Protein concentration in the supernatant was determined in other aliquots (38).

Immunoprecipitation. Aliquots of muscle lysate prepared as described above were incubated with end-over-end rotation overnight (4°C) with anti-AS160 before a slurry of prewashed protein A-agarose beads was added to the lysate and incubated for 1 h (4°C) and then centrifuged at 12,000 g for 15 min (4°C). Aliquots of the supernatant were removed before the beads were washed three times with ~1 ml of homogenization buffer and then washed three times with ~1 ml of Tris-buffered saline (TBS).

Immunoblotting. SDS loading buffer was added to muscle lysates or immunoprecipitated protein bound to anti-AS160 and protein A-agarose beads and was boiled before being subjected to polyacrylamide gel electrophoresis. Proteins were subsequently transferred to nitrocellulose overnight (4°C). The membrane was washed with TBS and blocked with 5% nonfat milk in TBS plus 0.1% Tween (TBST) for 1 h at room temperature. The membrane was then washed with 0.1% TBST and incubated with the appropriate primary antibody (4°C). The membrane was again washed with TBST before being incubated with the secondary antibody (horseradish peroxidase-conjugated anti-rabbit IgG) for 1 h at room temperature. The membrane was washed with TBST and then washed with TBS and finally developed with ECL or SuperSignal reagent. Protein bands were quantitated per milligram of total protein by densitometry (Alpha Innotech, San Leandro, CA). The mean values for sedentary muscles
incubated without insulin on each blot were normalized to equal 1.0, and then all samples on the blot were expressed relative to the normalized control value. For the IPEX and 4hPEX experiments, phosphorylation of AS160 was measured using the PAS antibody in samples that had undergone prior immunoprecipitation using anti-AS160. Phosphorylation of AS160 was also evaluated in the 4hPEX experiment using the PAS antibody with samples that had not undergone prior immunoprecipitation.

Statistical analysis. For the 4hPEX experiment, two-way ANOVA was used to identify significant main effects (exercise and insulin) and interactions (exercise-by-insulin) for glucose transport and protein phosphorylation. A Student-Newman-Keuls post hoc test was used to identify the source of significant variance. The insulin-stimulated increases (Δ-insulin) in glucose transport and protein phosphorylation were calculated by subtracting the values for muscles incubated without insulin from the respective values of paired muscles incubated with insulin. Student’s t-test was used to compare values from sedentary and IPEX groups and to compare sedentary and 4hPEX groups for AS160 abundance and Δ-insulin values. The Mann-Whitney rank sum test was used to compare two groups if data failed the test for normality. A Pearson product moment correlation was used to assess the relationship between glucose transport and phosphorylated proteins using values for the correlated parameters from the same muscle in the 4hPEX experiment and from paired muscles (glucose transport from incubated muscles and phosphorylated proteins from contralateral muscles freeze-clamped immediately after dissection) for the IPEX experiment. A P value ≤ 0.05 was considered statistically significant. Data are presented as means ± SE.

RESULTS

3-MG transport. Insulin-independent glucose transport was approximately fourfold greater (P < 0.001) for IPEX rats (0.977 ± 0.098 μmol·g⁻¹·15 min⁻¹, n = 5) compared with sedentary (SED) controls (0.257 ± 0.032 μmol·g⁻¹·15 min⁻¹, n = 6). In the 4hPEX experiment, there was a significant main effect of insulin (P < 0.001) and a significant exercise-by-insulin interaction (P < 0.005) for glucose transport (Fig. 1). As expected, the large increase in insulin-independent glucose transport that was evident IPEX was nearly completely reversed by 4hPEX: post hoc analysis indicated that insulin-independent glucose transport trended (P = 0.110) to be only ~1.3-fold greater for 4hPEX compared with sedentary values. Also as expected, post hoc analysis revealed that insulin-treated muscles had a greater glucose transport than paired muscles incubated without insulin for both the sedentary (P < 0.01) and 4hPEX (P < 0.001) groups. Furthermore, glucose transport in muscles incubated with insulin was 1.6-fold greater (P < 0.001, Newman-Keuls post hoc test) for the 4hPEX group compared with the sedentary controls. Calculated insulin-stimulated (Δ-insulin) glucose transport (values for insulin-treated muscle minus values from paired muscles without insulin) was 2.1-fold greater (P < 0.005) for the 4hPEX group compared with sedentary controls, indicative of the expected postexercise elevation in insulin action.

