

Impairments in Site-Specific AS160 Phosphorylation and Effects of Exercise Training

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The purpose of this study was to determine if site-specific phosphorylation at the level of Akt substrate of 160 kDa (AS160) is altered in skeletal muscle from sedentary humans across a wide range of the adult life span (18–84 years of age) and if endurance- and/or strength-oriented exercise training could rescue decrements in insulin action and skeletal muscle AS160 phosphorylation. A euglycemic-hyperinsulinemic clamp and skeletal muscle biopsies were performed in 73 individuals encompassing a wide age range (18–84 years of age), and insulin-stimulated AS160 phosphorylation was determined. Decrements in whole-body insulin action were associated with impairments in insulin-induced phosphorylation of skeletal muscle AS160 on sites Ser-588, Thr-642, Ser-666, and phospho-Akt substrate, but not Ser-318 or Ser-751. Twelve weeks of endurance- or strength-oriented exercise training increased whole-body insulin action and reversed impairments in AS160 phosphorylation evident in insulin-resistant aged individuals. These findings suggest that a dampening of insulin-induced phosphorylation of AS160 on specific sites in skeletal muscle contributes to the insulin resistance evident in a sedentary aging population and that exercise training is an effective intervention for treating these impairments. *Diabetes* 62:3437–3447, 2013

Skeletal muscle plays a prominent role in whole-body glucose regulation and is considered the primary target for insulin-mediated glucose uptake (1). In skeletal muscle, the binding of insulin to the insulin receptor initiates a signaling process that results in the translocation of the insulin-sensitive glucose transporter (GLUT4) to cell surface membranes and the facilitated diffusion of glucose into the cell (2). The complex nature of this process is evident by data indicating normal activation of proximal signaling components, including Akt, despite overt insulin-resistant conditions

imposed by lipid infusion (3), fasting (4), obesity (5), and diabetes (5). Such findings suggest that elements downstream of Akt may be more closely related to insulin action.

In skeletal muscle, the Akt substrate of 160 kDa (AS160, also known as TBC1D4), a Rab guanosine triphosphatase-activating protein (GAP), is currently recognized as the most distal signaling step associated with insulin-mediated glucose transport. In the basal state, the GAP guanosine triphosphatase-activating domain of AS160 is hypothesized to maintain Rab proteins in their inactive form, allowing AS160 to colocalize and retain GLUT4 in intracellular vesicles (6). In response to insulin, AS160 becomes phosphorylated on a number of Akt consensus sequences, suppressing its GAP activity and resulting in the translocation of GLUT4 to the plasma membrane (6–9). The functional importance of phosphorylated AS160 is evident because a mutation in one or more phosphorylation sites results in a reduction in insulin-stimulated GLUT4 translocation (9–11).

Insulin-stimulated AS160 phosphorylation may be impaired in insulin-resistant conditions because the insulin-induced phosphorylation of AS160 is diminished with type 2 diabetes (12,13), polycystic ovary syndrome (14), and fasting (4). Site-specific impairments in AS160 phosphorylation (Ser-318, Ser-588, and Ser-751) have recently been reported (13) with type 2 diabetes, suggesting that certain phospho-specific sites may have greater implications in insulin resistance. However, it is not evident whether the site-specific regulation of AS160 is evident and consistent across insulin-resistant conditions in human skeletal muscle.

The inhibitory mechanisms regulating AS160 phosphorylation remain obscure. In adipocytes, the transcriptional coregulator, receptor interacting protein 140 (RIP140), has been reported to interact with AS160, impeding the ability of Akt to phosphorylate AS160 (15). It remains unknown whether RIP140 impairs AS160 phosphorylation through a similar mechanism in the skeletal muscle of insulin-resistant individuals.

Endurance- (16–18) and strength-oriented (19,20) exercise training can both improve insulin sensitivity and are recommended as a means of intervention/prevention for insulin resistance. However, data examining the effect of exercise training on AS160 phosphorylation in human skeletal muscle is sparse. Some findings indicate that short-term endurance training (3 weeks or less) was not sufficient to increase insulin-stimulated AS160 phosphorylation in young, healthy (21), obese, nondiabetic (22), or diabetic individuals (22). Unfortunately, conclusions from these studies (21,22) are limited based on their use of the anti-phospho-Akt substrate (PAS) antibody, which is thought to only recognize phosphorylation of AS160 on site Thr-642 (9,23).

The insulin resistance typically evident in middle- to older-aged individuals is multifaceted and involves increases in

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overall and central adiposity and a reduction in cardiorespiratory fitness as well as the effect(s) of chronological age itself (1,24–31). The main objectives of the current study were to 1) determine if the insulin resistance evident in sedentary, middle- to older-aged individuals is associated with impaired site-specific phosphorylation of AS160 in human skeletal muscle and 2) to determine whether strength- and/or endurance-oriented exercise training could rescue these impairments.

RESEARCH DESIGN AND METHODS

Experimental design. Study 1 used a cross-sectional design encompassing younger to older individuals to determine if insulin action is associated with a decrement in insulin signaling at the level of AS160. Study 2 determined if a specific exercise training modality (endurance or strength training) could effectively ameliorate the insulin resistance evident in sedentary, insulin-resistant aged individuals by enhancing the most distal component of insulin signal transduction. Figure 1 provides an overview of the experimental design. **Study 1—Cross-sectional study examining insulin action and distal insulin signaling in human skeletal muscle**

Participants. Specifically recruited for this study were 73 participants (41 women, 32 men) comprising a wide age range (18–84 years). Physical characteristics of the subjects are provided in Table 1. All participants were nonsmokers and participated in less than 1 h/week of organized physical activity, as assessed by a standardized questionnaire. In an attempt to study a representative population, inclusion required that a participant's BMI be between the 25th and 75th percentile for his or her decade of age (32). Excluded were individuals with heart disease, diabetes, endocrine, and/or metabolic disorders, and those taking lipid-altering medication. Premenopausal women were tested during the early follicular phase of the menstrual cycle (days 1–6). Written informed consent was obtained, and the protocol was in accordance of the Declaration of Helsinki and approved by the East Carolina University Policy and Review Committee on Human Research.

Preliminary testing. Cardiorespiratory fitness was measured with an incremental, maximal treadmill test (33), with expired gases analyzed continuously (TrueMax 2400; ParvoMedics, Sandy, UT) to determine $\dot{V}O_{2\text{peak}}$. Body composition was measured by dual X-ray absorptiometry, and circumference measurements of the waist, hip, and thigh were obtained with a spring-loaded measuring tape.

Euglycemic-hyperinsulinemic clamp and muscle biopsies. Subjects reported to the laboratory at 0700 after a 12-h overnight fast. A 2-h euglycemic-hyperinsulinemic clamp was used to determine insulin action and elicit activation of insulin signaling, as previously described (18,34). Briefly, a primed insulin

(Humulin; Eli Lilly, Indianapolis, IN) infusion was performed for 10 min (starting at $313 \text{ mU} \cdot \text{m}^{-2} \cdot \text{min}^{-1}$), followed by a continuous infusion of insulin at a submaximal dosage of $100 \text{ mU} \cdot \text{m}^{-2} \cdot \text{min}^{-1}$. Blood samples were obtained every 5 min, centrifuged, and autoanalyzed for serum glucose (YSI 2300 STAT Plus Glucose and Lactate Analyzer; YSI Inc., Yellow Springs, OH), and the glucose infusion rate was adjusted as needed to maintain euglycemia. Blood plasma was obtained every 10 min and stored at -80°C for the subsequent analysis of plasma insulin (Access Immunoassay System; Beckman Coulter, Fullerton, CA). A steady-state M-value was determined from the final 20 min of the clamp (35).

