Original Article

Exercise-Induced Phosphorylation of the Novel Akt Substrates AS160 and Filamin A in Human Skeletal Muscle

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Skeletal muscle contraction stimulates multiple signaling cascades that govern a variety of metabolic and transcriptional events. Akt/protein kinase B regulates metabolism and growth/muscle hypertrophy, but contraction effects on this target and its substrates are varied and may depend on the mode of the contractile stimulus. Accordingly, we determined the effects of endurance or resistance exercise on phosphorylation of Akt and downstream substrates in six trained cyclists who performed a single bout of endurance or resistance exercise separated by ~7 days. Muscle biopsies were taken from the vastus lateralis at rest and immediately after exercise. Akt Ser^{473} phosphorylation was increased (1.8-fold; $P = 0.011$) after endurance but was unchanged after resistance exercise. Conversely, Akt Thr^{308} phosphorylation was unaltered after either bout of exercise. Several exercise-responsive phosphoproteins were detected by immunoblot analysis with a phospho-Akt substrate antibody. pp160 and pp300 were identified as AS160 and filamin A, respectively, with increased phosphorylation (2.0- and 4.9-fold, respectively; $P < 0.05$) after endurance but not resistance exercise. In conclusion, AS160 and filamin A may provide an important link to mediate endurance exercise–induced bioeffects in skeletal muscle. *Diabetes* 55:1776–1782, 2006

Skeletal muscle contractile activity stimulates multiple signaling molecules known to regulate metabolic and transcriptional events (rev. in 1,2). Akt/protein kinase B regulates metabolism, growth/muscle hypertrophy, and cell survival (3–6) and is activated by numerous growth factors and cellular stress (7). In humans, cycling exercise induces an intensity-dependent increase in Akt activity that is accompanied by increases in phosphorylation of Akt on activating residues (Thr^{308} and Ser^{473}) (8). Akt is also phosphorylated in rat skeletal muscle in response to in vitro contraction (9–11), in situ contraction (9,12), or treadmill running (13). In contrast to these observations, several investigations provide evidence against activation or phosphorylation of Akt in human (14) or rodent skeletal muscle in response to endurance (12,15,16) or resistance (17) exercise or after in vitro or in situ contraction (18–20). These conflicting results may be in part due to differences arising from the various modes and intensities of exercise used, fiber-type differences between species, and tissue sampling time points. Accordingly, the first aim of this study was to determine the effects of two different modes of exercise on Akt phosphorylation in human skeletal muscle.

Several Akt substrates have been identified as putative links between insulin signaling and metabolic or gene regulatory responses (21). Using a phospho-Akt substrate antibody (PAS) that recognizes the Akt phosphomotif R*X*R*XX*pS/T (where *X* is any amino acid), several insulin-elicited phosphoproteins (pp250, pp160, pp105, pp75, pp47, pp43, and pp32) were detected in 3T3-L1 adipocytes (22). Insulin also leads to the phosphorylation of several Akt substrates in human (23) and rodent (11,24) skeletal muscle. The Akt substrate pp250 has a predicted GTPase-activating protein (GAP) domain for Rheb and/or Rap at its COOH terminus (25); however, the biological role of this protein is largely unknown. The 160-kDa phosphoprotein (AS160) also contains a GAP domain for Rabs, which are small G-proteins required for vesicle trafficking processes (26). Expression of AS160 with two or more in vivo phosphorylation sites mutated inhibited insulin-stimulated GLUT4 translocation (26) by blocking exocytosis of the GLUT4-containing vesicles at a step before fusion with the plasma membrane (27). Thus, AS160 is the most proximal step identified thus far in the insulin signaling cascade to glucose transport. Interestingly, in vitro contraction of rat epitrochlearis muscle elicits an increase in the phosphorylation of at least three Akt substrates; pp250, pp180, and pp160 (AS160) (11). Many of the physiologically relevant substrates for Akt remain to be identified. Accordingly, a second aim of this investigation was to evaluate the effects of exercise on phosphorylation of Akt substrates in human skeletal muscle.

RESEARCH DESIGN AND METHODS

Six male cyclists (age 28.7 ± 6.1 years, body weight 74.7 ± 7.6 kg, estimated peak oxygen uptake [$\dot{V}O_{2peak}$] 65.2 ± 6.4 ml · kg^{-1} · min^{-1}) with a prolonged history (8.5 ± 2.7 years) of endurance training volunteered to participate in this study. At the time of investigation, subjects were riding 425 ± 292 km/week (range 200–800 km) and did not participate in any form of

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AMPK, AMP-activated protein kinase; GAP, GTPase-activating protein; PAS, phospho-Akt substrate antibody; PKC, protein kinase C; PPO, peak power output.

