Original Article

Distinct Signals Regulate AS160 Phosphorylation in Response to Insulin, AICAR, and Contraction in Mouse Skeletal Muscle

Henning F. Kramer,[1] Carol A. Witczak,[1] Nobuharu Fujii,[1] Niels Jessen,[1] Eric B. Taylor,[1] David E. Arnolds,[1] Kei Sakamoto,[1] Michael F. Hirshman,[1] and Laurie J. Goodyear[1,2]

Insulin and contraction increase GLUT4 translocation in skeletal muscle via distinct signaling mechanisms. Akt substrate of 160 kDa (AS160) mediates insulin-stimulated GLUT4 translocation in L6 myotubes, presumably through activation of Akt. Using in vivo, in vitro, and in situ methods, insulin, contraction, and the AMP-activated protein kinase (AMPK) activator AICAR all increased AS160 phosphorylation in mouse skeletal muscle. Insulin-stimulated AS160 phosphorylation was fully blunted by wortmannin in vitro and in Akt2 knockout (KO) mice in vivo. In contrast, contraction-stimulated AS160 phosphorylation was only partially decreased by wortmannin and unaffected in Akt2 KO mice, suggesting additional regulatory mechanisms. To determine if AMPK mediates AS160 signaling, we used AMPK α2-inactive (α2i) transgenic mice. AICAR-stimulated AS160 phosphorylation was fully inhibited, whereas contraction-stimulated AS160 phosphorylation was partially reduced in the AMPK α2i transgenic mice. Combined AMPK α2 and Akt inhibition by wortmannin treatment of AMPK α2 transgenic mice did not fully ablate contraction-stimulated AS160 phosphorylation. Maximal insulin, together with either AICAR or contraction, increased AS160 phosphorylation in an additive manner. In conclusion, AS160 may be a point of convergence linking insulin, contraction, and AICAR signaling. While Akt and AMPK α2 activities are essential for AS160 phosphorylation by insulin and AICAR, respectively, neither kinase is indispensable for the entire effects of contraction on AS160 phosphorylation. *Diabetes* 55:2067–2076, 2006

From the [1]Metabolism Research Division, Joslin Diabetes Center, Boston, Massachusetts; and the [2]Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts.

Address correspondence and reprint requests to Laurie J. Goodyear, Section Head, Metabolism, Joslin Diabetes Center, One Joslin Pl., Boston, MA 02215. E-mail: laurie.goodyear@joslin.harvard.edu.

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α2i, α2-inactive; AMPK, AMP-activated protein kinase; aPKC, atypical protein kinase C; AS160, Akt substrate of 160 kDa; EDL, extensor digitorum longus; GAP, GTPase-activating protein; PAS, phospho-Akt substrate; PI3-K, phosphatidylinositol 3-kinase.

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Insulin and exercise share the capacity to increase glucose uptake into skeletal muscle and positively regulate glucose homeostasis in healthy individuals and people with type 2 diabetes. Each stimulus, alone and in combination, promotes migration of intracellular GLUT4 proteins to the cell membrane, thereby facilitating glucose uptake from blood into muscle for storage and/or utilization (1–3). Insulin exerts these partitioning effects through a well-characterized signaling network involving its transmembrane insulin receptor, insulin receptor substrate proteins, and downstream engagement of phosphatidylinositol 3-kinase (PI3-K). Briefly, activity of PI3-K catalyzes the formation of phosphatidylinositol 3,4,5-triphosphate, which in turn recruits the serine/threonine kinase Akt to the cell membrane, where it can be phosphorylated by phosphoinositide-dependent kinase-1 (1). In addition, both PI3-K and its lipid products directly activate atypical protein kinase C (aPKC) isoforms (4). The resultant pools of activated Akt (5), particularly the Akt2 isoform (6), as well as aPKC (4), have been proposed to be critical for insulin-mediated GLUT4 translocation from endosomal storage sites to the sarcolemma. However, the intermediate substrates connecting these signals with the GLUT4 machinery are only beginning to emerge.

Akt substrate of 160 kDa (AS160) is a Rab GTPase-activating protein (GAP) that modulates GLUT4 trafficking in insulin-sensitive 3T3-L1 adipocytes (7) and L6 myoblasts (1). The most striking structural features of AS160 are two phosphotyrosine binding domains, a COOH-terminal Rab-GAP domain, as well as six phosphorylation motifs (R*X*R*XX*S*/T*) targeted by, but not exclusive for, Akt (Ser^{318}, Ser^{341}, Ser^{570}, Ser^{588}, Thr^{642}, and Ser^{751}) (8,9). In addition, a yeast two-hybrid screen recently identified an AS160 calmodulin binding region positioned just NH_2-terminal to the GAP domain (10). Under basal conditions, AS160 exists primarily in an unphosphorylated state and retains GLUT4 vesicles intracellularly through the activity of its GAP domain (9,11). When cells are treated with insulin, AS160 is rapidly phosphorylated at multiple Akt phospho-motifs (8). Both 3T3-L1 adipocytes (7) and L6 GLUT4-myc myoblasts (1) transfected with a constitutively active mutant AS160 (incapable of being phosphorylated at these regulatory sites) exhibit significantly reduced insulin-induced GLUT4 translocation. AS160 phosphorylation therefore appears to suppress and/or alter the intrinsic GAP activity of the protein such that exocytosis of GLUT4 vesicles is permitted.

Muscle contractions and exercise also potently stimulate glucose uptake but in a manner independent of and distinct from insulin (2,3). This insulin-independent mechanism is critical in the maintenance of whole-body glucose homeostasis, especially in people with type 2 diabetes, in whom there are defects in insulin action but normal or near-normal effects of exercise on glucose disposal. Multiple metabolic, chemical, and mechanical factors have been implicated in contraction-mediated GLUT4 translocation, though pinpointing a primary mechanism has remained elusive. A growing body of literature suggests that increases in the activity of AMP-activated protein kinase (AMPK), calmodulin-dependent protein kinases, and aPKC isoforms could all potentiate increases in glucose transport in response to exercise (12–14). In addition, Akt activity can increase with muscle contractions but not through class I_A PI3-K (15). These findings are compelling in light of a recent study (16) that reported increased AS160 phosphorylation with in vitro contractions in rat epitrochlearis muscles. This raises the possibility that AS160 operates as a common, downstream point of convergence mediating the effects of both insulin and contraction on GLUT4 translocation.

While Akt is known to phosphorylate AS160 at key regulatory phospho-motifs, it is possible that additional kinases phosphorylate AS160 at these sites and/or contraction-specific phosphorylation sites. For example, AMPK is a plausible candidate since it recognizes and phosphorylates substrates along consensus sequences similar to Akt (17–20), and AICAR treatment in rat muscle increases AS160 phosphorylation (16). In the current study, we first assessed the regulation of AS160 in response to insulin, contraction, and AICAR in mouse skeletal muscle. Once this was established, our primary objective was to determine the signaling network(s) mediating skeletal muscle AS160 phosphorylation.

