Increased Phosphorylation of Akt Substrate of 160 kDa (AS160) in Rat Skeletal Muscle in Response to Insulin or Contractile Activity

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In 3T3-L1 adipocytes, insulin-stimulated GLUT4 translocation requires phosphorylation of the protein designated Akt substrate of 160 kDa (AS160). Both insulin and contractions activate Akt in skeletal muscle. Therefore, we assessed the effects in skeletal muscle of each stimulus on phosphorylation of proteins, including AS160, on the Akt phosphomotif. Isolated rat epitrochlearis muscles were incubated with insulin (for time course and dose response), stimulated to contract, or incubated with 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) and used to assess the following: serine-phosphorylation of Akt (P-Akt), immunoreactivity with an antibody recognizing the Akt phosphomotif (α-phospho-[Ser/Thr] Akt substrate [PAS]), and PAS immunoreactivity of samples immunoprecipitated with anti-AS160. P-Akt peaked at 5 min of insulin, and PAS immunoreactivity subsequently peaked for proteins of 250 kDa (10 min) and 160 kDa (15 min). P-Akt, PAS-160, and PAS-250 increased significantly with 0.6 nmol/l insulin. Contractile activity led to increased P-Akt and PAS immunoreactivity of proteins of 160 and 250 kDa. The 160-kDa protein was confirmed to be AS160 based on elevated PAS immunoreactivity in AS160 immunoprecipitates. Wortmannin inhibited insulin (120 nmol/l) and contraction effects on AS160 phosphorylation. Incubation with AICAR caused increased phosphorylation of AMP-activated protein kinase and AS160 but not Akt. Our working hypothesis is that phosphorylation of these putative Akt substrates is important for some of the insulin and contraction bioeffects. *Diabetes* 54:41–50, 2005

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AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; AS160, Akt substrate of 160 kDa; KHB, Krebs-Henseleit buffer; PAS, phospho-(Ser/Thr) Akt substrate; PI, phosphatidylinositol; TBST, Tris-buffered saline plus Tween.

The signaling pathways that mediate insulin's many actions remain incompletely understood, but the following sequence has been well characterized: insulin binds to its receptor, leading to receptor autophosphorylation and activation of receptor tyrosine kinase, which in turn results in tyrosine phosphorylation of endogenous substrates including insulin receptor substrate proteins. These docking proteins engage downstream signaling molecules such as phosphatidylinositol (PI) 3-kinase (1–3). PI 3-kinase catalyzes the phosphorylation of phosphatidylinositol 4,5-bisphosphate on the D3 position of inositol, and the resultant PI 3,4,5-trisphosphate binds and activates more distal signaling proteins, including phosphoinositide-dependent kinase-1 and Akt, a serine/threonine kinase. Akt has been implicated as a key signaling protein for several of insulin's actions, including activation of glycogen synthesis, protein synthesis, and GLUT4 translocation to the cell surface, thereby increasing glucose transport (1–5). Identification of the intermediate signaling steps linking Akt to insulin's diverse actions remains incomplete.

Recent research using 3T3-L1 adipocytes demonstrated that insulin leads to the phosphorylation of multiple proteins that contain one or more consensus sequences for phosphorylation by Akt (6). Among these Akt substrates was a 160-kDa protein, named Akt substrate of 160 kDa (AS160), which contained six Akt consensus sequences that become phosphorylated in insulin-treated adipocytes. AS160 also includes a GTPase-activating domain for small G-proteins, known as Rabs, which participate in vesicular trafficking (7). A point mutation of two or more of the consensus phosphorylation sites for Akt resulted in a marked decline in insulin-stimulated GLUT4 redistribution to the cell surface. Thus, in 3T3-L1 adipocytes, phosphorylation of AS160 was strongly implicated as an intermediate step linking insulin's activation of Akt to increased glucose transport. More recently, Zeigerer et al. (8) demonstrated that AS160 is important for insulin's activation of GLUT4 vesicle exocytosis without altering insulin-mediated inhibition of GLUT4 internalization.

Insulin also activates Akt in skeletal muscle (9,10), and AS160 is expressed by this tissue (6), which is a major target for insulin action. An important question is this: Does insulin, in skeletal muscle, lead to increased phosphorylation of AS160 and/or other proteins containing the Akt phosphomotif? Accordingly, our first aim was to

characterize, in skeletal muscle, the time course and dose response for insulin on phosphorylation of substrates of Akt.

Several studies have indicated that, in addition to its activation by insulin, Akt can be activated in skeletal muscle by in vitro contractile activity or in vivo exercise (9,11,12). Therefore, the second major aim of this study was to determine if AS160 or other proteins containing the Akt phosphomotif become phosphorylated in response to muscle contraction. For both the insulin and contraction experiments, we also evaluated the effects of these stimuli on Akt phosphorylation, so that we could assess the relationship between phosphorylation of Akt and its putative substrates, and determined if wortmannin influenced their effects to assess the possible involvement of PI 3-kinase.

RESEARCH DESIGN AND METHODS

Antibodies. Serine-phosphorylated Akt was detected using anti-phospho-Akt antibody (anti-P-Akt) that was raised against a synthetic phospho-peptide corresponding to residues around Ser473 of mouse Akt (catalog number 559029 BD, Biosciences Pharmingen, San Diego, CA, or catalog number 9271, Cell Signaling Technology, Beverly, MA). An affinity-purified polyclonal antibody against the 12–amino acid (PTNDKAKAGNKP) COOH-terminal of mouse AS160 was used for its immunoprecipitation (6). Proteins phosphorylated on Akt phosphomotifs were detected using the rabbit anti–phospho-(Ser/Thr) Akt substrate (anti-PAS) antibody, a polyclonal affinity-purified antibody that recognizes several Akt phosphorylation motif peptide sequences [RXRXX(T*/S*)] (catalog number 9611; Cell Signaling Technology). This antibody was previously used to detect multiple proteins, including AS160, that were phosphorylated in insulin-stimulated 3T3-L1 adipocytes (6,7). Phosphorylated AMP-activated protein kinase (P-AMPK) was determined using an antibody that detects phosphorylation of AMPK at Thr^{172} (catalog number 2531; Cell Signaling Technology).

Animal treatment. Research protocols were approved by the University of Wisconsin-Madison Research Animal Review Committee and the University Committee on the Use and Care of Animals at the University of Michigan. Male Wistar rats purchased from Harlan (St. Louis, MO) were housed in a 12 h:12 h/light:dark cycle (lights off at 1800) and allowed free access to HarlanTeklad rodent food (Madison, WI) until 1700 the night before the experiment, when they were restricted to ≤5 g of food. The next day between 1030 and 1330, rats (150–190 g body wt) were anesthetized with an intraperitoneal injection of sodium pentobarbital (70 mg/kg wt). While rats were under deep anesthesia, both epitrochlearis muscles were rapidly dissected out.