A biopsy specimen was obtained from the vastus lateralis with the percutaneous muscle biopsy technique at baseline and at 60 min of the clamp. The 60-min time point was selected because we have previously reported that components of insulin signal transduction (PI3-kinase activation and Akt-Ser473 phosphorylation) appeared to be maximally activated at this time (36–38). Tissue samples were immediately frozen in liquid nitrogen for subsequent analyses.

Western blot and immunoprecipitation procedures. Skeletal muscle was homogenized and protein content determined as previously described (39,40). For Western blot analyses, muscle lysate (30–100 μg cellular protein) was separated by SDS-PAGE, electrotransferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA), and probed overnight with Cell Signaling (Beverly, MA) antibodies for PAS, phospho (p)Akt-Ser473 (recognizes Akt-Ser472/473/474), AS160, Akt2, and cyclooxygenase IV (COXIV). Membranes were also probed for pAS160-Thr642 (Millipore), pAS160-Ser666 (Millipore), pAS160-Ser588 (Symansis NZ Ltd, Timaru, New Zealand), GLUT4 (Affinity BioReagents, Golden, CO), RIP140 (Santa Cruz Biotechnology, Santa Cruz, CA), AS160 (Abcam, Cambridge, MA), and phospho-specific antibodies for AS160 at sites Ser-318 and Ser-751, as previously described (23,41). Proteins were visualized by horseradish peroxidase-conjugated IgG antibodies and ECL SuperSignal (Pierce Biotechnology, Rockford, IL) exposed to X-ray film. All samples were normalized to a control sample on each gel, and phosphorylation levels were additionally normalized to total protein after membranes were stripped, as previously reported (41), and reprobed with the corresponding antibody for total protein. For immunoprecipitation, lysates (200 μg) were incubated at 4°C overnight with Cell Signaling Technology antibodies for AS160 or Akt2 and for 3 h with protein A Sepharose beads (GE Healthcare Biosciences Corp., Piscataway, NJ). Supernatant portions from samples were removed and immunocomplexes analyzed with Western blotting.

Study 2—Effect of exercise training on distal insulin signaling

Participants. Of the 73 subjects recruited for the cross-sectional study, 45 volunteered for the experiment examining the effects of exercise training on insulin signal transduction. Inclusion criteria required participants to be ≤ 35 years of age (young) or ≥ 55 years of age (aged). These individuals were then randomized into a 12-week endurance- ($n = 12$ young, $n = 11$ aged) or strength-training ($n = 11$ young, $n = 11$ aged) program. Two individuals were subsequently excluded from the young, endurance group due to noncompliance (Fig. 1).

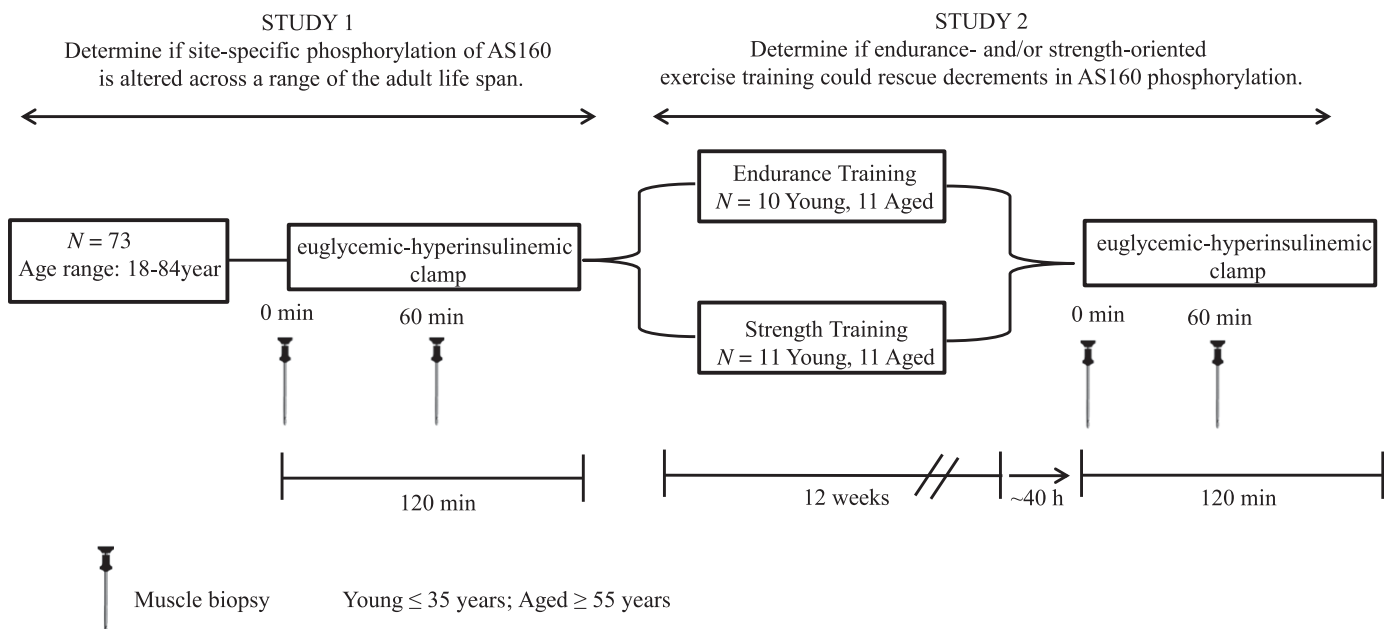


FIG. 1. Overview of experimental design. Young, ≤ 35 years (range 18–35); aged, ≥ 55 years (range 55–84).

TABLE 1
Participant characteristics for cross-sectional study

	Men (<i>n</i> = 32)	Women (<i>n</i> = 41)
Age	43.0 ± 4.3 (19–84)	45.0 ± 3.2 (18–76)
Mass (kg)	80.5 ± 2.1 (62.7–105.9)	68.4 ± 1.8 (52.3–97.7)*
BMI (kg/m ²)	25.6 ± 0.7 (19–34)	25.2 ± 0.6 (18–35)
Body fat (%)	23.0 ± 1.7 (8–39)	38.1 ± 1.3 (16–53)*
Trunk fat (%)	27.2 ± 2.1 (7.0–45.1)	39.1 ± 1.5 (14.3–54.8)*
Fat-free mass (kg)	57.6 ± 1.0 (48.3–72.4)	38.9 ± 0.8 (30.5–51.8)*
Waist-to-hip ratio	0.86 ± 0.02 (0.76–1.04)	0.76 ± 0.01 (0.66–0.92)*
VO ₂ peak (mL/kg/min)	34.8 ± 2.1 (12.6–58.7)	26.0 ± 1.2 (12.6–42.2)*
Fasting plasma glucose (mg/dL)	89.2 ± 1.4 (73–105)	88.1 ± 1.5 (71–118)
Fasting plasma insulin (μIU/mL)	5.5 ± 0.6 (1.5–14.4)	5.9 ± 0.8 (1.5–29.5)

Data are presented mean ± SEM (range). **P* < 0.005 vs. men.

Experimental protocol. All subjects performed preliminary cardiovascular, body composition, muscular strength, blood chemistry measurements, and a 3-day diet record, which were repeated during the final week of training (Tables 2 and 3). The euglycemic-hyperinsulinemic clamp with muscle biopsies was performed before exercise training and ~40 h after the final exercise training session (Fig. 1). All subjects completed a 24-h diet record the day before their pretraining euglycemic-hyperinsulinemic clamp and were then asked to duplicate this diet the day before their post-training test.