RESEARCH DESIGN AND METHODS

Antibodies. Total AS160 was detected using an affinity-purified mouse pan-AS160 antibody (7,8). AS160 phosphorylation was detected with a phospho-Akt substrate (PAS) antibody (Cell Signaling Technology, Danvers, MA), and a custom lot of anti–phospho-AS160 Thr^{642} antibody (44-1071G; Biosource International, Camarillo, CA). This latter antibody was purified via epitope-specific chromatography and targets the peptide fragment RRRAH[pT]FSHPPS on AS160. Its specificity was validated using mutant AS160 incapable of phosphorylation at T^{642}. AMPK Thr^{172} phosphorylation (Biosource International) and phosphorylation of Akt Thr^{308} (Cell Signaling Technology) were also determined. A horseradish peroxidase–conjugated anti-rabbit antibody (Amersham Biosciences, Piscataway, NJ) was used to bind and visualize detections of all primary antibodies.

Animals. Protocols for animal use were reviewed and approved by the Institutional Animal Care and Use Committee of the Joslin Diabetes Center and were in accordance with National Institutes of Health guidelines. Female ICR mice (aged 8–10 weeks) were purchased from Taconic (Hudson, NY). Male and female Akt2 knockout (KO) mice and wild-type littermates (C57BL/6N background; aged 10–14 weeks) were kindly provided by Dr. Morris Birnbaum (18), while α2-inactive (α2i) AMPK transgenic mice and wild-type littermates (FVB background; aged 10–14 weeks) were generated as described previously (21). All mice were housed in a 12:12-h light:dark cycle and fed standard laboratory diet and water ad libitum. Mice allocated for in vivo insulin protocols were fasted overnight (10:00 P.M. to 8:00 A.M.) before the morning of the experiment.

In vitro muscle incubations. Mice were killed by cervical dislocation, and extensor digitorum longus (EDL) muscles were rapidly dissected and preincubated in Krebs-Ringer bicarbonate buffer plus 2 mmol/l pyruvate for 20 min, as described (15). Incubations were conducted in the absence or presence of maximal insulin (50 mU/ml) for 20 min, AICAR (2 mmol/l) for 40 min, and/or contraction for 5 min (see below). For inhibition of PI3-K activity, muscles were coincubated with 100 nmol/l wortmannin dissolved in 0.05% DMSO. Muscles were immediately frozen in liquid N_2 following each treatment.

In vivo insulin administration. Fasted mice were assayed for basal blood glucose before intraperitoneal injections of 0.9% NaCl or maximal insulin (0.5 units/mouse). After 10 min, blood glucose concentrations were measured to confirm an insulin response. Mice were cervically dislocated, and hindlimb muscles were immediately removed and frozen in liquid N_2.

Treadmill exercise. All mice were initially familiarized to the exercise protocol by running on a rodent treadmill over 3 separate days, during which they performed 10 min of exercise at 0.4 (day 1), 0.6 (day 2), and 0.8 (day 3) mph, with 0% grade. Three days later, mice were divided into basal or exercise groups. Exercised animals performed 30 min of running at 0.8 mph and 20% grade, or the equivalent of a moderate work pace, while basal animals remained sedentary. Mice were immediately killed following the exercise or basal interval, and gastrocnemius muscles were harvested and frozen in liquid N_2.

In situ and in vitro muscle contractions. Hindlimb muscles from anesthetized mice (pentobarbital 90 mg/kg) were contracted in situ by electrically stimulating sciatic or peroneal nerves, as described (15). While one leg was left unstimulated (basal/sham control), the other leg was subjected to contractions for 5 or 10 min. Mice were immediately killed following the experiment, and muscles were harvested and frozen in liquid N_2. In vitro tetanic contractions were induced for 5 min using established parameters (15). Force production was captured by IWORX 118 USB hardware (CB Sciences, Dover, NH) and analyzed with LabScribe software (CB Sciences). In experiments utilizing α2i transgenic mice, force production between transgenic and wild-type littermates was normalized, as done previously (21). Briefly, transgenic EDL was contracted using maximal in vitro stimulation settings (mean of 14*g*/10 s), while wild-type muscles were only stimulated enough to match the force output generated by transgenic muscles (~50% of maximal force capacity). This precise voltage was determined using two to three brief contractions (pulse rate of 125 Hz for 180 ms) before the 5-min contraction protocol. Following contraction, muscles were immediately frozen in liquid N_2.

Tissue processing and immunoblotting. Frozen muscles were pulverized and homogenized as described previously (15), and protein concentrations were determined via Bradford assay. Equal amounts of skeletal muscle proteins (40–50 μg) were resolved by SDS-PAGE (22) for Western blot analysis (23). Antibody-bound proteins were visualized using enhanced chemiluminescence (Amersham Biosciences). Protein bands were scanned by ImageScanner (Amersham Biosciences) and quantitated by densitometry (FluorChem 2.0; Alpha Innotech, San Leandro, CA).

Statistical analysis. Data are expressed as means ± SE. Statistical analyses were performed using a paired Student's *t* test and one-way ANOVA. When ANOVA revealed significant differences, Tukey's post hoc test for multiple comparisons was performed. P values <0.05 were considered statistically significant.

RESULTS

Insulin-stimulated AS160 phosphorylation. We initially determined whether insulin regulates AS160 phosphorylation in isolated mouse skeletal muscle using two different antibodies. First, phosphorylation of AS160 at known insulin-responsive motifs was detected with the PAS antibody, which recognizes as many as six sites on AS160 bearing the amino acids R*X*R*XX*S*/T* (where "*" indicates residue of phosphorylation) (7,8). Note that PAS immunoreactivity is not exclusive for Akt phosphorylation events, as other arginine-sensitive kinases are able to phosphorylate substrates along similar PAS-detectable sequences (17,19,20,24,25). In addition, samples were also immunoblotted with a modification-state antibody, specific for phosphorylation at the AS160 Thr^{642} residue in mouse skeletal muscle. This latter motif is recognized by both antibodies and has been demonstrated to be the primary target of Akt in insulin-treated 3T3-L1 cells (7,8).

In vitro insulin treatment significantly increased AS160 phosphorylation in isolated mouse EDL muscle using both anti–phospho-AS160 antibodies ($P < 0.05$; Fig. 1*A* and *B*). To understand whether the insulin-stimulated increase in AS160 phosphorylation in vitro is mediated through activation of Akt, we cotreated EDL muscles with insulin and the PI3-K inhibitor wortmannin (100 nmol/l). Wortmannin completely inhibited insulin-stimulated Akt phosphorylation at Thr^{308} (Fig. 1*C*) and concomitantly suppressed