Muscle incubations for insulin stimulation. Epitrochlearis muscles used to study the effects of insulin were incubated in glass vials containing Krebs-Henseleit buffer (KHB) + 0.1% BSA supplemented with 8 mmol/l glucose (KHB + BSA + glucose) or were shaken 60 min in a water bath at 35°C with continuous gassing (95% O_2/5% CO_2). Temperature, shaking, and gassing remained constant throughout all subsequent incubations. After the initial incubation, some muscles were immediately blotted, rapidly trimmed of connective tissue, and freeze-clamped with liquid N_2–cooled aluminum tongs and then stored at −80°C until subsequent homogenization and analysis. These muscles were used for basal (no insulin) values. To determine the time course for insulin-stimulated phosphorylation of Akt and its putative substrates, other muscles were transferred to a vial containing KHB + BSA + glucose supplemented with 120 nmol/l insulin for 1, 2.5, 5, 10, 15, 30, or 60 min.

Other muscles, used to characterize insulin's dose response for signaling, underwent an initial 60-min incubation before being transferred to vials containing KHB + BSA + glucose and varying insulin concentrations (0, 0.15, 0.3, 0.6, 12, or 120 nmol/l) for a 30-min incubation period. Subsequently, all muscles were rapidly blotted, trimmed of connective tissue, and freeze-clamped with liquid N_2–cooled aluminum tongs then and stored at −80°C until subsequent homogenization and analysis.

The PI 3-kinase inhibitor wortmannin was dissolved in DMSO at 1 mmol/l and stored at −20°C until used. Muscles were incubated in vials containing KHB + BSA + glucose supplemented with wortmannin (final concentration of 500 nmol/l) or an equal volume of vehicle (DMSO; 0.05% final concentration) for 30 min at 35°C and then transferred to another vial with the same solution as the preceding vial but with or without insulin (120 nmol/l) supplementation for 30 min. Muscles were then frozen and stored as described above.

In vitro muscle contractions. Muscles dissected from other rats were used to study the effects of in vitro contractile activity. Both epitrochlearis muscles from each rat were mounted in a temperature-controlled bath. The distal end of the muscle was attached to a glass rod, and the proximal end was attached to a force transducer (Radnoti, Litchfield, CT) as previously described (13). The mounted muscles were preincubated for 30 min in KHB + 2 mmol/l Na pyruvate with continuous gassing of 95% O_2/5% CO_2 before the contraction protocol was begun. One muscle was then stimulated to contract (Grass S48 Stimulator; Grass Instruments, Quincy, MA) for 5 min in fresh KHB + 2 mmol/l Na pyruvate using a protocol previously described by Sakamoto et al. (9): pulse duration of 0.1 ms, pulse rate of 100 pulses/s, train duration of 10 s, train rate of 2/min, train duration of 10 s. The contralateral muscle remained attached in the bath for 5 min with passive tension set at 0.4 g but was not stimulated to contract. Immediately after the 5-min period of contraction or passive tension, the muscles were blotted, trimmed of connective tissue, frozen using liquid N_2–cooled tongs, and stored at −80°C until homogenization.

To determine the effect of wortmannin on contraction-stimulated phosphorylation of Akt and AS160, paired muscles were attached to the force transducer as described above and incubated for 30 min in KHB + 2 mmol/l Na pyruvate supplemented with wortmannin (100 or 500 nmol/l) or an equal volume of vehicle (DMSO). Muscles were then stimulated to contract as described above. Other muscles, serving as resting controls, were incubated with wortmannin or DMSO for the same duration as the contracting muscles before being blotted, trimmed, frozen, and stored at −80°C.

Muscle incubations for 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside stimulation. Paired muscles were incubated in vials containing KHB and 8 mmol/l glucose for 30 min at 35°C. Muscles were then transferred to another vial containing KHB + glucose, with or without 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) (2 mmol/l) for 40 min at 35°C. Subsequently, muscles were blotted, trimmed of connective tissue, frozen using liquid N_2–cooled tongs, and stored at −80°C until homogenization.

Homogenization. Frozen muscles were homogenized in 0.6 ml ice-cold homogenization buffer (20 mmol/l Tris-HCl, pH 7.4, 150 mmol/l NaCl, 1% NP40, 2 mmol/l Na_3VO_4, 10 mmol/l NaF, 2 mmol/l EDTA, 2 mmol/l EGTA, 2.5 mmol/l NaPP, 20 mmol/l β-glycerophosphate, 2 mmol/l PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 μg/ml leupeptin) using glass-on-glass tubes (Kontes, Vineland, NJ). Homogenates were subsequently rotated at 4°C for 1 h before being centrifuged (12,000*g* for 10 min at 4°C). A portion of the resultant supernatant was used to determine protein concentration by the bicinchoninic acid assay (14), and the remaining supernatant was stored at −80°C until it was further analyzed.

Immunoprecipitation. Homogenized muscle lysate (200–250 μg protein at 1 mg/1 ml) was incubated with 1.5–2 μg of anti-AS160 at 4°C with gentle rotation overnight, and then 100 μl of a 50% slurry of prewashed protein A agarose beads (Upstate, Lake Placid, NY) was added to the lysate + antibody mix. The lysate + antibody + bead mix was rotated at 4°C for 2 h before centrifugation at 2,600*g*, and the supernatant was aspirated. After washing (nine times with 300 μl homogenization buffer), the protein bound to the protein A beads was eluted with 50 μl 2 × SDS loading buffer and boiled before loading on a polyacrylamide gel.

Immunoblotting. After separation using SDS-PAGE, proteins were electrophoretically transferred to nitrocellulose, which was then rinsed with Tris-buffered saline plus Tween (TBST) (0.14 mol/l NaCl, 0.02 mol/l Tris base, pH 7.6, and 0.1% Tween), blocked with 5% nonfat dry milk in TBST for 1 h at room temperature, washed 3 × 5 min at room temperature, and treated with the primary antibody anti-PAS (1:1,000 in TBST + 5% BSA) overnight at 4°C. Blots were then washed 3 × 5 min with TBST; incubated with the secondary antibody, goat anti-rabbit IgG HRP conjugate (Upstate, Lake Placid, NY) (1:5,000 in TBST + 5% milk), for 1 h at room temperature; washed again 3 × 5 min with TBST; and developed with enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ).

Densitometry. Protein bands were quantified by the densitometric method (Bio-Rad GS-670; Bio-Rad, Hercules, CA). The amount of protein loaded in each lane for immunoblotting was within the linear range (i.e., for protein loaded vs. band density) using antibodies against P-Akt, PAS, and P-AMPK. Band densities were expressed relative to the respective basal values within each blot.