Akt and GSK3 phosphorylation. Muscle pSerAkt was not significantly different for IPEX rats (1.02 ± 0.10, n = 4) compared with sedentary controls (1.00 ± 0.21, n = 5). There was also no difference in pThrAkt for IPEX (0.82 ± 0.10, n = 5) vs. sedentary (1.0 ± 0.10, n = 6) groups. Consistent with the absence of an IPEX effect on Akt, the IPEX (1.10 ± 0.11, n = 4) and sedentary (1.00 ± 0.16, n = 6) groups did not differ (P = 0.649) for pGSK3, a substrate of Akt.

For the 4hPEX experiment, there was a significant main effect of insulin (P < 0.001), but not exercise, on pSerAkt (Fig. 2). Not surprisingly, post hoc analysis indicated that, in both sedentary (P < 0.001) and 4hPEX (P < 0.001) groups, pSerAkt was greater for insulin-treated muscles vs. muscles incubated without insulin. Consistent with earlier research, there was no significant exercise-by-insulin interaction for pSerAkt, and calculated Δ-insulin pSerAkt was unaltered by exercise.

For pThrAkt, there were significant main effects of both insulin (P < 0.001) and 4hPEX (P < 0.005; Fig. 3). As for pSerAkt, post hoc analysis indicated that pThrAkt was greater for muscles incubated with insulin vs. without insulin in both sedentary (P < 0.001) and 4hPEX (P < 0.001) groups. Post hoc analysis of muscles incubated in the absence of insulin did not indicate a significant difference (P = 0.414) in pThrAkt between the sedentary and 4hPEX groups. In contrast to pSerAkt, pThrAkt from insulin-stimulated muscles was greater (P < 0.001) for 4hPEX vs. sedentary controls, and the calculated Δ-insulin for pThrAkt was greater (P < 0.05) for 4hPEX compared with sedentary rats.

Fig. 1. Rate of 3-O-methylglucose (3-MG) transport from the 4-h postexercise (4hPEX) experiment. Paired epitrochlearis muscles, dissected from rats that were sedentary (SED, filled bars) or were anesthetized ~4-h postexercise (4hPEX; open bars), were incubated without insulin or with 50 μU/ml insulin. Data are means ± SE for 29 muscles per group. A: there were significant main effects of exercise (P < 0.001) and insulin (P < 0.001) and a significant interaction (exercise-by-insulin, P < 0.01). Post hoc analysis: *P < 0.001, SED with insulin vs. SED without insulin; †P < 0.001, 4hPEX with insulin vs. 4hPEX without insulin and 4hPEX with insulin vs. SED with insulin. B: insulin-stimulated increase (Δ-insulin) for glucose transport was calculated as 3-MG transport with insulin minus 3-MG transport for the contralateral muscle without insulin. ‡P < 0.005, 4hPEX Δ-insulin vs. SED Δ-insulin (Mann-Whitney rank sum test).
Consistent with the insulin effect on both pSerAkt and pThrAkt, there was a significant main effect of insulin (P < 0.05) on pGSK3 (Fig. 4). However, there was no significant main effect of 4hPEX (P = 0.767) or exercise-by-insulin interaction (P = 0.375) on pGSK3, and the Δ-insulin for pGSK3 was also not significantly different (P = 0.354) for 4hPEX animals compared with sedentary controls.

**AMPK and ACC phosphorylation.** Muscle pAMPK was greater (P < 0.01) for the IPEX group (1.44 ± 0.12, n = 5) compared with sedentary controls (1.00 ± 0.05, n = 6). The increased pAMPK was accompanied by increased (P < 0.05) phosphorylation of its substrate, ACC for the IPEX (2.82 ± 0.59, n = 5) vs. the sedentary (1.00 ± 0.31, n = 6) group. By 4hPEX, the exercise effects had reversed for both pAMPK (Fig. 5) and pACC (SED without insulin = 1.00 ± 0.18, n = 8; SED with insulin = 1.39 ± 0.35, n = 8; 4hPEX without insulin = 1.14 ± 0.28, n = 17; 4hPEX with insulin = 1.71 ± 0.29, n = 17). As expected, there was no effect of insulin on pAMPK (P = 0.211) or pACC (P = 0.108), regardless of exercise, and no significant Δ-insulin for pAMPK (P = 0.597) or pACC (P = 0.345).