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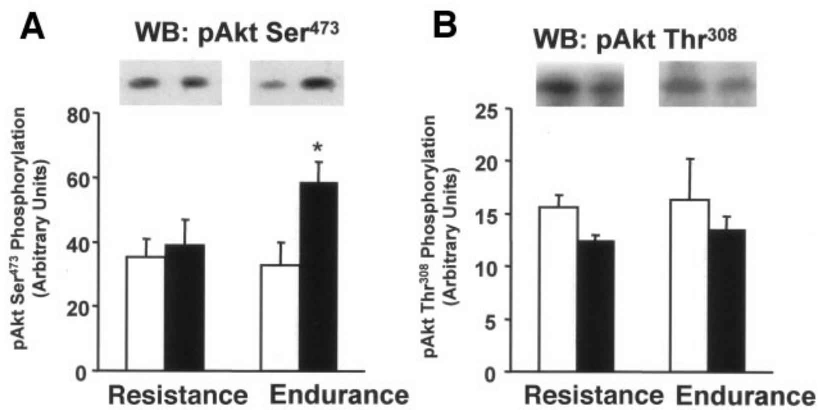

FIG. 1. Akt phosphorylation. Skeletal muscle from the vastus lateralis was obtained before (rest) (□) or immediately after (post) (■) resistance 12,000*g* or endurance exercise, and Akt Ser^{473} (*A*) and Thr^{308} (*B*) phosphorylation was determined. *Top panels*: Representative immunoblot. *Bottom panels*: Means ± SE (arbitrary units). *P* < 0.05 vs. rested.

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resistance/strength training. The experimental procedures and possible risks associated with the study were explained to each subject who then gave his written informed consent. The study was approved by the Human Research Ethics Committee of RMIT University.

The study consisted of a cross-over approach in which each subject performed two different exercise testing sessions separated by a minimum of 7 days. One exercise session was undertaken in the habitual training discipline (i.e., cycling), and the other session comprised a bout of unfamiliar exercise (intense resistance exercise, described subsequently). Muscle biopsies were taken at rest and immediately after the exercise session.

Preliminary testing

$\dot{V}O_{2peak}$. $\dot{V}O_{2peak}$ for each subject was predicted from the peak power output (PPO) attained during the maximal incremental cycling test to volitional fatigue (bicycle ergometer; Lode, Groningen, the Netherlands). We have previously reported robust correlations between PPO and $\dot{V}O_{2peak}$ for both well-trained and untrained individuals (28). The results from this maximal test were used to estimate the power output (~63% of PPO) that corresponded to ~70% of predicted $\dot{V}O_{2peak}$ for each subject (to be used in the subsequently described exercise testing sessions).

Maximal voluntary strength. Maximal concentric and eccentric strength for each subject was determined using seated leg extensions performed on an isokinetic dynamometer (Kin-Com, Chattanooga, TN). Quadriceps strength was determined during a series of three-repetition leg extension sets. Individual 1-RM was defined as the peak torque recorded during the concentric and eccentric contraction phases of the test protocol.

Diet and exercise control. Before each experimental trial (described subsequently), subjects refrained from vigorous physical activity for 24 h and were provided with standardized prepacked meals that consisted of 3 g carbohydrate kg^{-1} body wt, 0.5 g protein kg^{-1} body wt, and 0.3 g fat kg^{-1} body wt to be consumed as the final meal the evening before an experiment.

Experimental trials. On the morning of an experiment, subjects reported to the laboratory in a 10- to 12-h overnight-fasted state. After resting quietly in a supine position for 10 min, local anesthesia (2–3 ml 1% xylocaine [lignocaine]) was administered to the skin, subcutaneous tissue, and fascia of the vastus lateralis in preparation for muscle biopsies. A resting biopsy was taken by using a 6-mm Berström needle with suction applied (29). Approximately 100 mg muscle was removed and immediately frozen in liquid N_2. Immediately upon completion of an exercise testing session, a second biopsy was taken from the same leg (~5 cm distal from the resting sample) and frozen within 10–15 s of exercise cessation. Every attempt was made to extract tissue from approximately the same depth in the muscle. Samples were stored at −80°C until subsequent analysis.

Exercise testing sessions

Resistance exercise. Maximal voluntary isokinetic leg extensions were performed on a Kin-Com dynamometer (as described previously). After a 2× five-repetition submaximal warm-up, subjects performed eight sets of five repetitions at maximal effort. Each set was separated by a 3-min recovery period. Peak and mean torque were recorded for each leg extension set. Verbal encouragement and real-time visual feedback were given to subjects during each repetition.

Cycling exercise. Subjects performed 60 min of continuous cycling at a power output that elicited ~70% of individual predicted $\dot{V}O_{2peak}$.