Statistical analysis. Statistical analyses were done using Sigma Stat version 2.0 (San Rafael, CA). Data are expressed as means ±SE. One-way ANOVA was used to determine significant differences in protein phosphorylation in the insulin time course and dose-response experiments. When data failed the Levene Median test for equal variance, the Kruskal-Wallis nonparametric ANOVA on ranks was used. A P value ≤0.05 was considered statistically significant. As appropriate, parametric (Dunnett) and nonparametric (Dunn) post hoc methods were used to identify which insulin-treated groups were significantly different from basal. Two-way ANOVA was used to determine

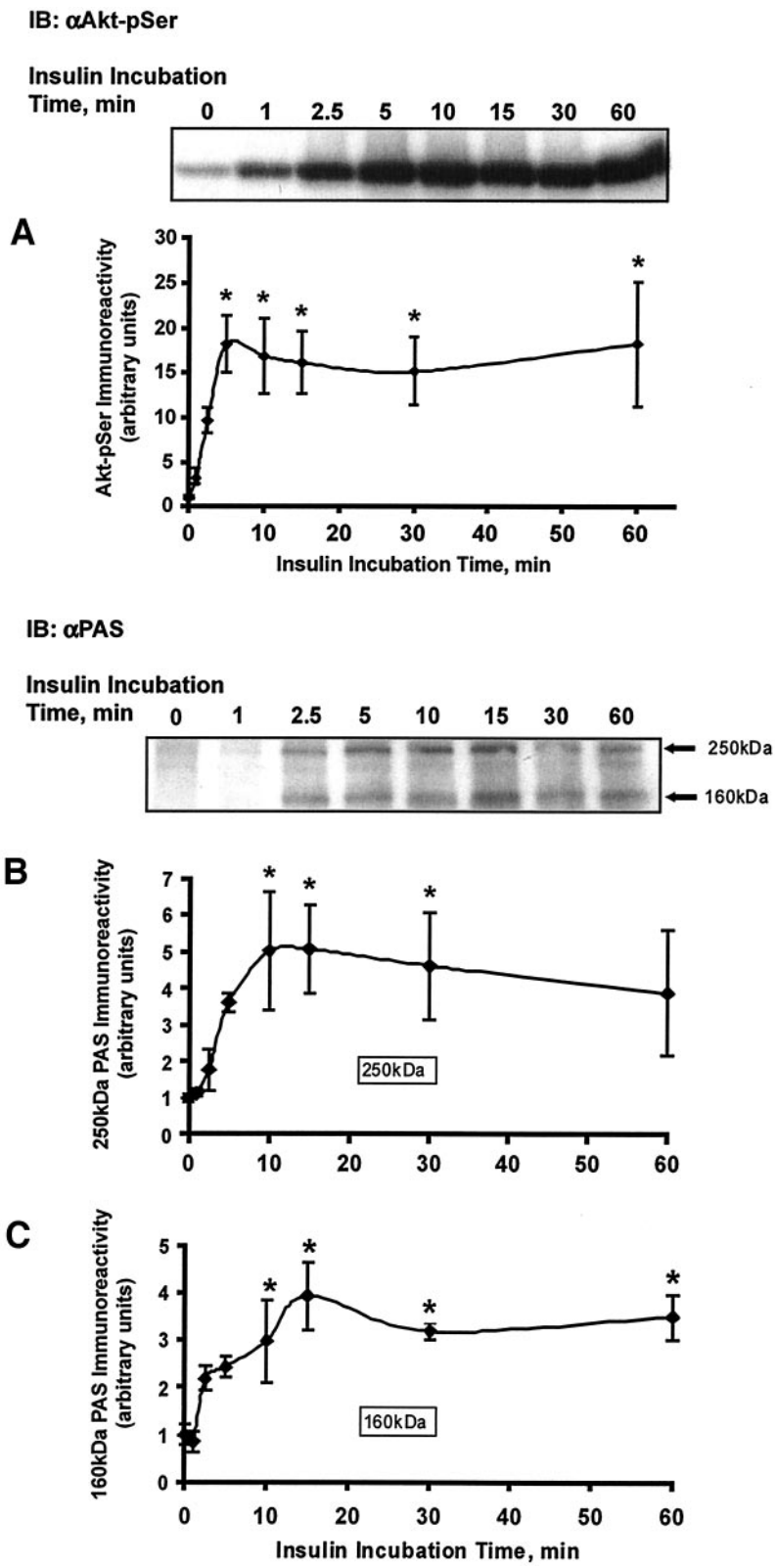

FIG. 1. Time course for insulin-stimulated (120 nmol/l) phosphorylation of Ser-Akt (*A*), PAS-250 (*B*), and PAS-160 (*C*). PAS-160 and PAS-250 were detected using the PAS-antibody against the Akt phosphomotif. Sample lanes were loaded with protein from supernatant of homogenates. Values (means ± SE, $n = 4$) are expressed relative to basal (no insulin). *$P < 0.05$ vs. basal. IB, immunoblotted.

significant differences in the experiments that evaluated wortmannin's effects on AS160 phosphorylation in insulin-stimulated or contraction-stimulated muscles, and the source of significant ($P \leq 0.05$) variance was detected with Tukey's post hoc test. For insulin-stimulated phosphorylation of AS160, a *t* test was used to compare basal and insulin-treated muscles. For contraction-stimulated effects on phosphorylation of Akt and its substrates and for AICAR-stimulated effects on phosphorylation of AMPK, Akt, and AS160, a paired *t* test was used to compare muscles stimulated by contraction or AICAR with their paired nonstimulated muscles. A paired *t* test was also used for phosphorylation of Akt in muscles stimulated by contraction or insulin compared with their contralateral muscles stimulated identically, but in the presence of wortmannin.

RESULTS

Time course of insulin. Akt-Ser phosphorylation tended to increase with 1 and 2.5 min of insulin exposure and increased significantly ($P < 0.05$) at 5 min of insulin