**AS160 abundance and phosphorylation.** AS160 abundance was not significantly different between the IPEX group (0.75 ± 0.19, n = 5) compared with its sedentary controls (1.00 ± 0.22, n = 5; P = 0.409) or for the 4hPEX group (0.89 ± 0.08, n = 10) vs. its sedentary controls (1.00 ± 0.13, n = 8; P = 0.486).

Phosphorylation of AS160 was evaluated for both the IPEX and 4hPEX experiment using the PAS antibody in samples that had been immunoprecipitated with anti-AS160 (PAS-AS160).

**Correlations.** After anesthetization, both epitrochlearis muscles were immediately dissected out of each animal in the IPEX group and their sedentary controls. One muscle was immediately freeze-clamped and subsequently used for PAS-AS160 determination, and the contralateral muscle was incubated without insulin to determine glucose transport rate. Insulin-independent glucose transport was significantly correlated (R = 0.801, P < 0.01, n = 10; Fig. 8) with PAS-AS160. Muscle pAMPK vs. insulin-independent glucose transport was significantly (P = 0.01) correlated (R = 0.764, n = 10) for the IPEX experiment, as was pAMPK vs. PAS-AS160 (R = 0.655, P < 0.05, n = 10), but not for only muscles incubated without insulin.
insulin \((P = 0.530)\) or for \(\Delta\)-insulin for AS160 phosphorylation vs. \(\Delta\)-insulin for glucose transport \((P = 0.564)\). There was also a significant correlation between the pThrAkt and glucose transport in the 4hPEX experiment \((R = 0.720, P < 0.0005, n = 20; \text{Fig. } 10A)\), and the correlation between \(\Delta\)-insulin for pThrAkt and \(\Delta\)-insulin for glucose transport was significant \((R = 0.665, P < 0.05, n = 10; \text{Fig. } 10B)\).

**DISCUSSION**

We evaluated the influence of prior exercise (IPEX or 4hPEX) on AS160, the most distal insulin-signaling protein that has been linked to regulation of GLUT4 translocation and glucose transport. An exercise protocol that improved insulin-independent glucose transport, IPEX, and insulin-stimulated glucose transport, 4hPEX, also caused an increase in phosphorylation of AS160, with unaltered AS160 abundance, at each time. Two kinases have been shown to phosphorylate AS160: Akt and AMPK. The elevated AS160 phosphorylation IPEX was accompanied by enhanced pAMPK and pACC (an AMPK substrate), but unaltered pSerAkt, pThrAkt, or pGSK3 (an Akt substrate), strongly suggesting that AMPK is important for the observed increase in AS160 phosphorylation that was observed IPEX. At 4hPEX, there was an insulin-independent increase in AS160 phosphorylation despite there being no exercise effect on pAMPK, pSerAkt, pThrAkt, pACC, and pGSK3 in muscles incubated without insulin. These results suggest that the persistent increase in AS160 phosphorylation at 4hPEX was a residual effect of increased pAMPK that was evident IPEX and/or the effect of unknown kinases or phosphatases that also regulate AS160. AS160 phosphorylation was correlated with glucose transport in the 4hPEX experiment. At 4hPEX, \(\Delta\)-insulin was increased for pThrAkt, and \(\Delta\)-insulin for pThrAkt was significantly correlated with \(\Delta\)-insulin for glucose transport.

Lienhard’s group \((19, 36)\) identified AS160 as an Akt substrate that becomes phosphorylated in 3T3-L1 adipocytes in response to insulin. They also provided evidence that insulin-stimulated phosphorylation of AS160 was essential for insulin-induced GLUT4 translocation and that AS160 has a functional Rab GTPase-activating domain that can specifically activate several members of the Rab family of G proteins that are found in GLUT4 vesicles \((28)\). McGraw and colleagues \((13, 48)\) found that insulin’s effect on GLUT4 movement via AS160 is specific for increasing GLUT4 exocytosis without slowing glucose transport.

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**Fig. 4.** Glycogen synthase kinase-3 phosphorylation (pGSK3) from the 4hPEX experiment. Paired epitrochlearis muscles, dissected from SED (filled bars) or 4hPEX (open bars) rats, were incubated without insulin or with 50 \(\mu\)U/ml insulin. Data are means \(\pm\) SE for 6 muscles per group. A: there was a significant main effect of insulin \((P < 0.05)\), but not exercise. B: there was no significant difference between SED and 4hPEX for calculated Δ-insulin of pGSK3.