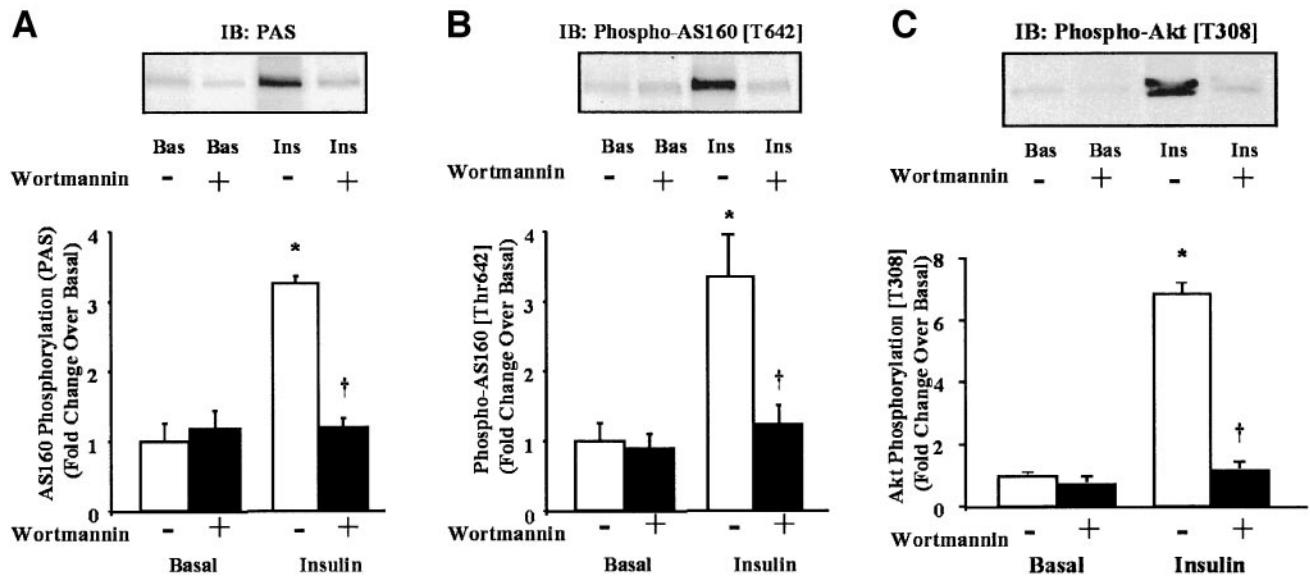

FIG. 1. Effects of in vitro insulin on AS160 phosphorylation in EDL muscle. EDL muscles were incubated in the absence (-) or presence (+) of 100 nmol/l wortmannin as described in RESEARCH DESIGN AND METHODS. Immunoblotting (IB) was with anti-PAS (*A*), anti–phospho-AS160 Thr^{642} (*B*), and anti–phospho-Akt Thr^{308} (*C*) antibodies. Data are means ± SE; *n* = 6/group. *P < 0.05 vs. basal.

AS160 phosphorylation (Fig. 1*A* and *B*). These results demonstrate the involvement of Akt in insulin-induced AS160 phosphorylation in vitro in mouse skeletal muscle.

We next assessed whether insulin stimulates AS160 phosphorylation in skeletal muscle in vivo in normal ICR mice. Ten minutes following maximal insulin injection, there was a significant increase in AS160 phosphorylation detected with both antibodies ($P < 0.05$; Fig. 2*A* and *B*). To discern the upstream mechanism(s) underlying these phosphorylation events, we utilized wild-type and Akt2 KO littermates. Akt2 KO mice are characterized by specific whole-body deletion of Akt2, the isoform primarily responsible for insulin-stimulated glucose transport in skeletal muscle (5,6). Insulin-stimulated AS160 phosphorylation was significantly blunted in Akt2 KO gastrocnemius muscle (Fig. 2*C* and *D*) and reflected the phosphorylation status of Akt (data not shown). There were no alterations in pan-AS160 expression between wild-type and Akt2 KO muscle (Fig. 2*C* and *D*), nor differences in proximal insulin signaling to insulin receptor substrate-1 and PI3-K (K.S., D.E.A., N.F., H.F.K., M.J. Birnbaum, M.F.H., L.J.G., unpublished observations). Taken together with the wortmannin data, these findings strongly support a model whereby Akt functions as the major upstream kinase regulating insulin-induced AS160 phosphorylation in mouse skeletal muscle.

Contraction-stimulated AS160 phosphorylation. We next examined whether contraction alters AS160 phosphorylation in mouse skeletal muscle using treadmill exercise, in situ contraction, and in vitro contraction protocols (Fig. 3*A*–*C*). Moderate-intensity treadmill running (Fig. 3*A*) and in situ muscle contractions (Fig. 3*B*) increased AS160 phosphorylation detected by immunoblots with both antibodies, although the magnitude of PAS-detected increases was significantly more pronounced than that detected with the phospho-Thr^{642}–specific probe (~2.5-fold vs. ~1.7-fold). High-intensity

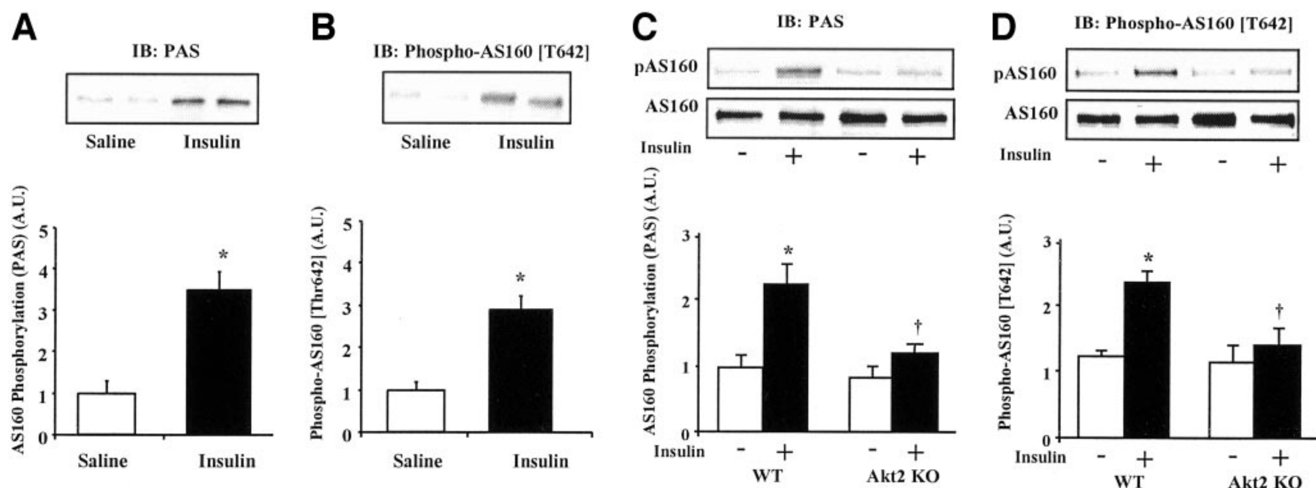

FIG. 2. Effects of in vivo insulin on AS160 phosphorylation in gastrocnemius muscle. Fasted ICR mice (*A* and *B*) and wild-type (WT) and Akt2 KO littermates (*C* and *D*) received saline or maximal intraperitoneal insulin injections and studied 10 min later. Immunoblotting (IB) was with anti-PAS (*A* and *C*), anti–phospho-AS160 Thr^{642} (*B* and *D*), and pan-AS160 (*C* and *D*) antibodies. Data are means ± SE; *n* = 6–8/group. *P < 0.05 vs. basal; †P < 0.05 vs. wild type.