Endurance training. The endurance-training program consisted of exercising on a graded treadmill, stationary cycle, or elliptical trainer within a target heart rate zone equivalent to 70–75% VO₂peak for a total of 180 min/week (three to four sessions per week). To ensure the appropriate workload during training, a VO₂peak test was performed after 6 weeks of training and workload adjusted appropriately.

Strength training. Strength training consisted of upper and lower body exercises, performed three times per week (~45 min/session). The upper body exercises included chest press, latissimus pull down, seated row, triceps pull down, and biceps curl. The lower body exercises included leg press, leg extension, and leg curl. Participants alternated between upper and lower body exercises to minimize fatigue, with 60–90 s rest between sets, and performed 10–12 repetitions to failure. When a subject could complete 12 repetitions on

two consecutive occasions, resistance was increased by ~5%. Two sets were completed for upper and lower body exercises during weeks 1–6, and during weeks 7–12, a third set was added for lower body exercises.

Statistics. Analyses were performed using SPSS version 18.0 software (SPSS Inc., Chicago, IL). Pearson correlation coefficients and stepwise regression were used to determine associations. Comparisons between young and aged individuals before and after exercise training and under basal and insulin-stimulated conditions were performed with repeated-measures ANOVA. Significant main effects and interactions were further analyzed using unpaired (age group) and paired (pre- vs. post-training and baseline vs. insulin-stimulated) contrast-contrast comparisons. Data are presented as means ± SEM. Statistical significance was defined as *P* ≤ 0.05.

RESULTS

Study 1—Cross-sectional study examining insulin action and distal insulin signaling

Subject characteristics. The women exhibited a lower body and fat-free mass, waist-to-hip ratio, VO₂peak, and elevated body and trunk fat percentage compared with the

TABLE 2
Changes in characteristics of young and aged individuals before and after endurance training

Variable	Young (<i>n</i> = 10)		Aged (<i>n</i> = 11)	
	Pretraining	Post-training	Pretraining	Post-training
Age (range)	24.4 ± 1.6 (18–34)		69.0 ± 2.2† (57–84)	
Sex (<i>n</i>)				
Female	5		5	
Male	5		6	
Insulin action				
M-value (mg/kg/min)	7.9 ± 0.8	9.8 ± 0.7*	5.7 ± 0.5†	6.3 ± 0.6†*
Body composition				
Mass (kg)	69.8 ± 2.2	69.2 ± 2.5	80.3 ± 4.2†	80.4 ± 4.4†
BMI (kg/m ²)	23.8 ± 0.9	23.5 ± 0.7	27.9 ± 1.1†	27.9 ± 1.2†
Body fat (%)	25.5 ± 3.7	23.6 ± 3.5*	38.4 ± 2.4†	37.3 ± 2.6†*
Lean body mass (kg)	47.4 ± 2.8	48.8 ± 3.1*	47.3 ± 3.3	47.8 ± 3.3*
Thigh circumference (cm)	48.7 ± 1.6	48.3 ± 1.5	50.1 ± 1.5	50.3 ± 1.8
Waist-to-hip ratio	0.78 ± 0.02	0.77 ± 0.02	0.88 ± 0.04†	0.87 ± 0.03†
Performance				
VO ₂ peak (mL/kg/min)	35.0 ± 1.6	41.7 ± 1.3*	19.9 ± 1.2†	22.3 ± 1.5†*
Peak isokinetic leg extension (N)	599 ± 72	612 ± 57	449 ± 47	468 ± 35
Peak isokinetic leg flexion (N)	396 ± 49	429 ± 50	252 ± 32†	272 ± 41†
1-RM leg press (kg)	119 ± 9	121 ± 10	96 ± 10	99 ± 12
1-RM chest press (kg)	49 ± 6	54 ± 8*	33 ± 3	37 ± 8*
Fasting blood chemistry				
Glucose (mg/dL)	84.3 ± 2.4	78.6 ± 2.8*	91.6 ± 3.1	89.1 ± 4.0*
Insulin (IU/mL)	4.6 ± 1.0	3.7 ± 1.1*	7.3 ± 1.2	4.3 ± 1.1*

Data are presented mean ± SEM (range) or as indicated. Statistics performed on absolute values. †*P* < 0.05 age main effect. **P* < 0.05 training main effect.

TABLE 3
Changes in characteristics of young and aged individuals before and after strength training

Variable	Young (<i>n</i> = 11)		Aged (<i>n</i> = 11)	
	Pretraining	Post-training	Pretraining	Post-training
Age (range)	23.6 ± 1.5 (20–35)		69.3 ± 2.7† (55–82)	
Sex (<i>n</i>)				
Female	4		6	
Male	7		5	
Insulin action				
M-value (mg/kg/min)	9.4 ± 0.8	10.8 ± 1.0*	6.1 ± 0.7†	6.9 ± 0.7†*
Body composition				
Mass (kg)	71.8 ± 3.5	73.3 ± 3.3*	76.4 ± 3.7	76.7 ± 3.7*
BMI (kg/m ²)	23.5 ± 0.7	25.3 ± 1.2	26.8 ± 1.1	26.9 ± 1.1
Body fat (%)	22.9 ± 3.7	22.1 ± 3.7	36.9 ± 2.6†	36.2 ± 2.7†
Lean body mass (kg)	53.0 ± 3.6	53.9 ± 3.4*	45.2 ± 3.3	46.2 ± 3.3*
Thigh circumference (cm)	50.9 ± 1.1	51.4 ± 1.0	49.0 ± 1.1	48.5 ± 1.5
Waist-to-hip ratio	0.76 ± 0.02	0.75 ± 0.02	0.87 ± 0.03†	0.87 ± 0.03†
Performance				
V _{O₂} peak (mL/kg/min)	38.2 ± 2.1	41.5 ± 2.8*	20.6 ± 1.8†	21.7 ± 1.9†*
Peak isokinetic leg extension (N)	718 ± 70	874 ± 73*	500 ± 43†	534 ± 36†
Peak isokinetic leg flexion (N)	413 ± 38	530 ± 41*	292 ± 24†	331 ± 29†*
1-RM leg press (kg)	121 ± 11	148 ± 17*	81 ± 12†	103 ± 9†*
1-RM chest press (kg)	54 ± 10	58 ± 9*	31 ± 5	37 ± 5*
Fasting blood chemistry				
Glucose (mg/dL)	84.1 ± 2.5	80.1 ± 2.8*	92.3 ± 2.8	89.5 ± 2.3*
Insulin (IU/mL)	4.8 ± 1.1	3.7 ± 0.9*	5.1 ± 1.1	3.7 ± 1.0*

Data are presented mean ± SEM (range) or as indicated. Statistics performed on absolute values. †*P* < 0.05 age main effect. **P* < 0.05 training main effect.

men (Table 1). Univariate correlations indicated that mass ($r = 0.26$, $P < 0.05$), BMI ($r = 0.45$, $P < 0.001$), body fat percentage ($r = 0.49$, $P < 0.001$), trunk fat ($r = 0.53$, $P < 0.001$), and waist-to-hip ratio ($r = 0.50$, $P < 0.001$) increased with age. Relative V_{O₂}peak (mL/kg/min) declined with age ($r = -0.78$, $P < 0.001$), whereas fasting blood glucose ($r = 0.40$, $P < 0.001$) increased with age.