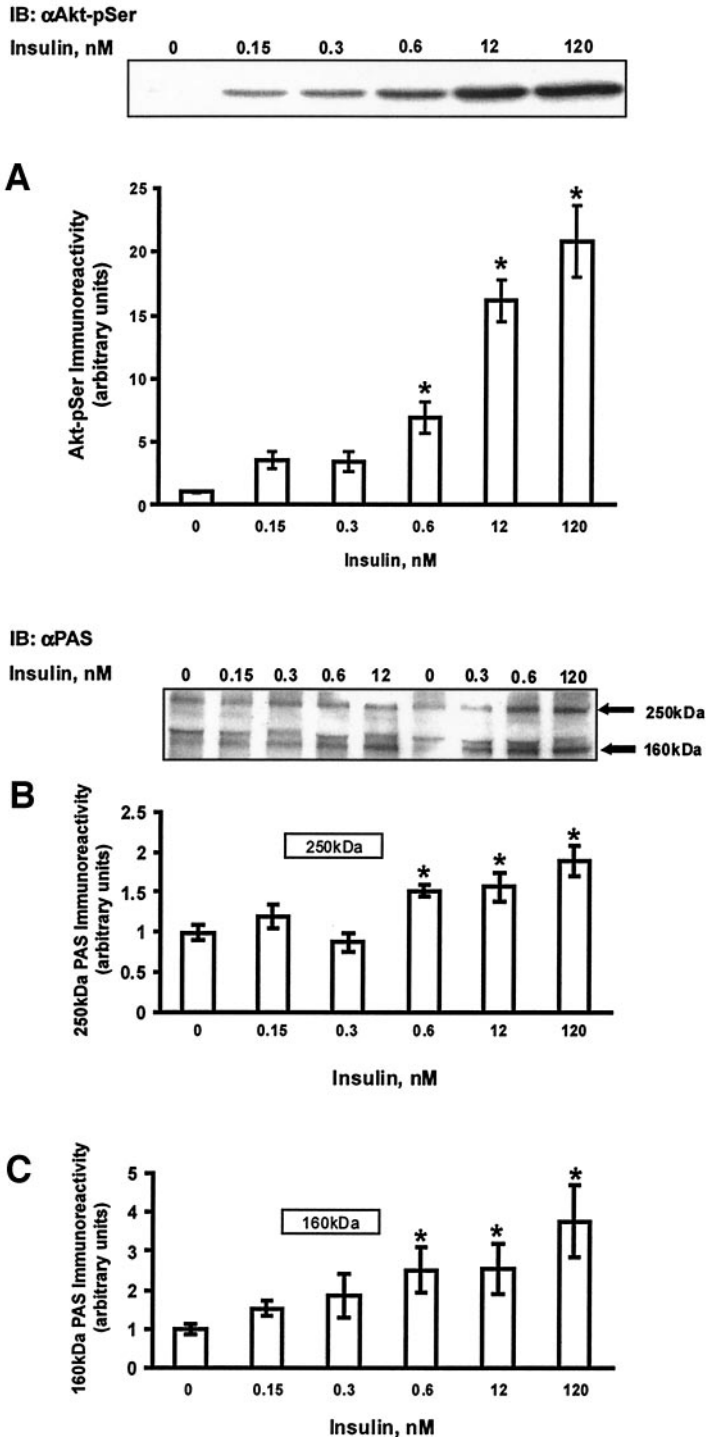

FIG. 2. Dose response for insulin-stimulated (0.15–120 nmol/l) phosphorylation of Ser-Akt (*A*), PAS-250 (*B*), and PAS-160 (*C*). Sample lanes were loaded with protein from supernatant of homogenates. Values (means ± SE, $n = 4–8$) are expressed relative to basal (no insulin). *$P < 0.05$ vs. basal. IB, immunoblotted.

incubation (18.2-fold above basal; Fig. 1*A*). Akt-Ser phosphorylation remained at approximately this level thereafter.

Two protein bands, migrating at ~160 kDa (PAS-160) and 250 kDa (PAS-250), consistently responded to insulin. The 160-kDa protein had a significant 3.0-fold increase in αPAS immunoreactivity at 10 min of insulin incubation ($P < 0.05$), and immunoreactivity peaked at 3.9-fold above basal at 15 min (Fig. 1*C*). With 10 min of insulin exposure, the αPAS immunoreactivity at 250 kDa peaked at 5.0-fold above basal ($P < 0.05$; Fig. 1*B*).

Insulin dose response. Akt-Ser phosphorylation was not significantly increased above basal with 0.15 or 0.3 nmol/l insulin, although there was a trend to increase at these insulin concentrations (Fig. 2*A*). A significant increase, sevenfold above basal, was evident with 0.6 nmol/l insulin ($P < 0.05$). Akt-Ser phosphorylation was even more dramatically elevated above basal with 12 nmol/l (16.2-fold) and 120 nmol/l (20.8-fold) insulin ($P < 0.05$).

The αPAS immunoreactivity at 160 kDa was 2.5-fold above basal in muscles incubated in 0.6 nmol/l insulin ($P < 0.05$; using nonparametric ANOVA and post hoc test; Fig. 2*C*). The 160-kDa αPAS immunoreactivity remained greater ($P < 0.05$) than basal with 12 nmol/l (2.6-fold) and 120 nmol/l (3.8-fold) insulin. The αPAS immunoreactivity

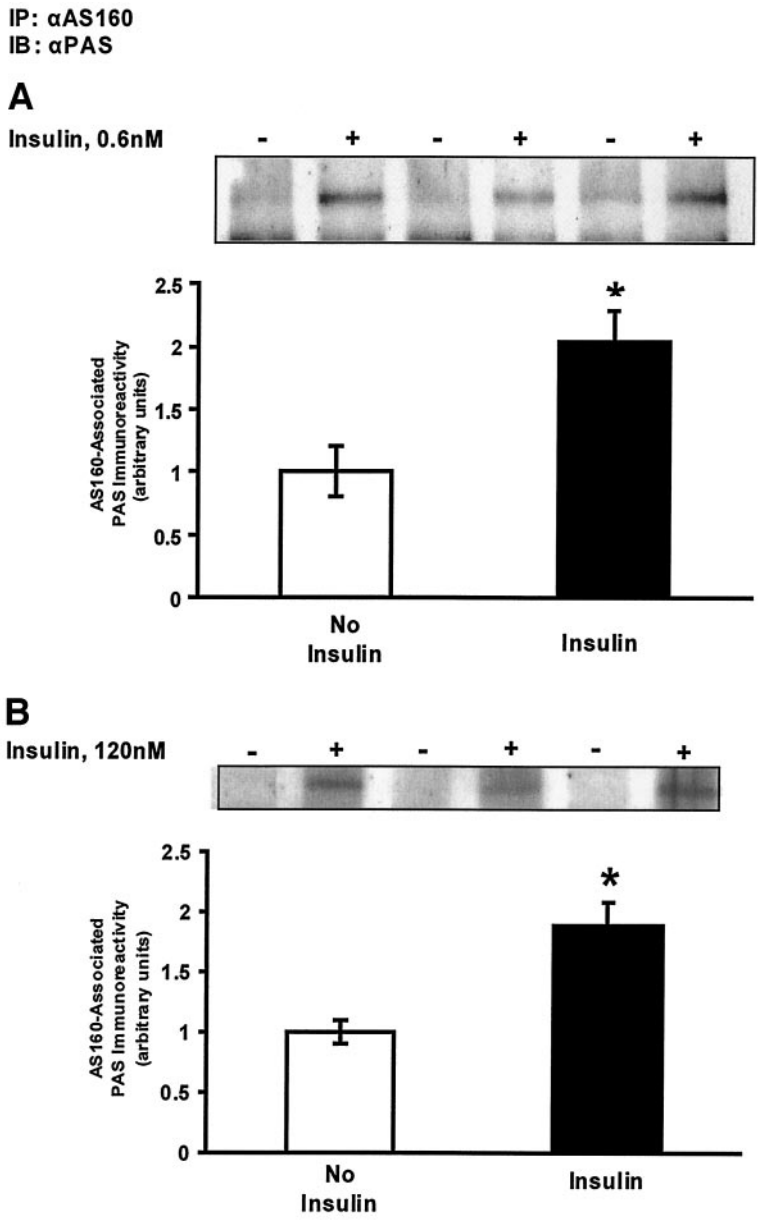

FIG. 3. Effect of insulin on phosphorylation of AS160. After incubation of muscles with varying insulin doses, samples were frozen, homogenized, and centrifuged. Supernatant was immunoprecipitated using anti-AS160. Phosphorylation on Akt phosphomotif was detected using PAS-antibody. *A*: Muscles were incubated without insulin or 0.6 nmol/l insulin. *B*: Muscles were incubated without insulin or 120 nmol/l insulin. Values (means ± SE, $n = 6–8$) are expressed relative to basal (no insulin). *$P < 0.01$ vs. basal. IB, immunoblotted; IP, immunoprecipitated.

at 250 kDa was 1.5-fold above basal in muscles incubated in 0.6 nmol/l insulin ($P < 0.05$), and this magnitude of increase was maintained with higher insulin concentrations (Fig. 2*B*).