**Fig. 5.** AMPK phosphorylation (pAMPK) from the 4hPEX experiment. Paired epitrochlearis muscles, dissected from SED (filled bars) or 4hPEX (open bars) rats, were incubated without insulin or with 50 \(\mu\)U/ml insulin. Data are means \(\pm\) SE for 5 muscles per group. A: there were no significant effects of exercise or insulin on pAMPK. B: there was no significant difference between SED and 4hPEX for calculated Δ-insulin of pAMPK.
endocytosis. Under basal conditions, a substantial portion of AS160 is reportedly associated with GLUT4 vesicles of 3T3-L1 adipocytes, and it is released upon insulin stimulation (24, 31). AS160 appears to also be important for insulin-stimulated glucose transport in skeletal muscle. Bruss et al. (6) and Arias et al. (3) demonstrated that insulin results in a rapid and dose-dependent increase in AS160 phosphorylation in rat epitrochlearis muscle. AS160 phosphorylation is also increased in human skeletal muscle upon physiological insulin stimulation in vivo, and this insulin effect is attenuated in muscle from insulin-resistant humans with type 2 diabetes mellitus, suggesting an association between glucose uptake and AS160 phosphorylation in skeletal muscle (21). In vivo glucose uptake was reduced in insulin-stimulated mouse muscles overexpressing the 4P mutant of AS160, which cannot be phosphorylated on four key Akt phosphomotifs (23). Taking these findings together with the compelling results for 3T3-L1 adipocytes, it seems likely that AS160 participates in insulin-stimulated glucose uptake in skeletal muscle. Thus, the enhanced AS160 phosphorylation after exercise has the potential to play a role in the improved insulin sensitivity.

Exercise and insulin can independently increase skeletal muscle glucose transport by leading to increased cell surface GLUT4 using distinct signaling pathways. The demonstration that AS160 phosphorylation can be increased in rat epitrochlearis muscle by either insulin stimulation without contractile activity or by in vitro contractile activity in the absence of insulin raised the possibility that AS160 may play a role in regulating GLUT4 trafficking with each stimulus (6). AS160 phosphorylation is also increased in skeletal muscle immediately after in vivo exercise by humans (11) and mice (22). These results are consistent with findings for rats from the present study, in which AS160 phosphorylation was increased for IPEX compared with sedentary controls. Furthermore, AS160 phosphorylation was directly correlated with insulin-independent glucose transport in the IPEX experiment, providing suggestive evidence that the increased AS160 phosphorylation may be important for the elevated glucose transport. Correlative findings cannot establish a causal relationship, but consistent with the idea that AS160 phosphorylation is important for contraction-stimulated glucose uptake, Goodyear’s group [Kramer et al. (23)] found that in vivo glucose uptake...
During and after electrically stimulated contractile activity was reduced in mouse muscle overexpressing the 4P AS160 mutant compared with control muscles undergoing an identical contraction protocol. However, a low dose of wortmannin that was sufficient to completely prevent the contraction-induced increase in AS160 phosphorylation in isolated rat epitrochlearis muscle (6) has no effect on contraction-stimulated glucose transport (25, 47). In addition, although contraction-stimulated glucose transport was normal for AMPK\(^{H9251}\) knockout (17) and only partially reduced for AMPK\(^{H9251}\) kinase-dead (29) animals, the in vitro contraction-stimulated increase in AS160 phosphorylation observed in wild-type controls was absent from each of these genetically modified models (40). These results suggest that increased AS160 phosphorylation, at least on sites recognized by the PAS antibody, is not essential for contraction-stimulated glucose transport in isolated muscle. It is important to recognize that the PAS antibody does not appear to have equal immunoreactivity against all of AS160’s Akt phosphomotifs (20), so PAS immunoreactivity may not always precisely reflect phosphorylation on all Akt-dependent sites. Although insulin-independent glucose transport was correlated with AS160 phosphorylation in the IPEX experiment, the association should be interpreted cautiously because a role for AS160 in exercise-stimulated glucose transport remains to be established.

Earlier studies have characterized exercise and contraction effects on AS160 phosphorylation only immediately after contractile activity, so the persistent elevation in AS160 phosphorylation in muscles at 4hPEX was a novel observation. The increased AS160 phosphorylation was accompanied by insulin-independent glucose transport of the 4hPEX group that tended to be \~30\% greater than sedentary control values, comparable to previous reports for an \~20–50\% increase in insulin-independent glucose transport of isolated rat epitrochlearis at \~4hPEX that has sometimes (10, 43), but not always (9, 16), achieved statistical significance.