Insulin action and AS160 phosphorylation. The euglycemic-hyperinsulinemic clamp increased ($P < 0.001$) plasma insulin concentration from fasting ($5.0 \pm 0.4 \mu\text{IU/mL}$) to $160 \pm 6 \mu\text{IU/mL}$. Whole-body insulin action (M-value) declined with age ($r = -0.52$, $P < 0.001$; Supplementary Fig. 1). Sex had no effect on this relationship; therefore, data from men and women were combined for subsequent analyses.

Stepwise linear regression analysis was used to determine variables (age, body weight, BMI, percentage body fat, percentage trunk fat, waist-to-hip ratio, V_{O₂}peak, and fasting insulin) that independently predicted insulin action. Age and BMI were both independent predictors of insulin action (total adjusted $R^2 = 0.44$, $P < 0.001$).

Insulin infusion increased ($P < 0.001$) phosphorylation of all five AS160 phospho-specific sites and PAS by ~2- to 8-fold and of Akt2 Ser-473 by ~30-fold. As presented in Fig. 2, whole-body insulin action was positively related with AS160 phosphorylation when determined by PAS ($r = 0.33$, $P < 0.01$) and on sites Ser-588 ($r = 0.34$, $P < 0.05$), Thr-642 ($r = 0.33$, $P < 0.05$), and Ser-666 ($r = 0.32$, $P < 0.05$). Insulin action was not related to AS160 phosphorylation of Ser-751 or Ser-318 sites or with Akt2 Ser-473 phosphorylation ($n = 61$, $r = 0.15$, $P > 0.05$).

Stepwise linear regression analysis was used to determine variables (age, BMI, percentage body fat, percentage trunk fat, waist-to-hip ratio, V_{O₂}peak, fasting glucose, and insulin) that independently predicted insulin-induced

phosphorylation of AS160. Chronological age was the sole predictor of insulin-induced phosphorylation of AS160 Ser-666 ($R^2 = 0.12$, $P < 0.05$). Body fat was determined to be the best predictor of insulin-induced phosphorylation of AS160 PAS ($R^2 = 0.12$, $P < 0.01$) and AS160 Ser-588 ($R^2 = 0.10$, $P < 0.05$), whereas fasting insulin was determined to be the best predictor of insulin-induced phosphorylation of AS160 Thr-642 ($R^2 = 0.10$, $P < 0.05$).

Age was not related to the ability of insulin to phosphorylate Akt2 Ser-473 ($r = -0.18$, $P > 0.05$; data not shown). Chronological age was negatively related to the insulin-stimulated phosphorylation of AS160 determined by PAS ($r = -0.25$, $P < 0.05$) and sites Ser-588 ($r = -0.28$, $P < 0.05$), Thr-642 ($r = -0.28$, $P < 0.05$), and Ser-666 ($r = -0.30$, $P < 0.05$; Supplementary Fig. 2). Basal phosphorylation levels of AS160 Thr-642 increased with age ($r = 0.30$, $P < 0.05$; data not shown). Age had no other effect on AS160 basal phosphorylation levels or protein content. GLUT4 protein content demonstrated a trend for a negative relationship with age ($r = -0.24$, $P = 0.06$; Supplementary Fig. 2).

RIP140. Total RIP140 protein content was determined in 19 young (range 18–35 years) and 20 insulin-resistant aged (range 57–84 years) individuals, and the amount of RIP140 complexed with AS160 was determined in a subset of 11 young (range 18–26 years) and 9 insulin-resistant aged (range 56–82 years) individuals. There were no differences in total RIP140 protein content between young and aged individuals (Fig. 3A), but insulin-resistant aged individuals had a higher amount of RIP140 complexed with AS160 ($P < 0.03$, Fig. 3B) compared with their young counterparts. As presented in Fig. 3C, the amount of RIP140 complexed with AS160 exhibited a trend ($r = -0.42$, $P = 0.06$) to be negatively related to insulin action.

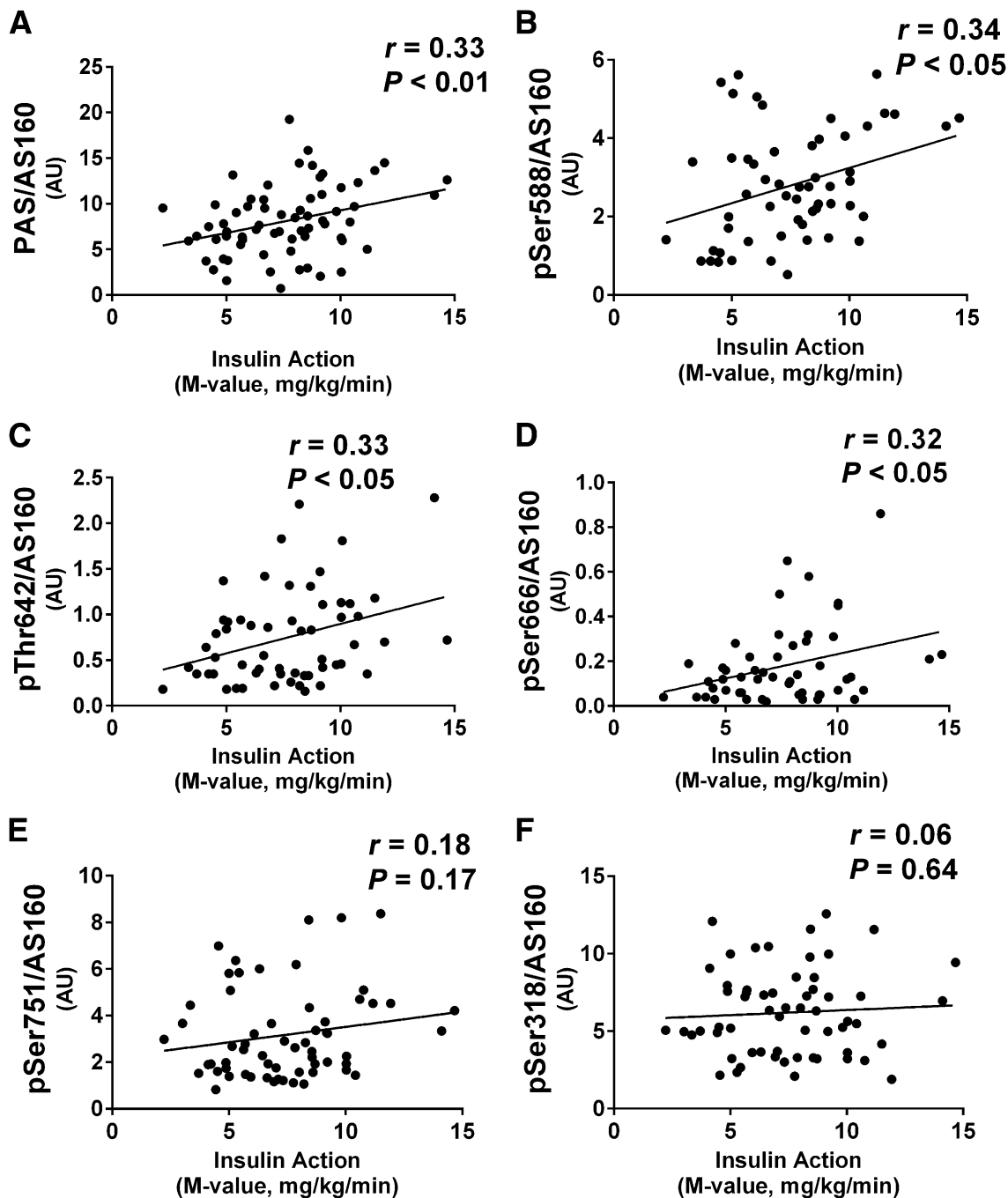


FIG. 2. Relationships among insulin-stimulated phosphorylation levels of skeletal muscle AS160 PAS ($n = 67$) (A), Ser-588 ($n = 56$) (B), Thr-642 ($n = 57$) (C), Ser-666 ($n = 52$) (D), Ser-751 ($n = 61$) (E), and Ser-318 ($n = 60$) (F) with whole-body insulin action. A: For PAS, AS160 was immunoprecipitated and then blotted with the PAS antibody. B–F: All other blots used phospho-specific antibodies. Values, in AU were normalized to a control sample run on each blot and then presented relative to total AS160 protein. AU, arbitrary units.