Analytical procedures

Tissue processing. Muscle biopsies (40–50 mg) were freeze-dried overnight and subsequently dissected under a microscope to remove visible blood, fat, and connective tissue. Muscles were homogenized in ice-cold homogenization buffer (20 mmol/l Tris [pH 7.8], 137 mmol/l NaCl, 2.7 mmol/l KCl, 1 mmol/l $MgCl_2$, 1% Triton X-100, 10% [wt/vol] glycerol, 10 mmol/l NaF, 1 mmol/l EDTA, 5 mmol/l sodium pyrophosphate, 0.5 mmol/l Na_3VO_4, 1 μg/ml leupeptin, 0.2 mmol/l phenylmethyl sulfonyl fluoride, 1 μg/ml aprotinin, 1 mmol/l dithiothreitol, 1 mmol/l benzamidine, and 1 μmol/l microcystin). Homogenates were rotated for 30 min at 4°C. Samples were subjected to centrifugation (12,000*g* for 15 min at 4°C), and protein concentration was determined in the supernatant using the BCA protein assay kit (Pierce, Rockford, IL). An aliquot of muscle homogenate (30 μg protein) was mixed with Laemmli buffer containing β-mercaptoethanol and subjected to SDS-PAGE. Each subject provided a biopsy before and after both types of exercise. Samples were compared in parallel, and representative gels of one such subject are shown.

Cell culture. Skeletal muscle biopsies (rectus abdominus) were obtained with the informed consent of from three male donors during scheduled abdominal surgery. Subjects had no known metabolic disorders (mean age 55 ± 4 years, BMI 26 ± 1.5 kg/m^2, and fasting blood glucose 5.2 ± 0.3 mmol/l). The Ethical Committee at the Karolinska Institutet approved the study protocols. Human skeletal muscle satellite cells were isolated and differentiated into myotubes, as previously described (30). Cultures were incubated for 30 min in the absence or presence of insulin (120 nmol/l) and lysed in homogenization buffer as described above.

Western blot analysis. Proteins were separated by SDS-PAGE (5–12% gradient or 7.5% resolving gel), transferred to polyvinylidine fluoride membranes, and blocked with Tris-buffered saline with 0.02% Tween containing 7.5% milk for 1 h. Membranes were incubated overnight with anti–phospho-Akt (Ser^{473}), anti–phospho-Akt (Thr^{308}), anti-PAS, anti-Akt, anti–phospho-filamin A (Ser^{2,152}) (Cell Signaling Technology, Beverly, MA), anti–filamin A (Santa Cruz Biotechnology), anti–filamin C, or anti–phopsho-filmain C (Ser^{2,213}) (gift from Sir Philip Cohen, University of Dundee, Scotland). Total AS160 protein expression was determined using an antibody provided by Gustav E. Lienhard (Dartmouth Medical School, Hanover, NH). Membranes were washed in Tris-buffered saline with 0.02% Tween and incubated with appropriate secondary horseradish peroxidase–conjugated antibodies (Bio-Rad, Richmond, CA). Immunoreactive proteins were visualized by enhanced chemiluminescence (ECL or ECL plus; Amersham, Arlington Heights, IL) and quantified by densitometry using Molecular Analyst Software (Bio-Rad).

Immunoprecipitation. Homogenized muscle lysate (350 μg protein) were immunoprecipitated with 3.5 μg anti-AS160 or anti–filamin A antibody at 4°C with gentle rotation overnight. Samples were incubated with an equal mixture of protein A Sepharose (Amersham) and protein G agarose (Sigma-Aldrich, St. Louis, MO) for 3 h at 4°C and subsequently washed three times with homogenization buffer and four times with PBS. The immunocomplex was suspended in Laemmli buffer containing β-mercaptoethanol. All the samples were heated at 95°C for 5 min and subjected to SDS-PAGE.

Statistics. Differences between values at rest and immediately after exercise were determined using a paired *t* test. Critical level of significance was established at $P < 0.05$. Results are presented as means ± SE.

RESULTS

Exercise responses. The power output at 70% of predicted $\dot{V}O_{2peak}$ was 242 ± 11 W. The average force during the resistance training session was 190 ± 4 N. All subjects completed both of the prescribed exercise testing sessions.