Akt was the first serine/threonine kinase that was linked to AS160 phosphorylation. Muscle levels of pSerAkt and pThrAkt were not altered from sedentary controls in muscles sampled at IPEX. The lack of an effect of in vivo exercise on Akt activation at IPEX is in agreement with most previous research on rats (27, 42) and mice (45), although one study of rats (34) found greater Akt activation determined IPEX compared with sedentary values. Electrically stimulated contractions by rat skeletal muscle can induce Akt activation (6, 33–35), although enhanced Akt activation has not always been detected with in vitro contractions (5, 26, 37). The IPEX and sedentary groups in the current study had similar levels for phosphorylation of GSK3, an Akt substrate, consistent with the lack of an IPEX effect on Akt phosphorylation. In summary,
the increased insulin-independent glucose transport by epitrochlearis muscles at IPEX in the current study cannot be attributed to increased Akt phosphorylation.

The absence of a significant effect of exercise on pSerAkt or pThrAkt in muscles incubated without insulin, in both IPEX and 4hPEX groups, suggests that other mechanisms account for exercise effects on AS160 phosphorylation in the absence of insulin. AS160 was first recognized to be an Akt substrate, but incubation of isolated rat epitrochlearis muscle with the AMP activator 5-aminooimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) can also induce an increase in AS160 phosphorylation despite unchanged Akt phosphorylation, implicating AS160 as a substrate for AMPK (6). This interpretation is consistent with the finding that recombinant AMPK can phosphorylate AS160 in a cell-free assay (40). The AMPK activator AICAR also results in AS160 phosphorylation of phosphorylate AS160 in a cell-free assay (40). The AMPK phosphorylation is consistent with the finding that recombinant AMPK can phosphorylate as a substrate for AMPK (6). This interpretation is consistent with the finding that recombinant AMPK can phosphorylate AS160 in a cell-free assay (40). The AMPK activator AICAR also results in AS160 phosphorylation of AMPK knockdown mouse cells, and AICAR’s effect on AS160 phosphorylation was markedly diminished in muscles from AMPK knockdown mouse cells (40). In addition, muscles from transgenic mice expressing α2-inactive AMPK had no AICAR-stimulated AS160 phosphorylation and a partial decline in contraction-stimulated AS160 phosphorylation (22). There is strong evidence that AS160 is a substrate of AMPK, and that AMPK can account for at least a portion of contraction-induced AS160 phosphorylation.

The half-time for reversal of AMPK activity after contraction was ~8 min for isolated rat epitrochlearis muscles (30) and ~40 min for rat hindlimb muscles after in situ stimulation via the sciatic nerve (41). Accordingly, it is not surprising that the increase in phosphorylation of AMPK and its substrate ACC observed IPEX was completely reversed in muscle 4hPEX. However, it is conceivable that AMPK’s effects on AS160 phosphorylation may persist after reversal of the increased pAMPK, so it is possible that the increased pAMPK evident IPEX contributed to the persistent increase in AS160 phosphorylation determined at 4hPEX. Alternatively, exercise may induce a persistent activation of other kinases that are not currently known to phosphorylate AS160.

Phosphorylation of Akt on both Thr308 and Ser473 is required for maximal activation, but Akt activity can reportedly be increased by its phosphorylation only on the Thr308 site (18). At 4hPEX, pSerAkt of insulin-stimulated muscles was not influenced by prior exercise, consistent with previously reported results (14). However, muscle pThrAkt was greater for insulin-stimulated muscles from 4hPEX compared with sedentary rats, and Δ-insulin for pThrAkt was increased in the 4hPEX group. Nonetheless, there was no evidence that exercise led to amplification of insulin-stimulated AS160 phosphorylation; i.e., there was no effect of exercise on Δ-insulin AS160 phosphorylation and no exercise-by-insulin interaction for AS160 phosphorylation. There was also not a significant effect of prior exercise on phosphorylation of GSK3, another Akt substrate, in insulin-stimulated muscles. One of our original hypotheses predicted that exercise would lead to increased insulin-stimulated AS160 phosphorylation, but the results do not support this prediction. Is it still possible that the exercise-induced increase in AS160 phosphorylation that was found regardless of insulin concentration plays a role in the increased insulin-stimulated glucose transport at 4hPEX? One speculative scenario would be that after exercise the GLUT4 that had cycled to the cell surface membranes is redistributed to intracellular vesicles that are associated with little or no AS160; e.g., perhaps AS160 must be dephosphorylated to rebind with GLUT4 vesicles. Regardless of the mechanism, it seems possible that a lack of AS160 associated with GLUT4-containing vesicles after exercise would favor increased susceptibility for subsequent insulin-stimulated GLUT4 translocation. In this context, the persistent, insulin-independent increase in AS160 phosphorylation that was evident at 4hPEX could potentially act together with other molecular events that are triggered when the muscle is exposed to insulin to induce greater insulin-stimulated glucose transport.