Insulin-induced phosphorylation of AS160. To confirm that the αPAS immunoreactivity in the 160-kDa band was AS160, samples were immunoprecipitated using an antibody against AS160 and then immunoblotted with αPAS. With this approach, muscles that had been incubated with 0.6 nmol/l insulin had a 2.0-fold increase in AS160 phosphorylation over basal ($P < 0.01$; Fig. 3*A*). A similar increase above basal (1.9-fold) was found with 120 nmol/l insulin ($P < 0.01$; Fig. 3*B*).

Wortmannin effect on insulin-stimulated muscles. As expected, Akt-Ser phosphorylation for muscles incubated with 120 nmol/l insulin (15.30 ± 2.91; relative to basal = 1.00) was markedly inhibited ($P < 0.001$) in paired muscles ($n = 4$) incubated with insulin + wortmannin (1.96 ± 1.09). The insulin-stimulated (120 nmol/l) increase (5.1-fold above basal) in AS160 phosphorylation ($P < 0.01$) was also inhibited by wortmannin (Fig. 4).

Contraction-induced phosphorylation of Akt-Ser, Akt substrates, and AS160. Akt-Ser phosphorylation was 2.5-fold higher in muscles stimulated to contract compared with sham-treated contralateral controls ($P < 0.05$; Fig. 5*A*). Probing with αPAS revealed two putative Akt substrates that consistently responded to in vitro contraction with increased immunoreactivity: *1*) protein of ~160 kDa had a 1.5-fold increase compared with controls ($P < 0.01$; Fig. 5*D*), and *2*) protein of ~250 kDa had a 2.6-fold increase compared with controls ($P < 0.001$; Fig. 5*B*). In addition, αPAS immunoreactivity of a third protein band of ~180 kDa tended ($P = 0.07$) to be 1.6-fold greater in muscles after contractions compared with noncontracting controls (Fig. 5*C*). To confirm that the 160-kDa band was AS160, samples were immunoprecipitated with αAS160 before immunodetection using αPAS (Fig. 6). This analysis revealed that phosphorylation of AS160 was in-

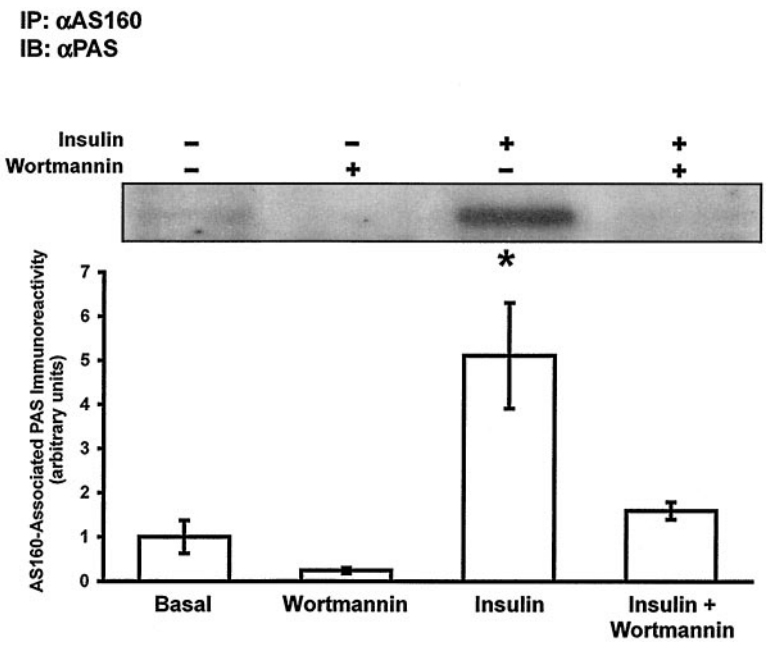

FIG. 4. Effect of wortmannin on insulin-stimulated AS160 phosphorylation. Supernatant from homogenized muscles was immunoprecipitated using anti-AS160. Phosphorylation on Akt phosphomotif was detected using PAS antibody. Values (means ± SE, $n = 4$) are expressed relative to basal (no insulin or wortmannin). *$P < 0.01$ vs. all other groups. IB, immunoblotted; IP, immunoprecipitated.

creased 3.7-fold above basal after contractile activity ($P < 0.05$).

To assess the possibility that passive tension led to increased phosphorylation of Akt or its putative substrates, we compared muscles that underwent passive tension treatment with other muscles that were incubated without fixed ends in glass vials (i.e., the treatment for the basal groups in the insulin experiments). No differences in protein phosphorylation were found between the passive tension and basal groups (data not shown).

Wortmannin effect on contraction-stimulated muscles. Akt-Ser phosphorylation in contraction-stimulated muscles (3.69 ± 0.84; relative to basal = 1.00) was eliminated ($P < 0.05$) in paired muscles ($n = 6$) that underwent contraction in the presence of 500 nmol/l wortmannin (1.13 ± 0.33). Wortmannin at either 100 or 500 nmol/l eliminated the 2.4-fold contraction-stimulated increase above basal in AS160 phosphorylation; therefore, pooled data from both concentrations are shown in Fig. 7 (contraction without wortmannin vs. all other groups; $P < 0.001$).

AICAR effect on muscles. As expected, muscles incubated with AICAR had a large (9.1-fold) increase ($P < 0.005$) in phosphorylated AMPK compared with paired controls (Fig. 8*A*). There was no evidence that AICAR-treated muscles compared with paired control muscles differed for Akt-Ser phosphorylation (Fig. 8*B*); however, AICAR treatment resulted in a 1.7-fold increase ($P < 0.05$) in AS160 phosphorylation (Fig. 8*C*).