Akt phosphorylation. Akt phosphorylation before and after cycling or resistance exercise was assessed (Fig. 1). Akt Ser^{473} phosphorylation increased 1.8-fold immediately

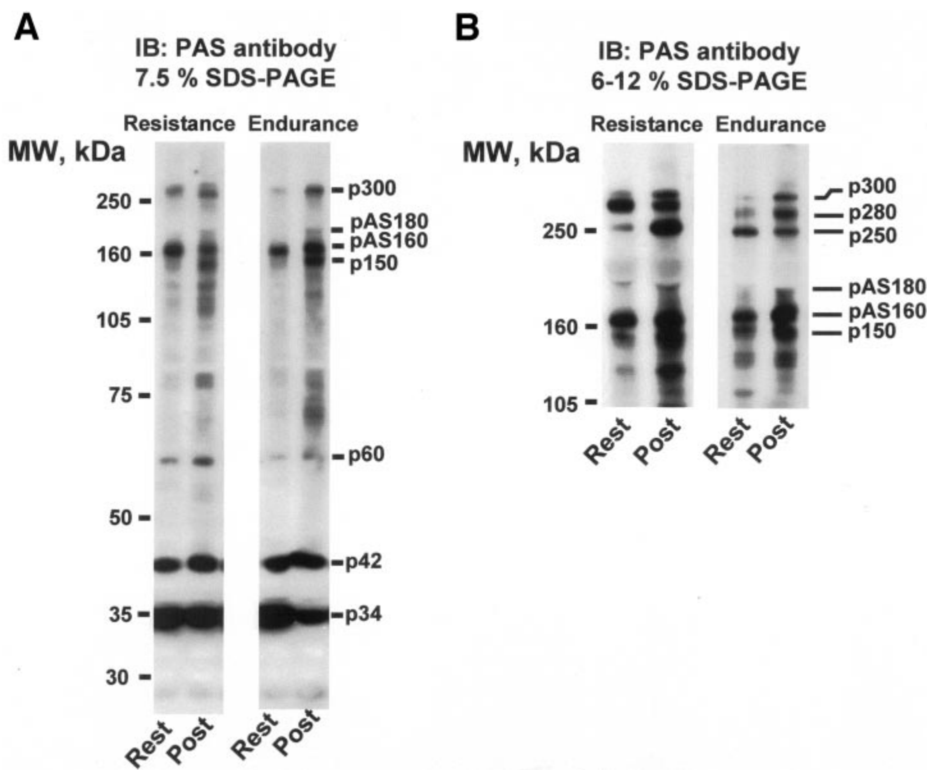

FIG. 2. Protein phosphorylation profiles. Skeletal muscle from the vastus lateralis was obtained at before (rest) or immediately after (post) resistance or endurance exercise, and lysates were subjected to either 7.5% (*A*) or 6–12% (*B*) PAGE followed by immunoblot analysis using a PAS antibody. Molecular weights of the phosphorylated proteins as indicated on the right of the immunoblot were established using a Rainbow molecular weight marker (Amersham). MW, molecular weight.

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after cycling (P = 0.01) and was unchanged after resistance exercise. In contrast, Akt Thr[308] phosphorylation was unaltered in response to either bout of exercise.

Contraction-induced phosphorylation of Akt substrates. The effect of cycling or resistance exercise on phosphorylation of Akt substrates was determined in human skeletal muscle (Fig. 2). Several exercise-responsive proteins were detected in crude lysates after immunoblot analysis with the PAS antibody. Phosphoproteins of 300, 250, 180, 160, 150, 42, and 34 kDa were among the most predominant immunoreactive proteins identified (Fig. 2*A*). Four of these proteins (pp300, pp160, pp180, and pp150) were clearly phosphorylated in response to exercise (Fig. 2*B*). We further identified pp160 and pp300 as AS160 and filamin A, respectively.

Identification of pp160 as AS160. To confirm that pp160 was AS160, immunoprecipitation experiments were performed. Lysate prepared from human skeletal muscle obtained at rest or after exercise was incubated overnight with an antibody raised against the COOH terminus of mouse AS160. Lysates, immunoprecipitated samples, and immunodepleted supernatants were immunoblotted with the PAS antibody (Fig. 3*A*). The membranes were stripped and re-blotted with an anti-AS160 antibody. AS160 was detected in the immunoprecipitated samples and depleted from the post-immunoprecipitated samples (Fig. 3*A*). Cycling exercise increased phosphorylation of AS160 twofold ($P < 0.05$; Fig. 3*B*). In contrast, AS160 phosphorylation was unaltered after resistance exercise. AS160 protein expression was unaltered after either cycling or resistance exercise (Fig. 3*C*).