It is also possible that the enhanced pThrAkt contributed to the elevated insulin-stimulated glucose transport observed at 4hPEX. To date, AS160 is the only Akt substrate that has been implicated in insulin-mediated GLUT4 translocation, but it is conceivable that the elevated pThrAkt in insulin-stimulated muscles at 4hPEX played a role in the increased glucose transport by acting on an Akt substrate other than AS160. Furthermore, in 3T3-L1 adipocytes, there is evidence that insulin’s effect on GLUT4 exocytosis may be mediated by a combination of processes, including Akt- and AS160-dependent, Akt-dependent but AS160-independent, and Akt-independent events (15). Thus, the improved insulin-stimulated glucose transport at 4hPEX may also involve insulin-stimulated processes that are both Akt and AS160-dependent. For example, insulin’s Akt- and AS160-dependent effects on GLUT4 redistribution appear to be limited to GLUT4 exocytosis to surface membranes (13, 15, 48). It is feasible that prior exercise influences insulin’s effect on GLUT4 endocytosis, thereby increasing glucose transport.

In conclusion, the results of the current study demonstrate that enhanced AS160 phosphorylation IPEX was not the result of increased Akt phosphorylation; rather, it was likely secondary to greater AMPK activation. In addition, insulin-independent glucose transport was significantly correlated with AS160 phosphorylation in the IPEX study. The increased insulin-stimulated glucose transport at 4hPEX was accompanied by increased insulin-stimulated pThrAkt without elevated pSerAkt or pAMPK. There was also a persistent exercise-induced elevation in AS160 phosphorylation at 4hPEX, regardless of insulin concentration, despite there being no exercise effect on pAMPK or pAkt in muscles incubated without insulin at that time. Although AS160 phosphorylation and pThrAkt were each directly correlated with glucose transport in muscles at 4hPEX played a role in the increased glucose transport by acting on an Akt substrate other than AS160. Furthermore, in 3T3-L1 adipocytes, there is evidence that insulin’s effect on GLUT4 exocytosis may be mediated by a combination of processes, including Akt- and AS160-dependent, Akt-dependent but AS160-independent, and Akt-independent events (15). Thus, the improved insulin-stimulated glucose transport at 4hPEX may also involve insulin-stimulated processes that are both Akt and AS160-dependent. For example, insulin’s Akt- and AS160-dependent effects on GLUT4 redistribution appear to be limited to GLUT4 exocytosis to surface membranes (13, 15, 48). It is feasible that prior exercise influences insulin’s effect on GLUT4 endocytosis, thereby increasing glucose transport.

In conclusion, the results of the current study demonstrate that enhanced AS160 phosphorylation IPEX was not the result of increased Akt phosphorylation; rather, it was likely secondary to greater AMPK activation. In addition, insulin-independent glucose transport was significantly correlated with AS160 phosphorylation in the IPEX study. The increased insulin-stimulated glucose transport at 4hPEX was accompanied by increased insulin-stimulated pThrAkt without elevated pSerAkt or pAMPK. There was also a persistent exercise-induced elevation in AS160 phosphorylation at 4hPEX, regardless of insulin concentration, despite there being no exercise effect on pAMPK or pAkt in muscles incubated without insulin at that time. Although AS160 phosphorylation and pThrAkt were each directly correlated with glucose transport in muscles from rats in the IPEX experiment, only insulin-stimulated pThrAkt was increased for the 4hPEX group compared with sedentary controls. On the basis of the findings of the current study, we propose three working hypotheses. 1) Enhanced AS160 phosphorylation, secondary to elevated pAMPK during and shortly after exercise, plays a role in the greater insulin-independent glucose transport IPEX. 2) The elevated AS160 phosphorylation at 4hPEX is attributable to a residual effect of the increased pAMPK that was evident IPEX. 3) The increased insulin-stimulated glucose transport at 4hPEX is secondary, at least in part, to the combined effects of the persistent insulin-independent increase in AS160 phosphorylation and the increased insulin-stimulated pThrAkt. Additional experiments will be needed to test these new hypotheses.
REFERENCES