DISCUSSION

There were two major findings of this study. First, in isolated rat epitrochlearis muscles, insulin led to a rapid increase in phosphorylation of Akt, with a subsequent elevation in phosphorylation of two potential Akt substrate proteins, including AS160, and these effects were induced by physiological insulin levels. Second, in vitro contraction elicited an increase in the phosphorylation of Akt-Ser and phosphorylation of two potential Akt substrate proteins, including AS160. Both insulin-stimulated and contraction-stimulated phosphorylations of AS160 were inhibited by wortmannin, concomitant with inhibition of Akt-Ser phosphorylation. Insulin and contraction also led to increased phosphorylation of a 250-kDa protein on Akt phosphomotifs.

The time course for insulin's effects on Akt phosphorylation was slightly more rapid than the results reported by Song et al. (10), likely because of the higher incubation temperature in our experiments (35°C compared with 30°C). The significant increase in Akt-Ser phosphorylation with 0.6 nmol/l insulin is in agreement with results from Derave et al. (15), who found that Akt activity was also increased in perfused rat hindlimb with 0.6 nmol/l insulin. The current study appears to be the first published characterization of the insulin dose response for Akt-Ser phosphorylation in isolated skeletal muscle.

Immunoblotting with αPAS revealed that two proteins, ~250 kDa (PAS-250) and ~160 kDa (PAS-160), became phosphorylated in response to insulin, with peak values at 10 and 15 min, respectively. Akt-Ser phosphorylation increased more rapidly (peaked at 5 min), consistent with a mechanism whereby insulin-stimulated Akt-Ser phosphorylation was required for subsequent phosphorylation of PAS-250 and PAS-160. The significantly increased phosphorylation of PAS-250 and PAS-160 with 0.6 nmol/l insulin indicates that these modifications may be physiologically relevant for insulin action in skeletal muscle.

Using the PAS antibody with 3T3-L1 adipocytes, Kane et al. (6) identified seven insulin-responsive putative Akt substrates, including proteins of 160 and 250 kDa. We confirmed that the 160-kDa protein that responded to insulin in skeletal muscle was AS160, consistent with the results in adipocytes. The 250-kDa protein in 3T3-L1 adipocytes was recently identified by mass spectrometry and found to include a predicted GTPase-activated domain for Rheb and Rap (16). Future research will be needed to determine if the insulin-responsive 250-kDa protein in skeletal muscle corresponds to the insulin-responsive 250-kDa protein found in 3T3-L1 adipocytes. Gridley et al. (16)

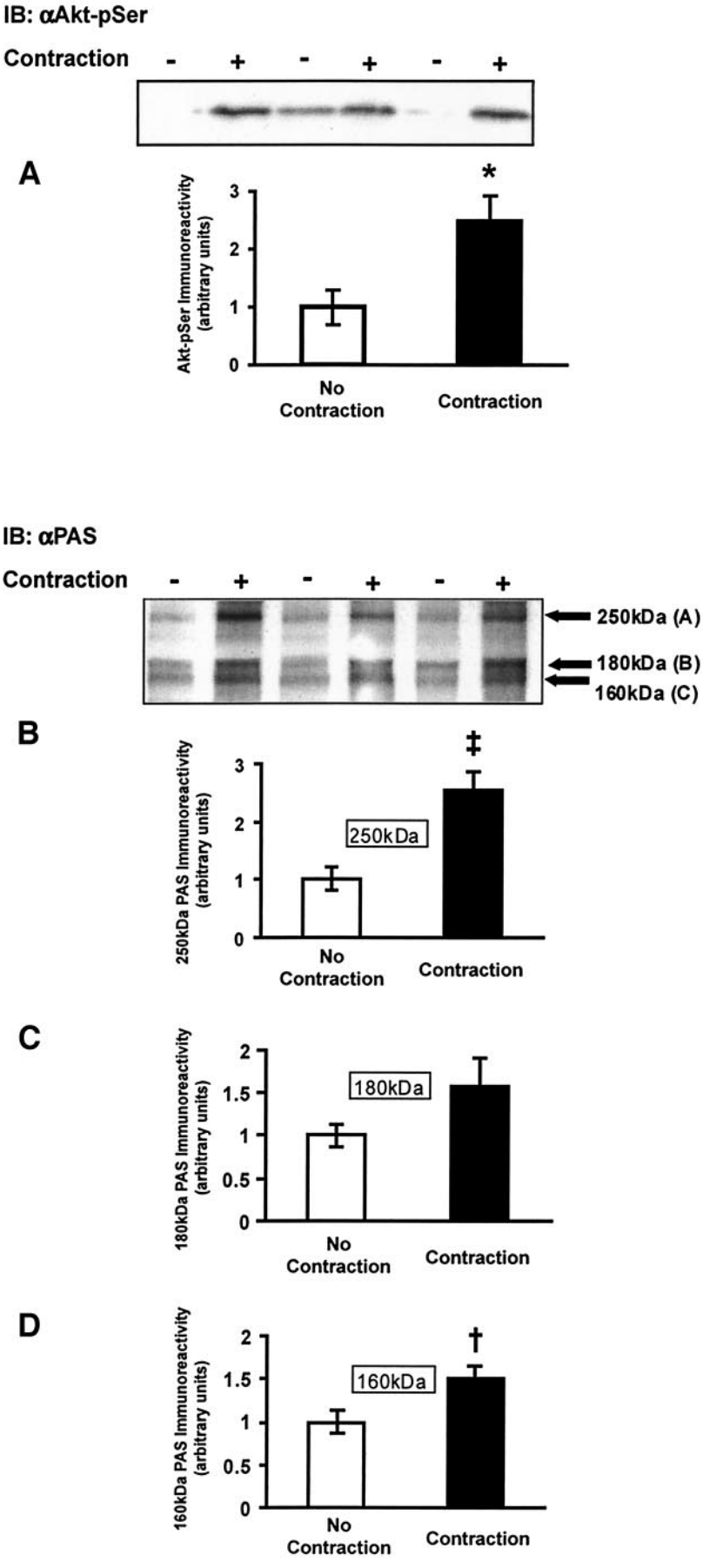

FIG. 5. Effect of in vitro contraction on phosphorylation of Ser-Akt (*A*), PAS-250 (*B*), PAS-180 (*C*), and PAS-160 (*D*). Sample lanes were loaded with protein from supernatant of homogenates. Values (means ± SE, $n = 8$) are expressed relative to the mean of paired muscles not stimulated to contract (no contraction). *$P < 0.05$ vs. no contraction; ‡$P < 0.001$ vs. no contraction; †$P < 0.01$ vs. no contraction. IB, immunoblotted.