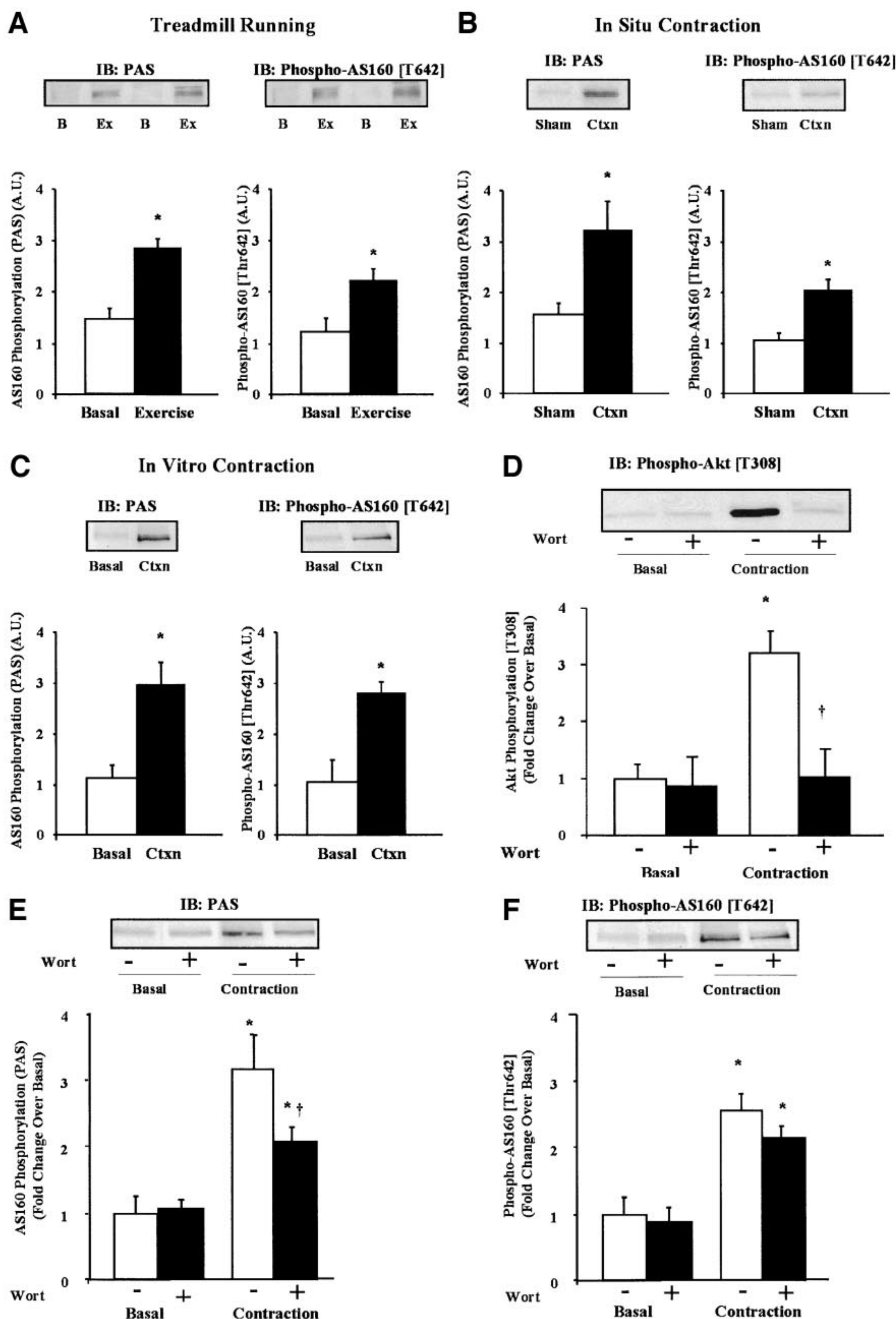

FIG. 3. Effects of exercise/contractions on AS160 phosphorylation in mixed gastrocnemius and EDL muscles. *A*: Mice performed moderate treadmill exercise for 30 min (Exercise) or remained sedentary (Basal), after which gastrocnemius muscles were studied for signaling. *B*: One gastrocnemius muscle from anesthetized mice contracted in situ (Ctxn) for 10 min, while the contralateral leg served as a sham-operated control (Sham). *C–F*: EDL muscles were incubated (-) or (+) 100 nmol/l wortmannin for 30 min and thereafter rested (Basal) or stimulated to contract for 5 min (Ctxn). Immunoblotting (IB) was with anti–phospho-Akt Thr^{308} (*D*), anti-PAS (*A, B, C,* and *E*), and anti–phospho-AS160 Thr^{642} (*A, B, C,* and *F*) antibodies. Data are means ± SE; n = 5–8/group. *P < 0.05 exercise/contraction vs. basal; †P < 0.05 vs. contraction alone.

tetanic contractions in vitro in isolated mouse EDL muscles (Fig. 3*C*) produced greater increases in AS160 phosphorylation, approximately threefold over basal with both antibodies. Overall, each model of muscle contraction/exercise increased AS160 Thr^{642} phosphorylation and potentially multiple PAS motifs that are also regulated by insulin stimulation.

To determine whether Akt mediates contraction-induced AS160 phosphorylation, we stimulated EDL muscles to contract in vitro in the absence or presence of wortmannin. Our laboratory and other groups (12,15,16) have reported enhanced Akt phosphorylation with contraction in skeletal muscle. As shown in Fig. 3*D*, 5 min of tetanic contractions elicited significant increases in Akt Thr^{308} phosphorylation ($P < 0.05$), which was abolished in the presence of wortmannin. In contrast, wortmannin only partially reduced contraction-stimulated AS160 phosphorylation detected with PAS (Fig. 3*E*) and did not significantly decrease phosphorylation at Thr^{642} (Fig. 3*F*). Thus, even in the absence of upstream Akt activity, AS160 is still phosphorylated in response to muscle contractions in vitro.

In addition to determining the effects of Akt inhibition in vitro, we also performed in vivo studies to examine whether contraction-mediated signaling to AS160 is preserved in the absence of Akt2 (Fig. 4). Wild-type and Akt2 KO mice were stimulated to generate hindlimb contractions in situ. As a control for contraction efficacy, phosphorylation of the contraction-sensitive kinase AMPK at Thr^{172} was significantly increased in both wild-type and Akt2 KO mice (Fig. 4*A*). Akt Thr^{308} phosphorylation was significantly increased in wild-type, but not Akt2 KO, mice (Fig. 4*B*). Despite the lack of Akt Thr^{308} phosphorylation in the Akt2 KO mice, both genotypes responded to contraction with comparable increases in AS160 phosphorylation ($P < 0.05$) using both antibodies (Fig. 4*C* and *D*). These data indicate that Akt activation cannot fully explain contraction-induced AS160 phosphorylation, in contrast to insulin. Another contraction-sensitive kinase(s) appears to converge upon and phosphorylate AS160 at key regulatory phospho-motifs.