Identification and characterization of an ~300-kDa phosphoprotein. Multiple phosphoproteins were identified in the region of 300 to 250 kDa (Fig. 2*B*). Recently, filamin C, a 300-kDa phosphoprotein, has been identified as an insulin-stimulated Akt substrate in L6 skeletal muscle cells (31). Thus filamin isoforms were evaluated as candidates for pp300. Lysates were prepared from skeletal muscle biopsies obtained before (rest) or after exercise. Basal and insulin-stimulated primary human skeletal muscle cells (myotubes) prepared and cultured as previously described (30) were used as a positive control for filamin C phosphorylation. Lysates, immunoprecipitated samples, and immunodepleted supernatants from exercise-stimulated (Fig. 4*A*) or insulin-stimulated (Fig. 4*B*) muscle were immunoblotted with PAS, *p*–filamin C, anti–filamin C, and anti–filamin A antibodies. The PAS antibody failed to detect phosphorylation of filamin C in the anti–filamin C immunoprecipitates from exercise-stimulated muscle (Fig. 4*A*). Immunoblot analysis with an anti–phospho-filamin C–specific antibody revealed that filamin C is phosphorylated in response to insulin (Fig. 4*B*) but not exercise (Fig. 4*A*). Filamin A was absent from filamin C immunoprecipitates of exercise-stimulated (Fig. 4*A*) or insulin-stimulated (Fig. 4*B*) muscle. Thus, filamin C is phosphorylated in response to insulin but not exercise. To determine whether filamin A is pp300 filamin A, lysates obtained from skeletal muscle biopsies obtained at rest or after exercise were immunoprecipitated with anti–filamin A and immunoblotted with either PAS or anti–filamin A antibodies (Fig. 4*C*). Exercise increased filamin A phosphorylation, as detected by the PAS antibody (Fig. 4*C*). Filamin A was enriched in immunoprecipitated samples and depleted from post-immunoprecipitated samples. Thus, pp300 was identified as filamin A. Cycling exercise increased filamin A phosphorylation 4.9-fold ($P < 0.05$) (Fig. 4*D*). Resistance exercise tended to increase filamin A phosphorylation (1.9-fold, P = 0.09), but this effect was not statistically significant (Fig. 4*D*). Protein expression of filamin A was unaltered after either cycling or resistance exercise (Fig. 4*E*). However, the residues on filamin A that are recognized by the PAS antibody are unknown. Filamin A has a well-characterized phosphorylation site at Ser[2,152]. To determine whether exercise increases phosphorylation on

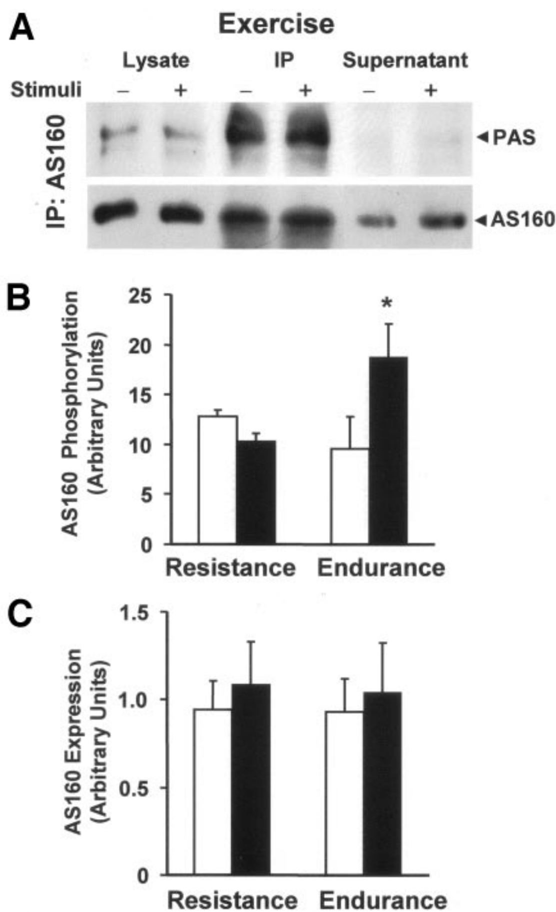

FIG. 3. Identification of AS160 as an exercise-responsive phosphoprotein. *A*: *Top panel* shows skeletal muscle lysates, AS160 immunoprecipitates (IP), and AS160 immunodepleted supernatants subjected to immunoblot analysis with anti-PAS. *Bottom panel* shows the same membrane probed for AS160 protein expression. *B*: AS160 phosphorylation as detected by immunoblot analysis using anti-PAS of skeletal muscle biopsies obtained before (rest) (□) or after (post) (■) resistance or endurance exercise. *C*: AS160 protein expression in skeletal muscle as described in *B*. Values in graphs are means ± SE (arbitrary units). $^*P < 0.05$ vs. rested.

this site, samples were subjected to immunoblot analysis using a phosphospecific anti–filamin A ($Ser^{2,152}$) antibody. Filamin A $Ser^{2,152}$ phosphorylation was unchanged after exercise (data not shown), providing evidence that exercise leads to phosphorylation of a novel residue (other than $Ser^{2,152}$). Thus, we provide the first evidence that filamin A is phosphorylated in response to exercise.

In addition to AS160 and filamin A, pp180 and pp150 were identified as exercise-responsive proteins (Fig. 2*B*). Endurance and resistance exercise increased phosphorylation of pp150 threefold (Fig. 5*A*). Endurance and resistance exercise also tended to increase pp180 phosphorylation, although this effect failed to reach statistical significance (Fig. 5*B*). The identity and function of these proteins is currently unknown.