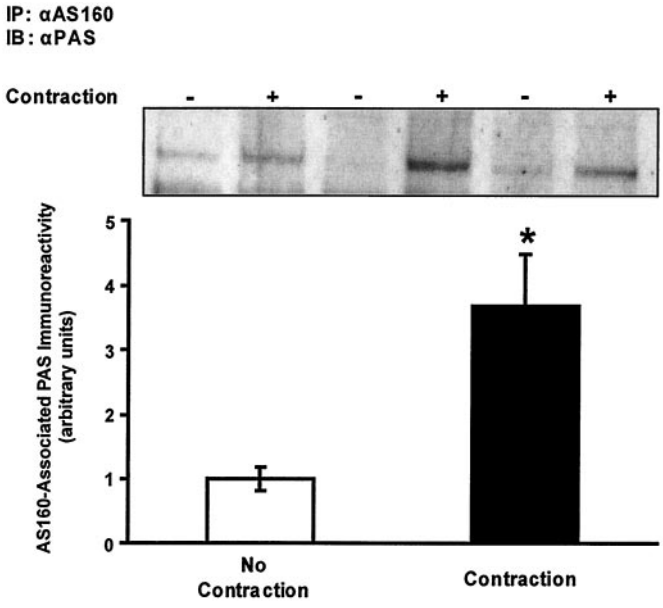

FIG. 6. Effect of contraction on phosphorylation of AS160. Immediately after contractile activity, stimulated muscles and noncontracting controls were frozen, homogenized, and centrifuged. Supernatant was immunoprecipitated using anti-AS160. Phosphorylation on Akt phosphomotif was detected using PAS antibody. Values (means ± SE, $n = 7$) are expressed relative to the mean of muscles not stimulated to contract (no contraction). *$P < 0.05$ vs. no contraction. IB, immunoblotted; IP, immunoprecipitated.

identified a 105-kDa protein of unknown function that, in 3T3-L1 adipocytes, responded to insulin with elevated immunoreactivity against the PAS antibody, and they found the protein to be highly abundant in skeletal muscle, although they did not assess insulin's effects in muscle. We consistently observed strong αPAS immunoreactivity at ~100 kDa that did not respond to any of our interventions (data not shown).

As expected, wortmannin inhibited the effect of insulin on Akt-Ser phosphorylation. Wortmannin also blocked insulin-stimulated phosphorylation of AS160, in support of the idea that AS160 was phosphorylated by Akt. Furthermore, taken together, our results are consistent with the hypothesis that AS160 phosphorylation is important for insulin-stimulated glucose transport in skeletal muscle, as it is in 3T3-L1 adipocytes.

Our data in epitrochlearis muscles that were stimulated to contract confirm the findings of Sakamoto et al. (9) for rat extensor digitorum longus muscles: in both muscles, in vitro contraction induced a rapid increase in Akt-Ser phosphorylation. While immunoblotting with αPAS, we identified two putative Akt substrates (160- and 250-kDa proteins) that were significantly phosphorylated in response to in vitro contraction. Immunoprecipitation with αAS160 and subsequent immunodetection using αPAS demonstrated that the contraction-responsive protein of 160 kDa was AS160.

Wortmannin blocked contraction-stimulated Akt-Ser phosphorylation in agreement with observations of Sakamoto et al. (9), who found that wortmannin (100 or 500 nmol/l) eliminated the contraction-induced increase in Akt activity. Contractile activity does not increase class I_A (9) or class II (17) PI 3-kinase activity, leading Sakamoto et al. to suggest that muscle contractions may activate class I_B PI 3-kinase, leading ultimately to Akt phosphorylation (9). Regardless of the mechanism for activating Akt, we found

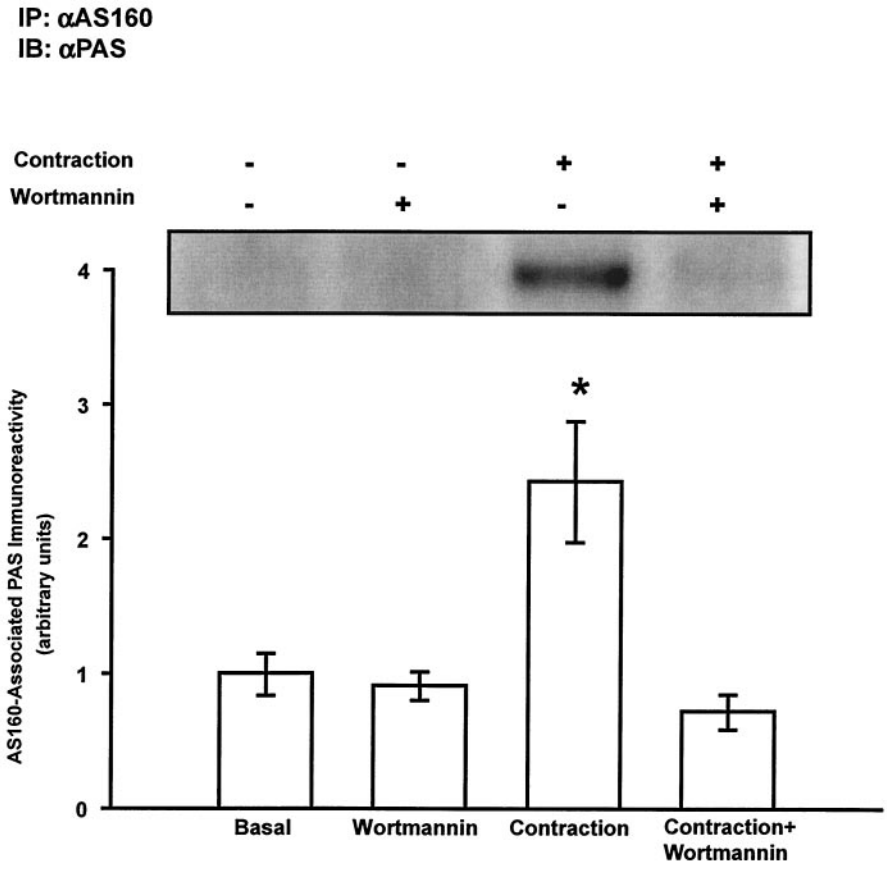

FIG. 7. Effect of wortmannin (100 or 500 nmol/l) and contraction on phosphorylation of AS160. Supernatant from homogenates was immunoprecipitated using anti-AS160. Phosphorylation on Akt phosphomotif was detected using the PAS antibody. Wortmannin at either 100 or 500 nmol/l eliminated the contraction-stimulated increase in phosphorylation of AS160; therefore, the figure represents the pooled data for both concentrations. Values (means ± SE, $n = 7–8$) are expressed relative to basal (no contraction or wortmannin). *$P < 0.001$ vs. all other groups. IB, immunoblotted; IP, immunoprecipitated.