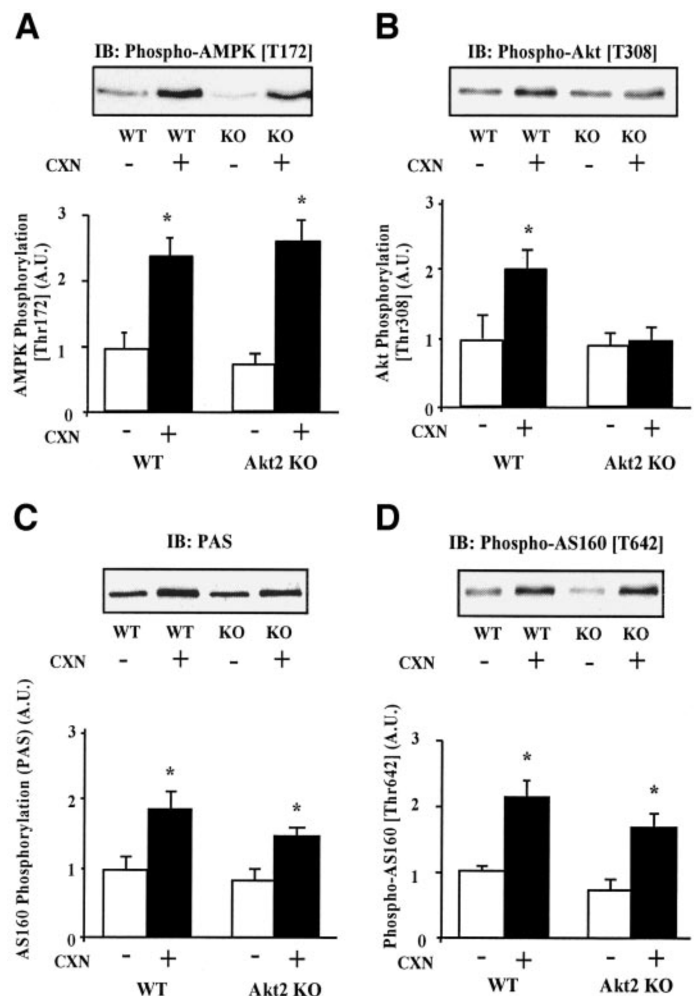

FIG. 4. Effects of in situ contraction on AS160 phosphorylation in Akt2 KO muscle. Anesthetized wild-type and Akt2 KO tibialis anterior muscles were stimulated to contract in situ. Immunoblotting (IB) was with anti–phospho-Akt Thr^{308} (*A*), anti–phospho-AMPK Thr^{172} (*B*), anti-PAS (*C*), and anti–phospho-AS160 Thr^{642} (*D*) antibodies. Data are means ± SE; n = 5–6/group. *$P < 0.05$ vs. sham.

AICAR-stimulated AS160 phosphorylation. Our results, both in vitro and in vivo, provide evidence for the existence of an Akt-independent mechanism for contraction-stimulated AS160 phosphorylation. To determine whether AMPK regulates AS160 phosphorylation in vitro, we incubated isolated EDL muscle in the absence or presence of the AMPK activator AICAR (Fig. 5). As expected, AICAR significantly increased AMPK Thr^{172} phosphorylation (Fig. 5*A*) but had no effect on Akt phosphorylation (Fig. 5*B*). AS160 phosphorylation detected with the anti-PAS antibody revealed significant increases (Fig. 5*C*), but there was not significant increase in phosphorylation when probing with the anti–phospho-AS160 Thr^{642} antibody (Fig. 5*D*). Moreover, PAS-detectable increases in AS160 phosphorylation were not affected by wortmannin. These data suggest that AICAR-stimulated increases in AS160 phosphorylation apparently occur at serine-terminal PAS phospho-motifs and are wortmannin insensitive.

To investigate whether AMPK α2 mediates AICAR-induced AS160 phosphorylation, we utilized wild-type mice and transgenic littermates overexpressing a skeletal muscle–specific α2i AMPK. These mice lack virtually all AMPK catalytic activity in skeletal muscle and exhibit no AICAR-stimulated glucose transport (21). AICAR incubation significantly increased AS160 phosphorylation detected by PAS immunoblotting wild-type EDL muscles, but this effect was fully blunted in AMPK α2i transgenic muscles (Fig. 6*A*). AICAR did not increase AS160 Thr^{642} phosphorylation in either wild-type or α2i AMPK transgenic mice (Fig. 6*B*). Total AS160 protein expression was not different between wild-type and transgenic mice (Fig. 6*C* and *D*). These data strongly suggest that AMPK activation directly or indirectly regulates AS160 serine-terminal PAS phosphorylation events associated with AICAR stimulation.

Contraction-stimulated AS160 phosphorylation in AMPK α2i transgenic mice. Based on our AICAR data, we next investigated whether contraction-stimulated AS160 phosphorylation is similarly abolished in α2i AMPK transgenic mice. We contracted tibialis anterior muscles from wild-type and α2i AMPK transgenic mice in situ via peroneal nerve stimulation. This contraction protocol increases AMPK α2 activity by 1.5-fold in wild-type mice, an effect completely abolished in α2i AMPK transgenic mice (21). Conversely, this protocol does not increase AMPK α1 activity in wild-type (21,26) or AMPK α2i transgenic mice (21). Contraction significantly increased AS160 phosphor-

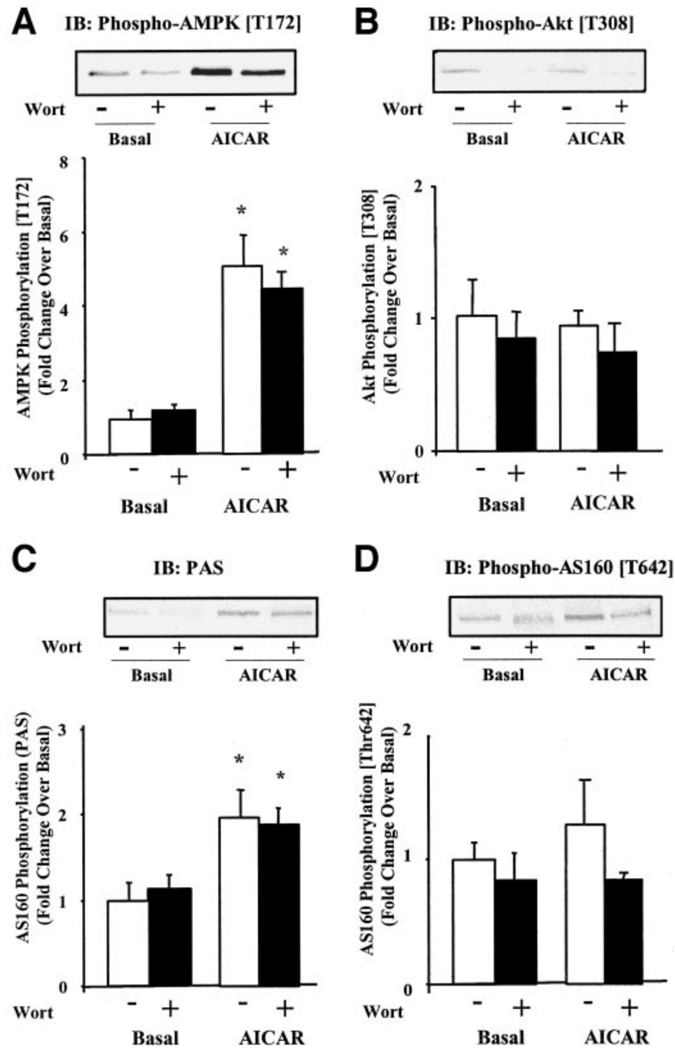

FIG. 5. Effects of in vitro wortmannin (Wort) and AICAR on AS160 phosphorylation in EDL muscle. EDL muscles were incubated (-) or (+) 100 nmol/l wortmannin for 20 min and thereafter rested (Basal) or treated with 2 mmol/l AICAR for 40 min (AICAR). Immunoblotting (IB) was with anti–phospho-AMPK Thr^{172} (*A*), anti–phospho-Akt Thr^{308} (*B*), anti-PAS (*C*), and anti–phospho-AS160 Thr^{642} (*D*) antibodies. Data are expressed as means ± SE; $n = 6–8$/group. *$P < 0.05$ vs. basal.