Study 2—Effects of exercise training on insulin action and distal insulin signaling

General adaptations to exercise training. Differences in whole-body insulin action (M-value), body composition, $\dot{V}O_2$ peak, and fasting blood chemistries were evident between the young and aged groups before initiating the 12 weeks of exercise training (Tables 2 and 3). With endurance training, there was a reduction in body fat and an increase in lean body mass, which resulted in no change in overall body mass (Table 2). Endurance training also increased insulin action (M-value), $\dot{V}O_2$ peak, and 1-repetition maximum (RM) chest press and decreased fasting

plasma glucose and insulin (Table 2). Strength training increased 1-RM leg press, 1-RM chest press, and peak isokinetic leg flexion strength in young and insulin-resistant aged participants and also increased lean and total body mass and $\dot{V}O_2$ peak (Table 3). Insulin action also increased irrespective of group, whereas fasting plasma glucose and insulin concentrations decreased in response to strength training (Table 3). Skeletal muscle COXIV protein, a marker of mitochondrial content, increased in response to endurance training (~30%) but not with strength training (Supplementary Fig. 3). Analysis of 3-day diet records revealed no significant changes in caloric

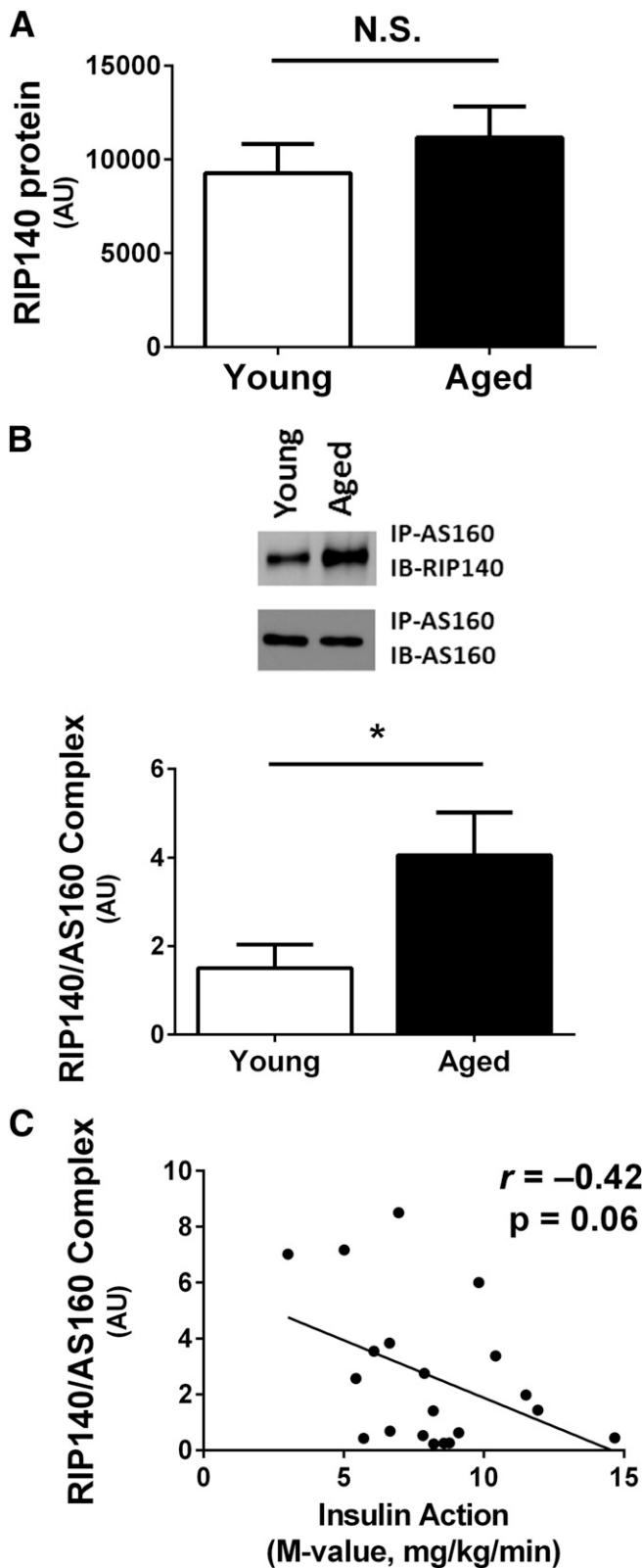


FIG. 3. Quantification of total RIP140 (A) in the skeletal muscle of young ($n = 19$, 18–35 years) vs. aged ($n = 20$, 57–84 years) individuals and the quantification of RIP140/AS160 complex (B) in the skeletal muscle of young ($n = 11$, 18–26 years) vs. aged ($n = 9$, 57–82 years) individuals. C: Relationship of RIP140/AS160 complex with insulin action ($n = 20$). B and C: For determination of RIP140/AS160 complex, whole-cell muscle lysates were immunoprecipitated (IP) with AS160 and immunoblotted (IB) with RIP140 (complex formation) and AS160 (total AS160 immunoprecipitated). Values are in AU and data expressed as mean \pm SEM. AU, arbitrary units; N.S., nonsignificant. * $P \leq 0.05$.

intake (total, protein, carbohydrate, and fat) during the training period.

AS160 phosphorylation. In agreement with the correlation analyses (Supplementary Fig. 1), reductions in insulin-stimulated Ser-588, Thr-642, Ser-666 ($P < 0.05$), and PAS ($P = 0.06$) phosphorylation were evident in insulin-resistant aged individuals in the pretraining, sedentary state (Fig. 4). Endurance training increased insulin-stimulated AS160 phosphorylation of PAS by $\sim 60\%$ in young and $\sim 75\%$ in insulin-resistant aged individuals ($P < 0.05$, Fig. 5A), whereas AS160 Ser-588 phosphorylation increased $\sim 25\%$ in both groups ($P < 0.05$, Fig. 5B). There was a significant interaction ($P < 0.05$) for AS160 Thr-642 in response to endurance training as the aged individuals increased AS160 Thr-642 phosphorylation by $\sim 57\%$ ($P < 0.05$, Fig. 5C), whereas no significant changes were observed in the young individuals. There was a tendency ($P = 0.07$) for insulin-stimulated Ser-666 phosphorylation to increase with endurance training in the aged subjects (Fig. 5D). No changes with endurance training were evident in insulin-stimulated Ser-751 and Ser-318 (data not shown). Strength training increased insulin-stimulated AS160 phosphorylation of PAS by $\sim 75\%$ ($P < 0.01$, Fig. 6A) in both groups, whereas insulin-stimulated Thr-642 phosphorylation increased by $\sim 33\%$ and $\sim 73\%$ in young and aged individuals ($P < 0.05$, Fig. 6C), respectively. In addition, strength training increased insulin-stimulated Ser-666 phosphorylation of AS160 by $\sim 100\%$ (Fig. 6D) in the insulin-resistant aged group. There were no changes in Ser-588 (Fig. 6B), Ser-751 (data not shown), or Ser-318 (data not shown) with strength training.