DISCUSSION

Considerable effort has been focused on identifying the signaling pathways that mediate skeletal muscle glucose uptake in response to insulin or contraction/exercise (32–34). The protein kinase Akt has been implicated as an important target for mediating insulin action on glycogen synthesis, GLUT4 translocation and glucose transport, and gene regulatory responses (6). Insulin and contraction activate Akt in skeletal muscle (11). Accordingly, Akt substrates are candidates by which insulin and contraction promote metabolic and gene regulatory effects in skeletal muscle.

A novel 160-kDa phosphoprotein has been identified and characterized as an Akt substrate (AS160) in 3T3-L1 adipocytes (22) and skeletal muscle (11,23) by immunoblot analysis using a PAS. AS160 contains a GAP homology domain, which has been shown to regulate the GTPase activity of certain Rab proteins in vitro (35). The GTPase activity of AS160 is likely to be inhibited by phosphorylation and is required for insulin-induced GLUT4 translocation in 3T3-L1 adipocytes, likely by increasing vesicular exocytosis (26,27,36). AS160 is also phosphorylated in rat skeletal muscle in response contraction (11). Because contraction-induced AS160 phosphorylation in rat epitrochlearis muscle is completely abolished by wortmannin, this observation implicates regulation of AS160 via a phosphatidylinositol-3 kinase-phosphoinositide–dependent kinase-1–Akt signaling pathway. Although the signaling pathway by which contraction mediates glucose transport is incompletely resolved, this process is generally believed to occur in a phosphatidylinositol 3-kinase–independent manner and possibly involves activation of either protein kinase C (PKC) and/or AMP-activated protein kinase (AMPK) (32–34). Activation of CAMK by Ca^{2+} also contributes to the regulation of glucose transport in response to contraction (37). Exercise has multiple effects on metabolic and gene regulatory responses, and these responses are unlikely to be mediated by a singular pathway. Interestingly, the phosphorylation sites on AS160 are similar to consensus sequences of substrates that are phosphorylated by PKC isoforms and AMPK (38,39). Thus, AS160 may also be a substrate of PKC and AMPK.

Full activation of Akt requires phosphorylation of Thr^{308} and Ser^{473} residues (38). Phosphoinositide-dependent kinase 1 is an upstream kinase for Thr^{308}, whereas Ser^{473} is phosphorylated by the mammalian target of rapamycin–rictor complex (40). Ser^{473} phosphorylation precedes the phosphorylation of Thr^{308} and is essential for the recognition of Akt by phosphoinositide-dependent kinase 1. We have previously reported that acute moderate cycling exercise does not lead to a measurable increase in Akt activity (14). In the present study, we observed that an acute bout of endurance cycling led to a robust increase in Akt Ser^{473} phosphorylation and a concomitant increase in AS160 phosphorylation. The lack of Thr^{308} phosphorylation may suggest that Akt is not activated during the exercise protocol, and thus the effect of exercise on phosphorylation of proteins recognized by the PAS antibody be Akt independent. The exercise-induced effect on Akt and AS160 was transient, and phosphorylation was suppressed to resting levels 3 h after exercise (data not shown). In contrast to endurance exercise, a single bout of resistance exercise had little effect on either Akt or AS160 phosphorylation. Using tissue collected from an identical exercise-testing protocol as used in the current investigation, we have recently reported that an acute bout of cycling exercise performed by individuals with a history of endurance training failed to activate AMPK signaling (41). Thus, the exercise-induced activation of Akt Ser^{473} and AS160 may possibly occur via an AMPK-independent mechanism. Although AMPK has been identified as a positive regulator of glucose transport (42), recent results from transgenic and AMPK-knockout animals reveal that