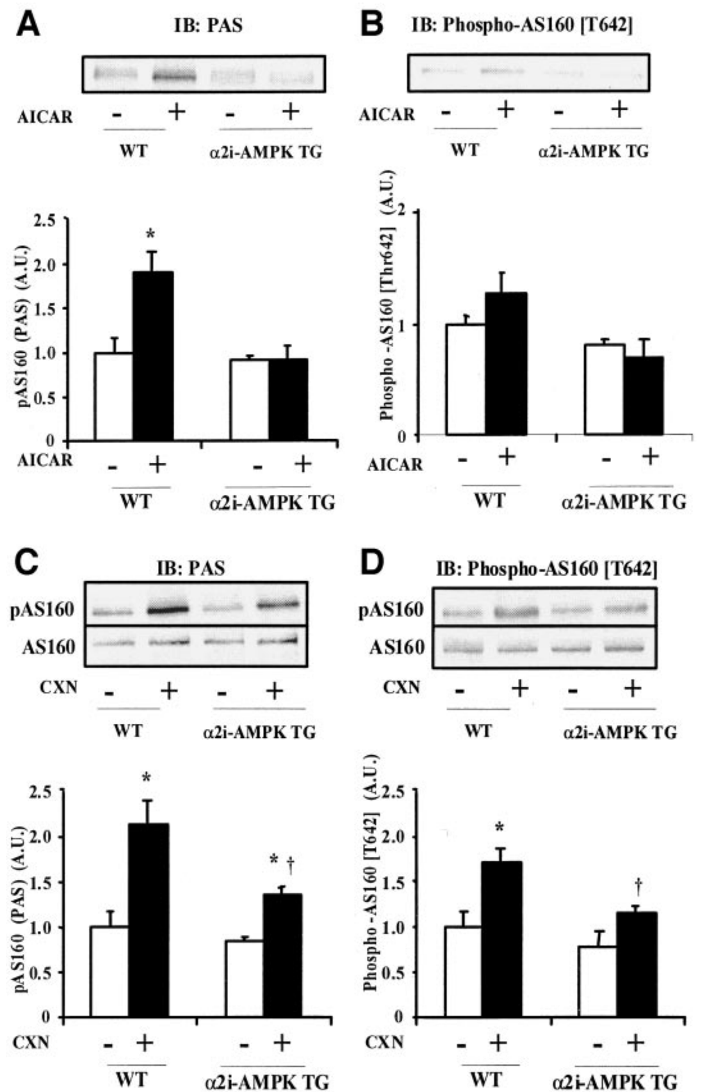

FIG. 6. Effects of in vitro AICAR and in situ contraction on AS160 phosphorylation in α2i AMPK transgenic (α2i-AMPK TG) muscle. *A* and *B*: EDL muscles were incubated (-) or (+) 2 mmol/l AICAR for 40 min. *C* and *D*: Tibilias anterior muscles were stimulated to contract in situ. Immunoblotting (IB) was with anti-PAS (*A* and *C*), anti–phospho-AS160 Thr^{642} (*B* and *D*), and anti–pan-AS160 antibodies. Data are means ± SE; $n = 4–8$/group. *$P < 0.05$ vs. basal/sham; †$P < 0.05$ vs. wild type (WT).

ylation in both wild-type and transgenic mouse tibialis anterior muscle in anti-PAS immunoblots (Fig. 6*C*). However, the increased AS160 phosphorylation in α2i AMPK transgenic mice was significantly reduced compared with wild-type littermates ($P < 0.05$). Phosphorylation at AS160 Thr^{642} exhibited a similar pattern, although transgenic mice only showed a trend toward increases in AS160 phosphorylation (Fig. 6*D*). Thus, AMPK α2 appears to be a primary regulator of contraction-specific AS160 phosphorylation events in mouse skeletal muscle, although the absence of AMPK α2 activity does not eliminate all contraction-stimulated increases in AS160 phosphorylation.

Removal of AMPK α2 catalytic activity decreases contraction-stimulated AS160 phosphorylation by ∼60%, whereas abolished Akt activity reduces contraction-stimulated PAS-detectable phosphorylation by 10–30%. We next examined whether contraction-mediated signaling to AS160 is completely inhibited when Akt and AMPK α2 activities are simultaneously blocked. EDL muscles from wild-type and α2i AMPK transgenic mice were stimulated to contract in vitro in the absence or presence of 100 nmol/l wortmannin. Although the mean AS160 phosphorylation assessed by PAS (Fig. 7*A*) or Thr^{642} (Fig. 7*B*) immunoblotting was ∼5–10% lower with combined Akt and AMPK α2 inhibition, these were not significantly different from the effects of AMPK α2 inhibition alone. Control immunoblots of Akt phosphorylation (Fig. 7*C*) confirm both the efficacy of the inhibitor as well as the contraction stimulus. Collectively, these results suggest that AMPK α2 is largely responsible for contraction-mediated AS160 phosphorylation at regulatory PAS motifs, although other kinases may also account for some residual increases in AS160 phosphorylation following contraction.

Additive effects of insulin and AICAR or contraction on AS160 phosphorylation. AICAR-stimulated AS160 phosphorylation appears to occur at serine-terminal phospho-motifs detected with PAS but not at Thr^{642}. Meanwhile, insulin and contraction treatments result in