Exercise training had no effect on AS160 protein content (data not shown). Basal phosphorylation levels of PAS were significantly reduced ($\sim 50\%$, $P < 0.01$) in response to strength training, which contributed to the significant training effect (Fig. 6A). Exercise training had no other effect on basal AS160 phosphorylation levels. Endurance training increased GLUT4 ($\sim 10\%$ for young and $\sim 15\%$ for aged, $P < 0.05$; Supplementary Fig. 4), whereas strength training had no effect. Multiple regression analysis indicated that exercise-induced changes in phosphorylation of PAS, Ser-588, Thr-642, and Ser-666 accounted for 28% of the variance in the improvement in insulin action ($P < 0.05$).

Before exercise training, Akt2 protein levels did not differ between the groups (data not shown). Endurance training increased Akt2 protein levels by $\sim 60\%$ in the young group ($P < 0.01$), with a similar trend in the aged individuals ($P = 0.08$). In response to strength training, Akt2 protein levels demonstrated a trend for an increase ($P = 0.08$). Neither age nor exercise training had an effect on Akt2 Ser-473 phosphorylation when normalized to protein content (Supplementary Fig. 5).

DISCUSSION

In the current study, we show for the first time that insulin-stimulated AS160 phosphorylation, measured by the PAS antibody, and specific phosphorylation at sites Ser-588, Thr-642, and Ser-666 are impaired in human skeletal muscle in conjunction with the decrement in insulin action typical with advancing age and a sedentary lifestyle (Figs. 2 and 4). Impaired insulin-mediated AS160 phosphorylation has been reported in other insulin-resistant conditions, including type 2 diabetes (12) and polycystic ovary syndrome (14), using the PAS antibody. The PAS antibody

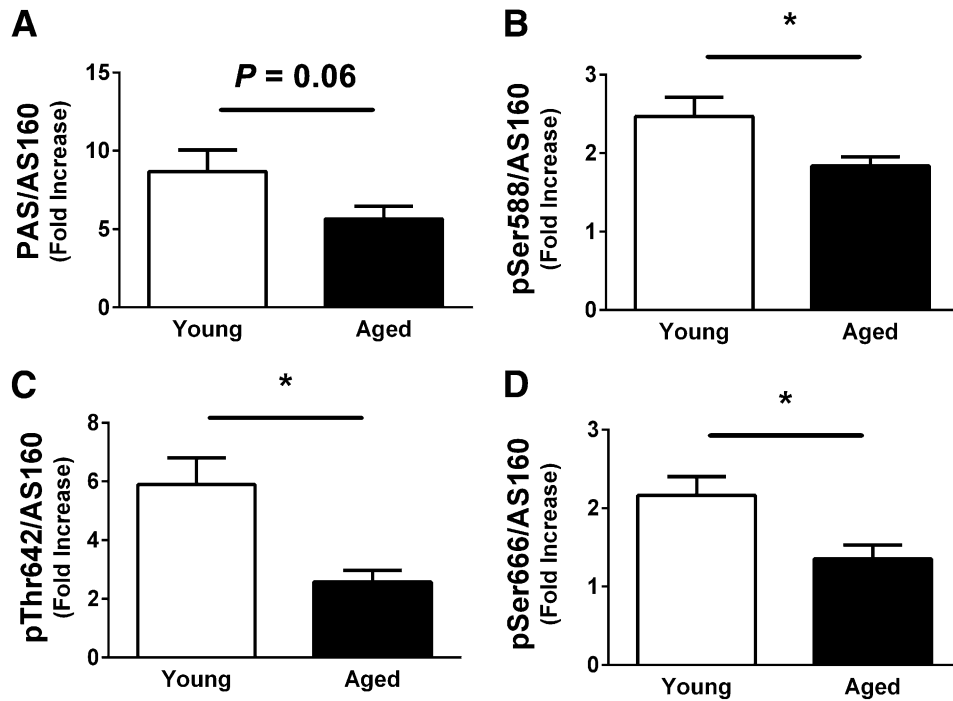


FIG. 4. Fold change (insulin stimulated over basal) in AS160 PAS (A), Ser-588 (B), Thr-642 (C), and Ser-666 (D) in young ($n = 18-21$, age range 18–35 years, white bars) and aged ($n = 19-22$, age range 55–84 years, black bars) individuals. Data are mean \pm SEM. * $P < 0.05$.

may recognize multiple phosphorylation sites on AS160; however, current research suggests this antibody is limited to only recognizing AS160 phosphorylation on Thr-642 (9,23). More recently, site-specific impairments were identified in patients with type 2 diabetes (Ser-318, Ser-588, and Ser-751) (13) and in healthy individuals after fasting-induced insulin resistance (Ser-588 and Ser-751) (4). The current data (Figs. 2 and 4), in combination with other data (4,13), provide the important information that an impairment in Ser-588 phosphorylation appears to be consistent in human skeletal muscle across conditions inducing insulin resistance. In contrast, other AS160 sites demonstrate differential phosphorylation patterns, possibly as a product of kinases and phosphatases being regulated by the severity or the pathology of insulin resistance. Collectively, these findings show that conditions of whole-body insulin resistance are linked with site-specific impairments in AS160 and provide novel insight into a signaling impairment located distally in the insulin-signaling cascade.

In an effort to investigate cellular mechanisms that could contribute to the impaired AS160 phosphorylation, we examined RIP140 expression and its association with AS160. In adipocytes, the binding of RIP140 to AS160 results in reduced glucose uptake, likely as a result of RIP140 impeding the ability of Akt to inactivate GAP activity on AS160 (15). The current finding that insulin-resistant aged individuals had a higher amount of RIP140 complexed with AS160 (Fig. 3B) provides novel evidence that the impairment in AS160 phosphorylation may be linked to the association of AS160 with RIP140, which in turn induces insulin resistance (Fig. 3C).

In an attempt to gain an understanding of factors that may regulate site-specific phosphorylation on AS160, we performed regression analyses using variables linked with insulin action. Body fat percentage was the best predictor

of Ser-588 phosphorylation, whereas basal plasma insulin levels proved to be the best predictors of Thr-642 phosphorylation, suggesting these phosphorylation sites may be differentially regulated. In agreement with these findings, *in vitro* experiments in adipocytes demonstrated that insulin-stimulated phosphorylation of Thr-642 occurs much more rapidly than Ser-588, and hierarchical clustering analysis revealed that Thr-642 did not cluster with Ser-588 (42). Taken together, this information provides insight into potential regulatory mechanisms of AS160 phosphorylation; however, we acknowledge that regression analyses only imply relationships and that additional variables not measured in the current study may also play a role in the regulation of site-specific AS160 phosphorylation.

Of the two Akt isoforms expressed in skeletal muscle (Akt1 and Akt2), Akt2 is considered crucial for glucose uptake in skeletal muscle (43). In relation to upstream signaling of AS160, we recognize that phosphorylation of both Akt Ser-473 and Thr-308 is required for the full activation of Akt; however, the current study was limited to Akt2 Ser-473 based on results in human skeletal muscle indicating that insulin-stimulated Akt2-Ser473 phosphorylation (as opposed to Akt-Thr308) was closely related to AS160-PAS phosphorylation and glucose uptake (43). Insulin-stimulated Akt2-Ser473 phosphorylation was not associated with insulin-resistant aged individuals in the current study; however, Sharma et al. (44), recently reported reduced insulin-stimulated Akt2-Thr308 phosphorylation in the soleus of aged (25-month) compared with adult (9-month) rats; therefore, we cannot conclusively state that all Akt sites were preserved with insulin resistance.