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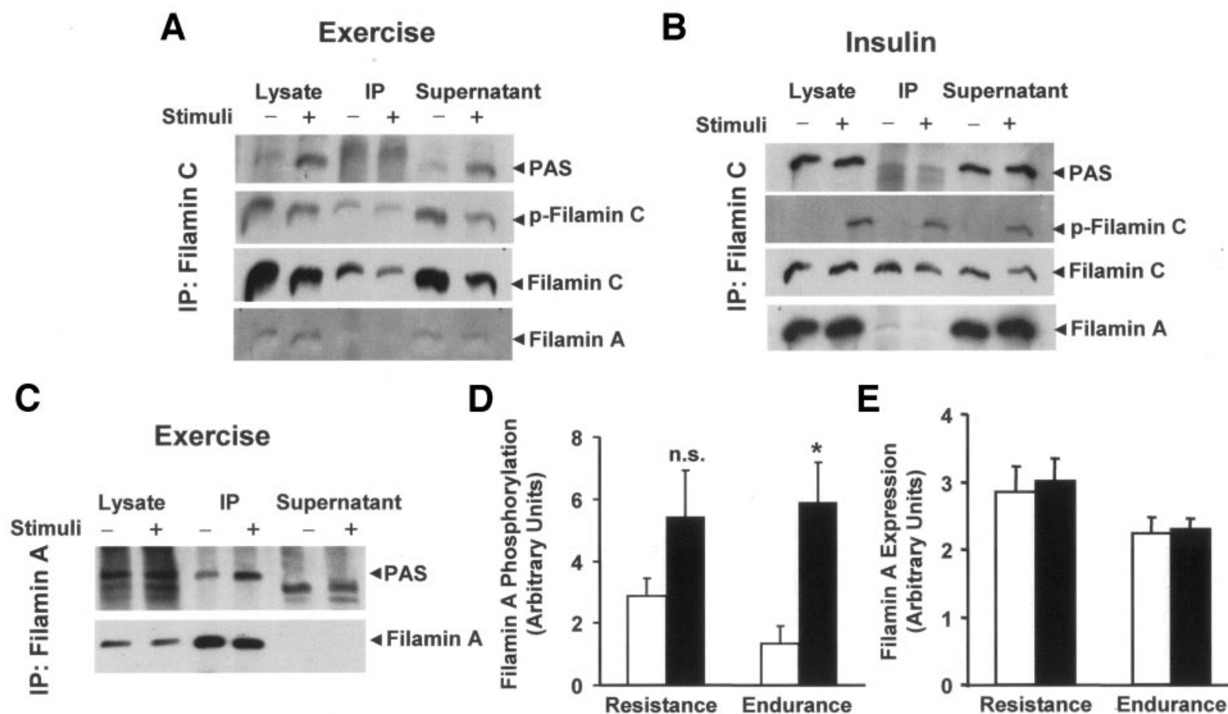

FIG. 4. Identification of filamin A as an exercise-responsive phosphoprotein. *A*: Skeletal muscle lysates obtained from biopsies taken before or after endurance exercise, filamin C immunoprecipitates (IP), and filamin C immunodepleted supernatants subjected to immunoblot analysis with anti-PAS, anti–phospho-filamin C, anti–filamin C, and filamin A antibodies, respectively. *B*: Human skeletal muscle cell lysates obtained before or after insulin (120 nmol/l) stimulation, filamin C immunoprecipitates, and filamin C immunodepleted supernatants subjected to immunoblot analysis with anti-PAS, anti–phospho-filamin C, anti–filamin C, and filamin A antibodies, respectively. *C*: *Top panel* shows skeletal muscle lysates obtained from biopsies taken before or after endurance exercise, filamin A immunoprecipitates, and filamin A immunodepleted supernatants subjected to immunoblot analysis with anti-PAS. *Bottom panel* shows same membrane probed for filamin A protein expression. *D*: Filamin A phosphorylation as detected by immunoblot analysis using anti-PAS of skeletal muscle biopsies obtained before (rest) (□) or after (post) (■) resistance or endurance exercise. *E*: AS160 protein expression in skeletal muscle as described in *D*. Values in graphs are means ± SE (arbitrary units). *$P < 0.05$ vs. rested. Filamin A phosphorylation tended to be increased in resistance exercise (1.9-fold, $P = 0.09$).

multiple signaling inputs are likely to contribute to the regulation of GLUT4 translocation in response to contraction (42–44). Because insulin-stimulated phosphorylation of AS160 is impaired in muscle from type 2 diabetic subjects (23), the identification of insulin-independent signaling proteins that stimulate glucose transport should provide worthwhile targets for pharmacological agents for the treatment of insulin resistance.

An important finding from the present study was the identification and characterization of filamin A, a ~300-kDa actin-binding protein that undergoes phosphorylation in skeletal muscle after endurance exercise. Filamin A is the most widely distributed isoform of a family of high–molecular weight dimeric proteins (45,46). Filamin is expressed as three highly related isoforms termed filamin A, B, and C (45) and is thought to stabilize and anchor three-dimensional actin filament networks with cell membranes (31). Actin filament remodeling promotes membrane ruffling in various cells, including myotubes, adipocytes, and fibroblasts. Moreover, disruption of actin cytoskeleton impairs GLUT4 translocation to cell surface of muscle and adipose cells (47,48). Thus, actin filament networks play an important role in GLUT4 trafficking. The rigidity or flexibility of actin filament network depends on the filamin-to-actin ratio such that an increase in the ratio of filamin to actin leads to tighter networks (49). Phosphorylation of filamin A may lead to actin remodeling and might be involved in membrane ruffle formation (50,51).