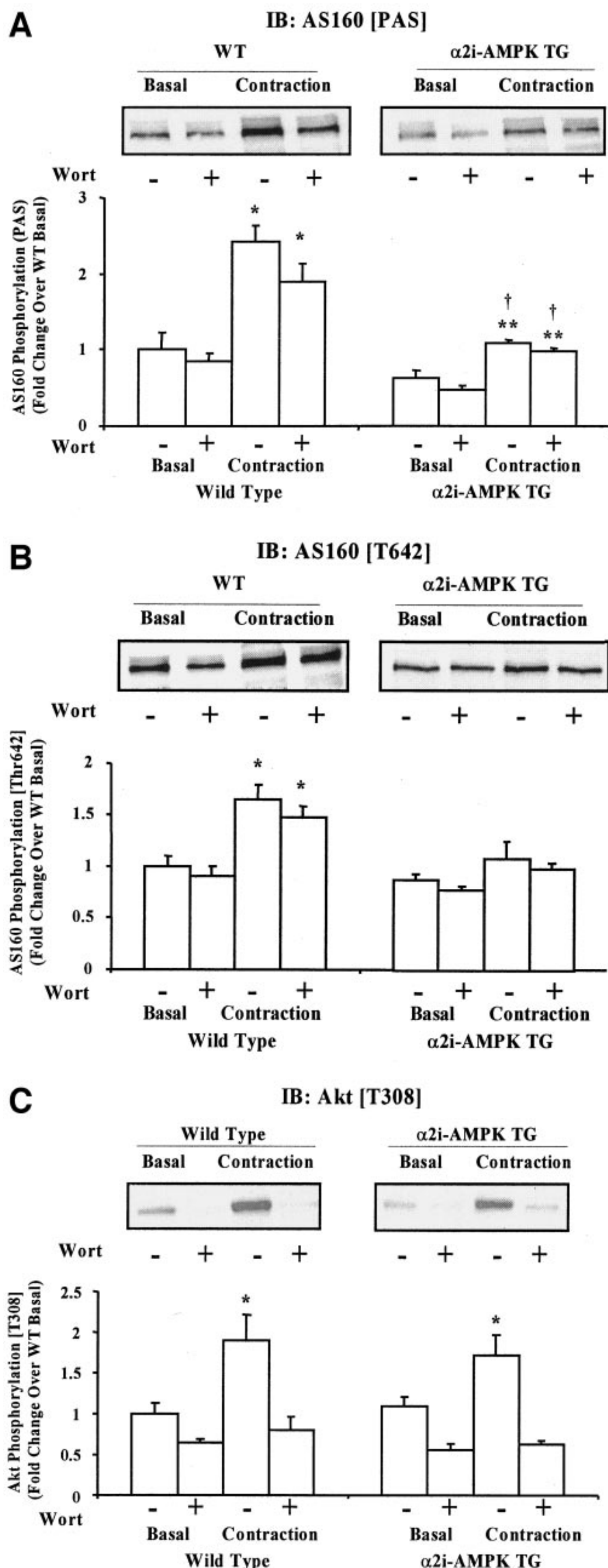

FIG. 7. Effects of in vitro wortmannin on contraction-stimulated AS160 phosphorylation in α2i AMPK transgenic (α2i-AMPK TG) muscle. EDL muscles were incubated (-) or (+) 100 nmol/l wortmannin for 30 min and thereafter rested (Basal) or stimulated to contract for 5 min (Contraction). Immunoblotting (IB) was with anti-PAS (*A*), anti–phospho-AS160 Thr^{642} (*B*), and anti–phospho-Akt Thr^{308} (*C*) antibodies. Data are means ± SE; n = 3–4/group. *P < 0.05 vs. wild-type (WT) basal; **P < 0.05 vs. TG basal; †P < 0.05 vs. wild-type contraction.

significant increases in Thr^{642} phosphorylation and possibly other PAS motifs. We therefore determined whether combined AICAR plus insulin and contraction plus insulin treatments generate greater increases in AS160 phosphorylation compared with either stimulus alone. Figure 8*A* and *B* show that AMPK Thr^{172} and Akt Thr^{308} phosphorylation were significantly increased, as expected, by AICAR plus insulin, respectively ($P < 0.05$). When samples were probed with anti-PAS (Fig. 8*C*), the combined AICAR plus insulin treatment resulted in the greatest magnitude of AS160 phosphorylation, with a significant increase above the independent effects of AICAR or insulin. In contrast, the increased AS160 phosphorylation at Thr^{642} observed with combined AICAR plus insulin closely resembled the effect of insulin alone (Fig. 8*D*). AS160 phosphorylation also increased in an additive manner during combined contraction plus insulin treatment (Fig. 8*E* and *F*). EDL muscles incubated in insulin and stimulated to contract in vitro (5 min) exhibited significantly greater increases in AS160 phosphorylation compared with contraction or insulin treatment alone, as assessed with both antibodies. Thus, independent effectors of skeletal muscle glucose metabolism appear to converge upon AS160 PAS motifs.

DISCUSSION

The purpose of this study was to determine whether independent Akt and AMPK signaling pathways converge on the novel regulator of insulin-stimulated glucose transport, the Rab-GAP AS160. Our findings demonstrate that insulin, contraction, and AICAR stimulate AS160 phosphorylation through distinct upstream signaling mechanisms in mouse skeletal muscle. Using Akt2 KO mice and α2i AMPK transgenic mice, we implicate convergent regulation of AS160 PAS phospho-motifs by both Akt and AMPK. Elucidation of the mechanisms regulating effectors of glucose transport common to both insulin and contraction signaling may yield information valuable for the treatment of type 2 diabetes.

Several important insights emerge from our data regarding the signaling mechanisms responsible for AS160 phosphorylation in mouse skeletal muscle. First, insulin increases AS160 phosphorylation at PAS motifs in an Akt-dependent manner both in vivo and in vitro. Second, exercise and muscle contractions also increase AS160 phosphorylation at PAS sites, principally through enhanced AMPK α2 activity. However, AMPK α2 alone does not account for all contraction-stimulated elevations. Third, AICAR increases AS160 phosphorylation in an AMPK α2–dependent manner and only at serine-terminal PAS motifs. Fourth, combined treatment of insulin plus AICAR and insulin plus contraction have additive effects on PAS-detectable AS160 phosphorylation.

A previous report in rat epitrochlearis muscle in vitro (16) has shown that wortmannin inhibits insulin-stimulated AS160 phosphorylation, and our findings using mouse muscle are consistent with these results. Wortmannin is a PI3-K inhibitor that blocks insulin-stimulated Akt phosphorylation, GLUT4 translocation, and ultimately glucose transport in skeletal muscle (27). As a PI3-K inhibitor, wortmannin could potentially abolish the activity of other PI3-K–responsive proteins that may interact with AS160 in addition to Akt. Our use of Akt2 KO mice provides strong evidence that Akt2, and not other wortmannin-sensitive kinases, is the primary regulator of insulin-stimulated AS160 phosphorylation in skeletal muscle. Interestingly,