Exercise training has long been recognized as a method to improve insulin action (16–19) (Tables 2 and 3). The effect of exercise training on insulin-stimulated AS160 phosphorylation has been sparsely addressed, particularly

Endurance Training

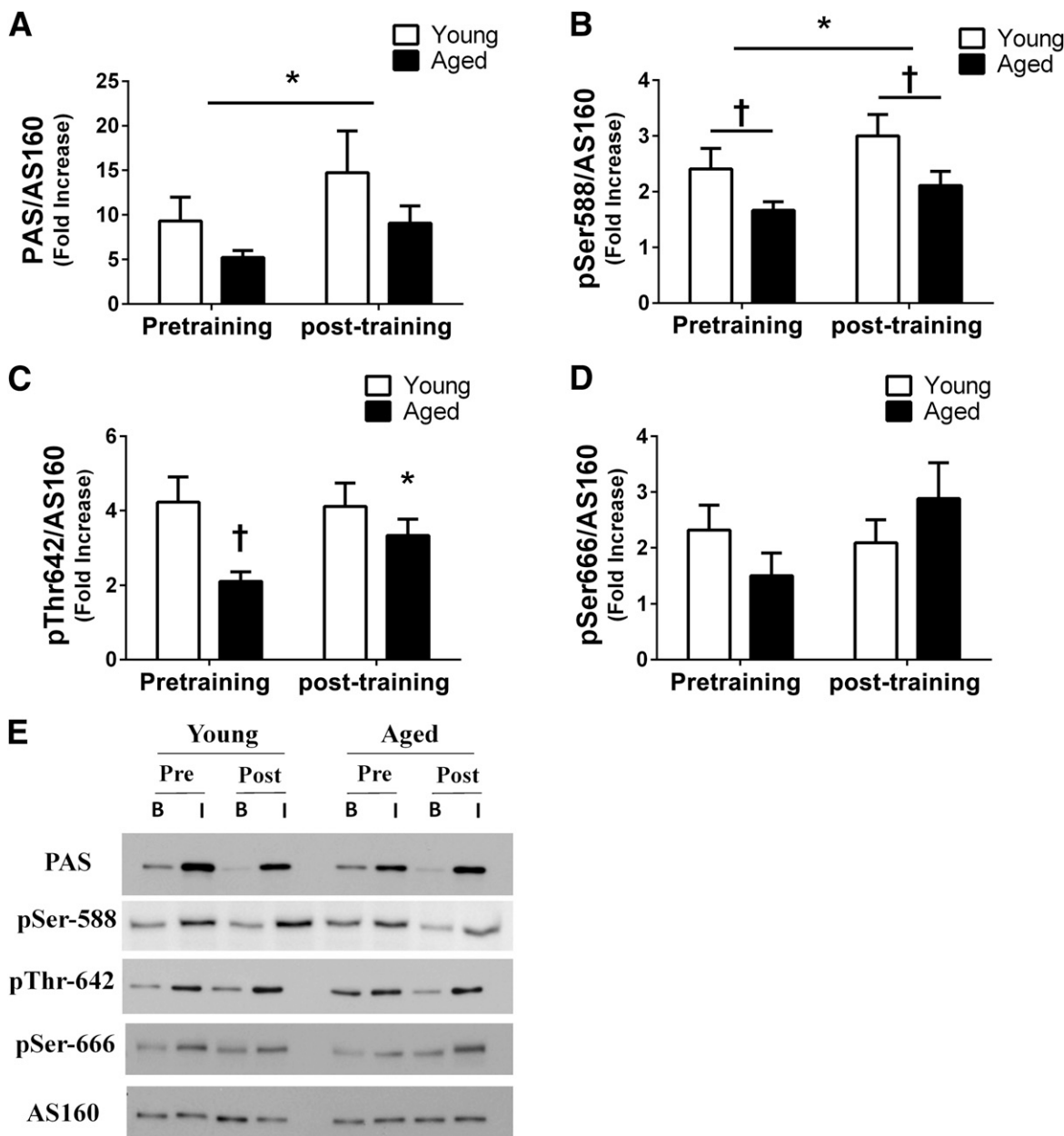


FIG. 5. Phosphorylation levels of skeletal muscle AS160 PAS ($n = 10$, young; $n = 11$, aged) (A), Ser-588 ($n = 10$, young; $n = 10$, aged) (B), Thr-642 ($n = 9$, young; $n = 9$, aged) (C), and Ser-666 ($n = 9$, young; $n = 8$, aged) (D) in response to insulin before and after 12 weeks of endurance training in young (age 24.4 ± 1.6 , range 18–34 years, white bars) and aged (age 69.0 ± 2.2 , range 57–84 years, black bars) individuals. Data are presented as fold change in phosphorylation levels normalized to total AS160 protein levels. The line above the bars represents the main effect for age (short bar) or training (long bar). Data are mean \pm SEM. E: Representative blots using AS160 phospho-specific antibodies and total protein in young and aged individuals under noninsulin (B) and insulin-stimulated (I) conditions. For PAS, AS160 was initially immunoprecipitated and then blotted with the PAS antibody. * $P < 0.05$ vs. pretraining. † $P < 0.05$ vs. young at that comparable time.

in regards to phospho-specific sites. Previous research reported that insulin-mediated AS160 phosphorylation increased in healthy young men after 3 weeks of one-legged endurance-oriented exercise training; however, these effects were negated when phosphorylation was normalized to AS160 protein content (21). In addition, O'Gorman et al. (22) reported that short-term endurance training (7 days) was not sufficient to increase insulin-stimulated AS160 phosphorylation in obese, nondiabetic, or diabetic individuals. However, conclusions from these studies (21,22) are limited based on the use of the PAS antibody.

A key finding in the current study was that decrements in specific insulin-stimulated AS160 phosphorylation sites were improved with exercise training (Figs. 5 and 6), with the exception of AS160 Ser-588 (Fig. 6B), which did not appear to be responsive to strength training (Fig. 6B). Vind et al. (13) previously reported increased insulin-induced AS160 phosphorylation on Ser-588 in type 2 diabetic patients, but not in nondiabetic control subjects, in response to 10 weeks of endurance training, suggesting that this site may be particularly responsive to endurance exercise in insulin-resistant populations. From our