In addition to actin, filamin A has more that 20 other binding partners, including membrane receptors, small GTPases, and stress-activated protein kinase (46). Filamin isoforms may also act as scaffolding proteins or tethering components of signaling pathways to enhance or suppress activation by particular agonists (31,45). For example, in

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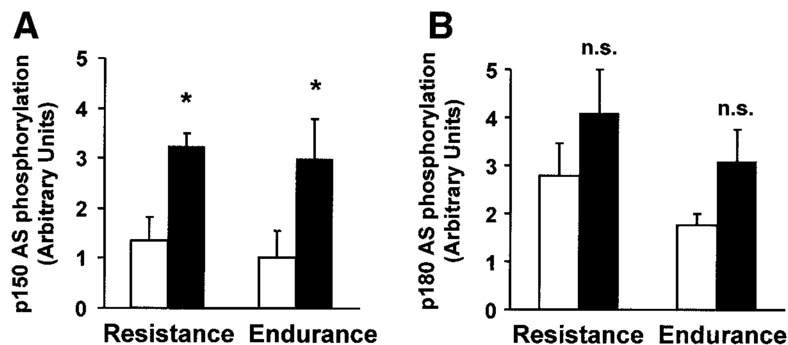

FIG. 5. Effect of exercise on pp150 and pp180. Anti-PAS immunoblot analysis was performed as described in Fig. 2*B* using lysates prepared from skeletal muscle obtained before (rest) (□) or after (post) (■) resistance or endurance exercise. Phosphoproteins of 150 (*A*) and 180 (*B*) kDa were quantified. Values in graphs are means ± SE (arbitrary units). *$P < 0.05$ vs. rested.

HepG2 cells, filamin A interacts with the insulin receptor to exert inhibitory tone along the mitogen-activated protein kinase cascade (52). Moreover, a role for filamin A as a scaffolding and actin cytoskeleton remodeling protein important for insulin receptor internalization has been proposed (53). Immunoprecipitation of cell lysates obtained from human skeletal muscle biopsies at rest or after exercise with anti–filamin A and subsequent immunoblot with the PAS antibody confirmed the phosphoprotein of 300 kDa as filamin A. However, the residues on filamin A that are recognized by the PAS antibody are unknown and do not appear to involve the well-characterized phosphorylation site at $Ser^{2,152}$, suggesting that exercise leads to phosphorylation of a novel residue (other that $Ser^{2,152}$).

The skeletal muscle–specific isoform, filamin C, has been identified and characterized as an insulin-responsive Akt substrate (31). Filamin C is phosphorylated in human muscle in response to insulin but not in response to exercise (data not shown), whereas filamin A is phosphorylated in response to endurance exercise but not insulin. Phosphorylation of filamin A may directly stimulate binding or cross-linking of actin filaments and regulate their binding with other partners to modulate signaling events mediating exercise-induced metabolic or gene regulatory responses.

In addition to AS160 and filamin A, several other exercise-responsive phosphoproteins, including pp250, pp180, pp150, pp42, and pp34, were detected in crude lysates of skeletal muscle after immunoblot analysis with the PAS antibody. Endurance but not resistance exercise primarily increased AS160 and filamin A phosphorylation. However, both forms of contraction tended to increase pp180 and significantly increased pp150 phosphorylation in skeletal muscle. The identity of these phosphoproteins is presently unknown. Interestingly, contraction of rat epitrochlearis muscle is associated with an increase in the phosphorylation of pp250 and pp180, as detected by immunoblot analysis using the PAS antibody (11). Although the identity of these proteins in rat skeletal muscle is also unknown, the authors proposed tuberous sclerosis complex 2, an inhibitor of cell growth and tumorigenesis, as a possible candidate for the 180-kDa protein. Further studies to identify exercise-responsive phosphoproteins in human skeletal muscle are currently underway.

Thus, analogous exercise-induced protein phosphorylation profiles are observed between human and rodent skeletal muscle. Similar experiments performed in insulin-stimulated 3T3-L1 adipocytes reveal that multiple phosphoproteins in addition to AS160, such as pp250, pp105, pp75, and pp47, can be detected by immunoblot analysis using the PAS antibody (22). Inhibitor studies and in vitro phosphorylation assays using recombinant Akt indicated that pp250, pp105, and pp47 are likely to be Akt substrates relevant for insulin action (25). Collectively, these studies reveal the PAS antibody has great utility for the identification of novel phosphoproteins involved in insulin- and exercise-mediated signaling cascades.

In conclusion, endurance exercise increases AS160 and filamin A phosphorylation in human skeletal muscle. The effects of exercise on protein phosphorylation occur primarily after endurance, rather than strength exercise, at least in subjects with a history of endurance activity. In contrast, pp150 phosphorylation was increased irrespective of training mode. AS160 and filamin A may provide a link to mediate exercise-induced metabolic and biological events in skeletal muscle.

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