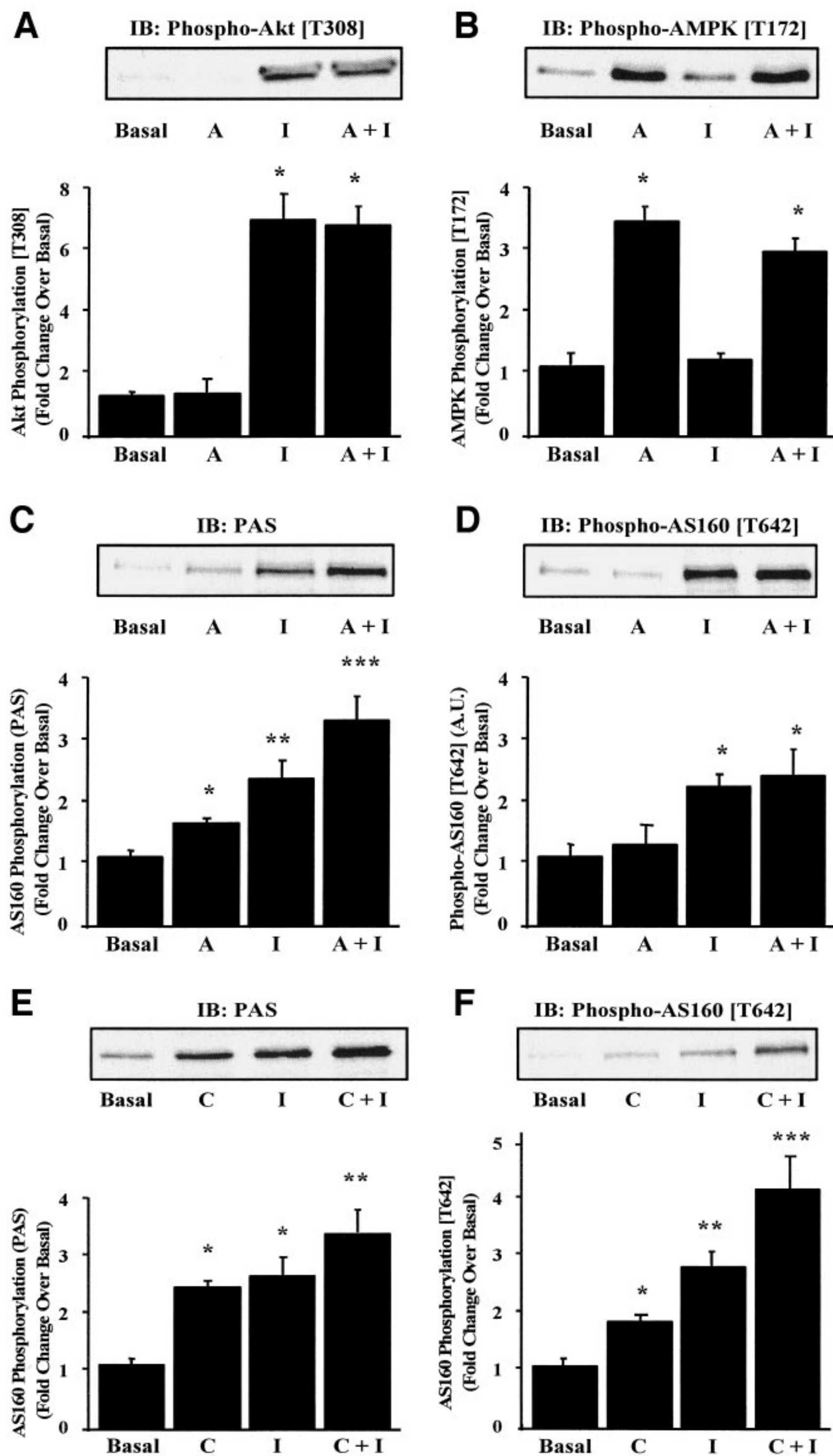

FIG. 8. Effects of combined in vitro treatments involving AICAR, insulin, and contraction on AS160 phosphorylation. EDL muscles were incubated with nothing (Basal), AICAR (A), insulin (I), combined AICAR + insulin (*A*+ I) or stimulated to contract (C) ± insulin (I) treatment. Immunoblotting (IB) was with anti–phospho-Akt Thr^{308} (*A*), anti–phospho-AMPK Thr^{172} (*B*), anti-PAS (*C* and *E*), and anti–phospho-AS160 Thr^{642} (*D* and *F*) antibodies. Data are means ± SE; *n* = 6–11/group. **P* < 0.05 vs. basal; ***P* < 0.05 vs. AICAR or contraction; ****P* < 0.05 vs. all other conditions.

Birnbaum and colleagues (6,18) have shown that Akt2 acts as the principle isoform regulating glucose transport in insulin-sensitive tissues. Absence of Akt2 results in impaired skeletal muscle and hepatic insulin sensitivity, effects that cannot be compensated for by residual or overexpressed Akt1 and/or 3 activities (28). Our data suggest that impairments in AS160 phosphorylation might be involved in the skeletal muscle insulin-resistant phenotype that characterizes Akt2 KO mice at submaximal insulin concentrations. Consistent with this hypothesis, type 2 diabetes in humans is associated with reduced insulin-stimulated AS160 phosphorylation (29).

The molecular mediators governing contraction-stimulated AS160 phosphorylation are more obscure, although our data suggest the AMPK system is a major contributory mechanism. We found a modest effect of wortmannin on contraction-stimulated AS160 phosphorylation in mouse skeletal muscle. These data differ from the full inhibition of contraction-stimulated AS160 phosphorylation with wortmannin reported previously (16). Differences in species (rat versus mouse), muscle fiber–type composition (epitrochlearis versus EDL), and immunoblotting may account for the divergent results. It is important to note, however, that wortmannin has no effect on contraction-

stimulated glucose uptake in muscle (12,27). If phosphorylation of AS160 is a central event for contraction-induced glucose transport in skeletal muscle, then our data indicating partial preservation of AS160 phosphorylation with concomitant contractions and wortmannin treatment appear reasonable. The dispensable nature of Akt2 for contraction-stimulated AS160 phosphorylation in vivo was confirmed in experiments employing Akt2 KO mice, since muscle contractions stimulated comparable increases in AS160 phosphorylation in both wild-type and Akt2 KO mice. This Akt2-independent means of signaling to AS160 may reflect the ability of Akt2 KO mice to increase glucose transport normally in response to contraction (K.S., D.E.A., N.F., H.F.K., M.J. Birnbaum, M.F.H., L.J.G., unpublished observations).

AMPK is one of multiple proteins that potently respond to contraction in skeletal muscle (12), and both AMPK and Akt phosphorylate substrates along similar anti–PAS-detectable epitopes (17,19,20,24). AICAR, a pharmacological activator of AMPK, increases AS160 phosphorylation at serine-terminal PAS motifs in both rat (16) and mouse skeletal muscle. Our use of α2i AMPK transgenic mice demonstrated that these AICAR-induced AS160 phosphorylation events require AMPK α2 activity. Intriguingly, many established AMPK substrates are phosphorylated along PAS-like epitopes ending in the amino acid serine. In addition, phosphofructokinase-2 (19) and Raf-1 (20) specifically contain PAS phosphorylation sites known to be targeted by both AMPK and Akt. AMPK may thus directly or indirectly phosphorylate AS160 at one or more serine-terminal PAS motifs.

Evidence from α2i AMPK transgenic mice also suggests an important role for AMPK in contraction-specific AS160 phosphorylation. α2i AMPK transgenic mice exhibit extreme diminutions in contraction-stimulated AS160 phosphorylation compared with wild-type littermates. However, subtle elevations in PAS-detectable AS160 phosphorylation still occur in response to contraction, indicating that other pathways may account for the residual or compensatory phosphorylation. Although we cannot rule out AMPK α1 activity as a source for AS160 phosphorylation in α2i AMPK transgenic mice, we think it is unlikely because the contraction protocol used does not increase α1 activity (21,26) and because AICAR-stimulated AS160 phosphorylation is fully inhibited in α2i AMPK transgenic mice despite normal AMPK α1 activation (21). Another putative upstream kinase family are aPKCs, which phosphorylate substrate sequences that are consistent with the PAS epitope (25) and are known to be activated by exercise in skeletal muscle (14). Thus, while downstream targets of PKC-mediating glucose transport are not currently known, it is certainly plausible that AS160 is one such substrate. Scansite analysis of mouse AS160 reveals multiple domains and phospho-motifs distinct from PAS (30). Indeed, absence of an observable increase in AS160 phosphorylation in immunoblots with anti-PAS or anti–phospho-AS160 Thr^{642} does not preclude the possibility of alternative phosphorylation events on AS160 with contraction.

In conclusion, the mechanisms leading to insulin-, AICAR-, and contraction-stimulated AS160 phosphorylation in mouse skeletal muscle are distinct. While insulin-stimulated AS160 phosphorylation is mediated by Akt, AICAR-stimulated AS160 phosphorylation occurs exclusively through the AMPK system. Contraction-stimulated AS160 phosphorylation events involve AMPK, possibly Akt, and other undefined kinase(s). Overall, all three stimuli increase AS160 phosphorylation at phospho-motifs recognized to be pivotal in L6 myotubes for insulin-stimulated GLUT4 translocation. AS160 and its phosphorylation status could be a central point of convergence for both insulin and contraction, making it an attractive molecule for prospective pharmacological or genetic manipulation.

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