Strength Training

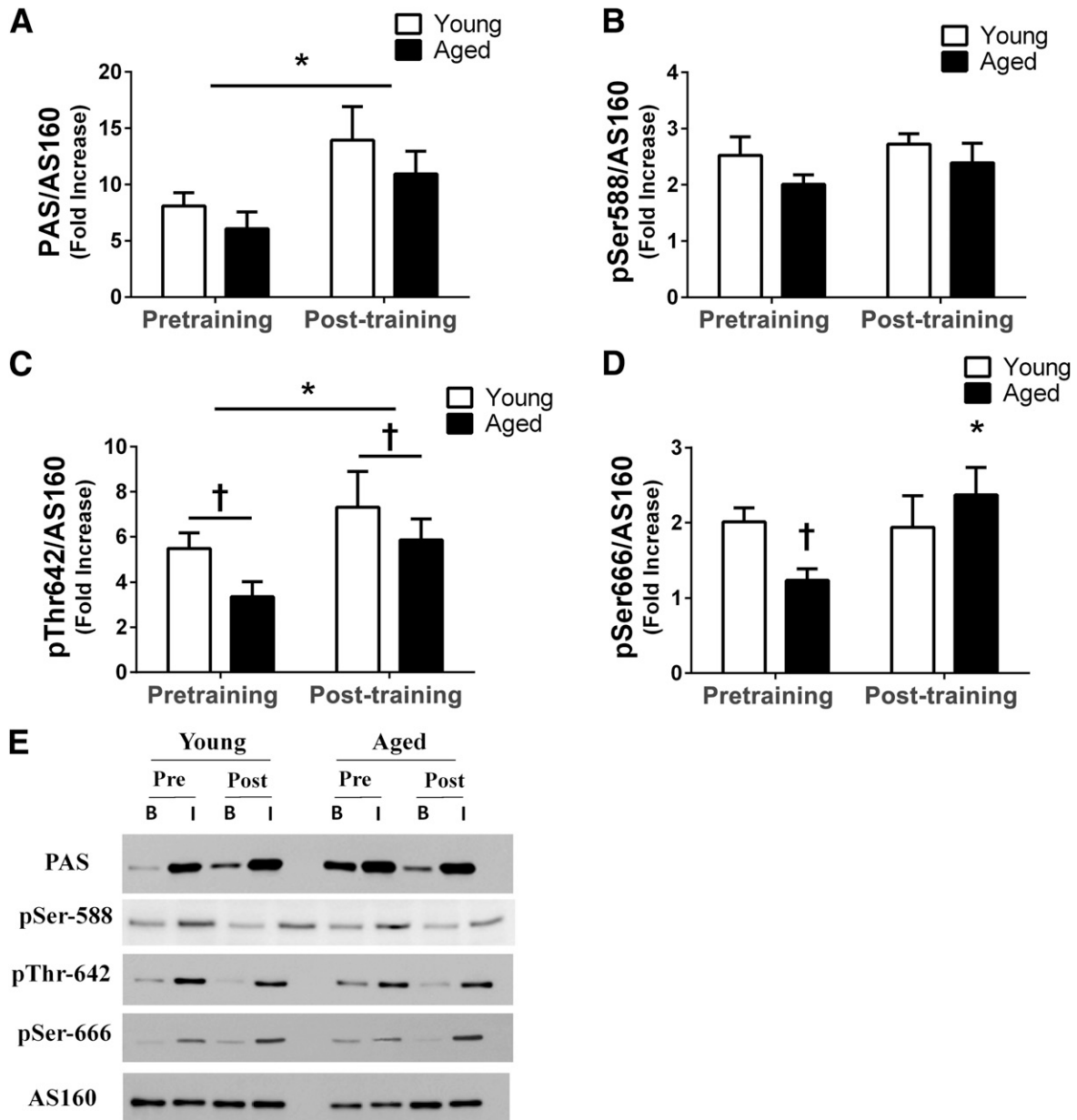


FIG. 6. Phosphorylation levels of skeletal muscle AS160 PAS ($n = 11$, young; $n = 11$, aged) (A), Ser-588 ($n = 11$, young; $n = 11$, aged) (B), Thr-642 ($n = 10$, young; $n = 10$, aged) (C), and Ser-666 ($n = 10$, young; $n = 10$, aged) (D) in response to insulin before and after 12 weeks of strength training in young (23.6 ± 1.5 years of age, range 20–35 years, white bars) and aged (69.3 ± 2.7 years, range 55–82 years, black bars) individuals. Data are presented as fold change in phosphorylation levels normalized to total AS160 protein levels. The line above the bars represents the main effect for age (short bar) or training (long bar). Data are mean \pm SEM. E: Representative blots using AS160 phospho-specific antibodies and total protein in young and aged individuals under noninsulin (B) and insulin-stimulated (I) conditions. For PAS, AS160 was initially immunoprecipitated and then blotted with the PAS antibody. * $P < 0.05$ vs. pretraining. † $P < 0.05$ vs. young at that comparable time.

cross-sectional data, we determined that body fat percentage was the best predictor of AS160 Ser-588 phosphorylation. Body fat percentage was reduced in response to endurance, but not strength training, which could explain why improvements in Ser-588 phosphorylation were only evident with this training modality. Protein kinase C ζ (PKC ζ) activity has been hypothesized to regulate AS160 Ser-588 phosphorylation (42), and endurance training has been reported to increase skeletal muscle PKC ζ activity (45). Although speculative, it is plausible to suggest that our endurance-training program improved PKC ζ activity,

which could in part explain improvements in insulin-stimulated AS160 Ser-588 phosphorylation.

Both modes increased PAS, Thr-642, and Ser-666 ($P = 0.07$ with endurance training) phosphorylation in the aged group, indicating the effectiveness of exercise in treating insulin resistance. The clinical relevance of our findings is that either endurance or strength training appears to improve insulin action through similar mechanisms in relation to insulin signaling at the level of AS160. This finding may provide relevant information in terms of therapeutic treatments for insulin-resistant conditions.

Consistent with other studies examining human subjects over a wide life span (1,24,26,30,46), our data demonstrated that whole-body insulin action declined with age (Supplementary Fig. 1). The nature of this age-related insulin resistance has been well-studied and likely involves a number of contributing factors, including increased abdominal adiposity (30), declining cardiorespiratory fitness (29,31), and chronological age itself (29), all of which were evident in our population (Table 1). Despite reports suggesting that the effect of chronological age is negated when adjusting for BMI (26), adiposity (27), or physical inactivity (28), our data indicate that chronological age was an independent predictor of whole-body insulin action, which is in agreement with the findings of at least one other study (29).

A limitation of the current study was that muscle fiber typing was not performed. Animal studies have reported greater age-related impairments in glucose uptake in slow-twitch compared with fast-twitch muscle (44,47), despite the apparent preservation of type I fiber cross-sectional area with aging (48). Likewise, 12 weeks of endurance or strength training has been associated with increases in type I (49) and type II (50) fiber area, respectively, independent of age (48,49). Therefore, the age- or exercise-related differences in AS160 phosphorylation in our current study could possibly have been influenced by changes in muscle fiber type. In addition, despite previous research suggesting maximal AS160 phosphorylation occurs at 60 min of an euglycemic-hyperinsulinemic clamp (36,38), we cannot exclude the possibility that the rate of site-specific AS160 phosphorylation was influenced by either age and/or exercise training.

In conclusion, the findings of the current study indicate for the first time that deficits in whole-body insulin action evident with the aging process and a sedentary lifestyle are associated with reduced insulin-stimulated phosphorylation of specific AS160 sites (Thr-642, Ser-588, Ser-666, and PAS). With respect to intervention/prevention, 12 weeks of endurance- or strength-oriented exercise training increased whole-body insulin action and rescued impairments in AS160 phosphorylation. Collectively, these findings suggest that decrements in the ability of insulin to phosphorylate specific sites on skeletal muscle AS160 contribute to insulin resistance and that exercise training is an effective treatment option to counteract these impairments.

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L.A.C. and J.A.H. conceived and developed the experiments. L.A.C. and J.V.M. collected and analyzed the exercise training data. L.A.C., C.A.N., D.N.C., M.S.D., C.J.T., and J.A.H. collected and analyzed euglycemic-hyperinsulinemic data. C.A.N., D.N.C., M.S.D., and J.A.H. obtained skeletal muscle biopsies. L.A.C. generated Western blot data. L.A.C., J.F.P.W.,

J.T.T., and J.A.H. analyzed Western blot data. L.A.C. and J.A.H. wrote the manuscript. All authors provided comments and approved the final version. L.A.C. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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