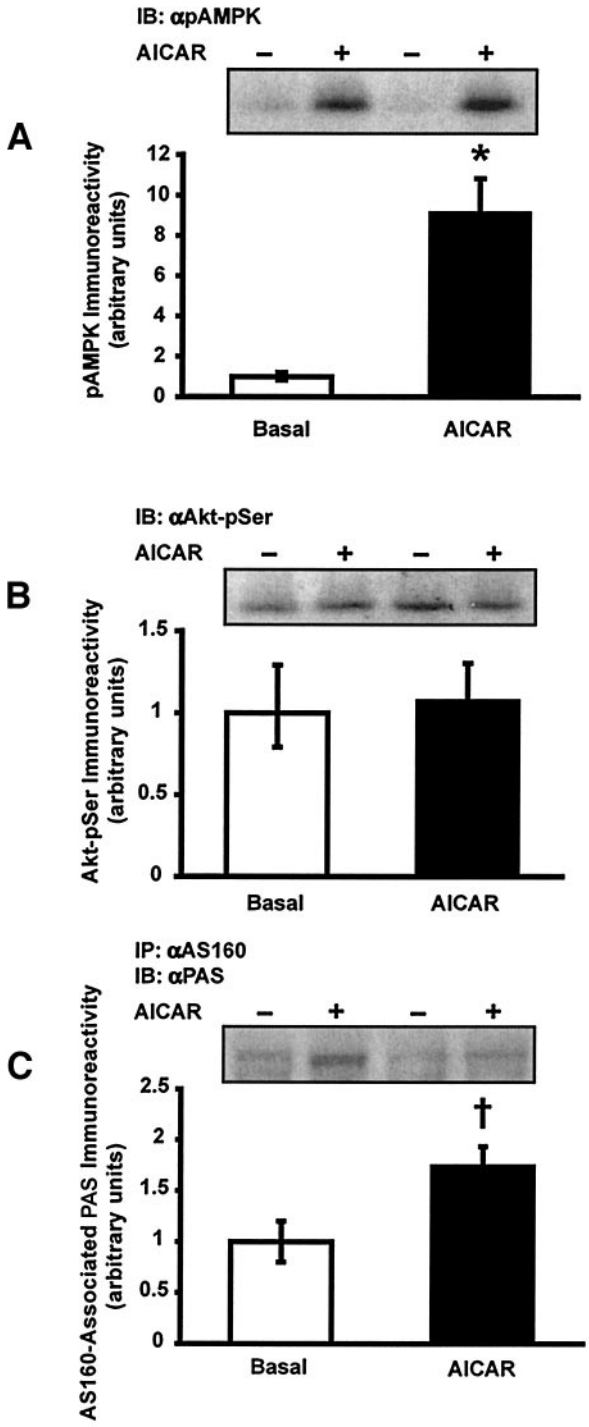

FIG. 8. Effect of AICAR on phosphorylation of AMPK (*A*), Akt-Ser (*B*), and AS160 (*C*). For AMPK and Akt-Ser, sample lanes were loaded with protein from supernatant of homogenates. For AS160 phosphorylation, supernatant from homogenates was immunoprecipitated using anti-AS160. Phosphorylation on Akt phosphomotif was detected using the PAS antibody. Values (means ± SE, $n = 8$ for AMPK, $n = 4$ for Akt, and $n = 4$ for PAS-160) are expressed relative to the mean of paired muscles without AICAR (basal). *$P < 0.005$ vs. basal; †$P < 0.05$ vs. basal. IB, immunoblotted; IP, immunoprecipitated.

that wortmannin also inhibited contraction-stimulated phosphorylation of AS160. This result is especially notable because wortmannin (100–2,000 nmol/l) does not inhibit contraction-stimulated glucose transport by isolated skeletal muscle (18–21). The phosphomotifs on AS160 identified by PAS immunoreactivity, which were responsive to contractile activity and inhibited by wortmannin, are evidently not essential for contraction-stimulated glucose transport. It remains possible that regulation of AS160 is relevant for contraction-stimulated glucose transport, e.g., by Akt-independent phosphorylation on sites not recognized by αPAS.

As expected, AICAR treatment resulted in a robust increase in AMPK phosphorylation, concomitant with no detectable change in Akt-Ser phosphorylation. Therefore, the effect of AICAR on AS160 phosphorylation was apparently attributable to AMPK activation and independent of Akt activation. In support of this interpretation, purified AMPK can phosphorylate purified AS160 in vitro, as detected with the PAS antibody (H. Sano, G.E.L., unpublished data). Both AMPK and Akt have been demonstrated to phosphorylate Ser^{466} of the cardiac isoform of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (22), providing a precedent for the idea that these kinases also share a common phosphorylation site on AS160. However, it is also possible that AMPK and Akt phosphorylate different AS160 sites that are immunoreactive with the PAS antibody.

Wortmannin does not inhibit the AMPK activity of purified enzymes (23) or the AMPK activity from $H\text{-}2K^b$cells stimulated by hyperosmotic stress (24). Thus, the wortmannin-induced inhibition of AS160 phosphorylation in contraction-stimulated muscles was likely the result of blocking the PI-3 kinase–phosphoinositide-dependent kinase-1–Akt signaling pathway and not inhibition of AMPK. However, it is unclear why a residual effect of AMPK on AS160 phosphorylation was not detected in the muscles incubated with wortmannin during contractile activity, which is known to activate AMPK (18,25).

An ~180-kDa protein tended to have a contraction-induced increase in immunoreactivity against PAS, but there was no detectable response to insulin in this protein, even at supraphysiological concentrations of insulin, either in isolated skeletal muscle or 3T3-L1 adipocytes (6,7), indicating that increased Akt-Ser phosphorylation was insufficient for increasing PAS immunoreactivity of this protein. It seems worthwhile to pursue the identity of PAS-180. One candidate is tuberin, a protein (molecular weight of 180–200 kDa) that is a substrate for Akt in HEK-293 and HeLa cells (26,27). Tuberin has multiple roles, including inhibition of cell growth and tumorigenesis, and phosphorylation of tuberin by Akt is thought to relieve tuberin-mediated cell growth inhibition (28). Additional studies will be needed to determine if PAS-180 is tuberin and, if so, to understand why insulin did not cause a detectable increase in its PAS immunoreactivity in isolated skeletal muscle.

In conclusion, two putative Akt substrates, PAS-250 and AS160, were phosphorylated in insulin-stimulated skeletal muscle with time courses and dose responses that are consistent with insulin's rapid physiological actions. Although the specific bioeffects influenced by the phosphorylation of these proteins in skeletal muscle have not been established, in light of results with 3T3-L1 adipocytes (6–8), it seems reasonable to suspect that phosphorylation of AS160 in skeletal muscle is part of the insulin signaling pathway leading to stimulation of GLUT4 trans-

location and glucose transport. AS160 phosphorylation was also increased by in vitro contractile activity or incubation with AICAR, although only contraction led to elevated Akt-Ser phosphorylation. The contraction-induced increase in AS160 phosphorylation was inhibited by wortmannin, indicating that contraction's effect on phosphorylation of AS160 was likely attributable to PI 3-kinase–mediated activation of Akt. Future research will be needed to fully understand these results, e.g., to determine if insulin, contractile activity, and AICAR act on distinct or overlapping pools of AS160; to identify the specific phosphomotifs on AS160, as detected by PAS immunoreactivity, that are modulated by each stimulus; and, ultimately, to determine the bioeffects influenced by AS160 and other novel Akt substrates in skeletal muscle.

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