Impairments in site-specific AS160 phosphorylation and effects of exercise training

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ABSTRACT

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 lifespan (18 to 84 years) 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 individuals encompassing a wide age range (n = 73, ages 18-84) and insulin-stimulated AS160 phosphorylation 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 (PAS), but not Ser-318 or Ser-751. Twelve weeks of either 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.
INTRODUCTION

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 which 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 a 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, Akt substrate of 160 kDa (AS160, also known as TBC1D4), a Rab GTPase-activating protein (GAP), is currently recognized as the most distal signaling step associated with insulin-mediated glucose transport. In the basal state, the GTPase activating domain of AS160 is hypothesized to maintain Rab proteins in their inactive form, allowing AS160 to co-localize 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, resulting in the translocation of GLUT4 to the plasma membrane (6-9). The functional importance of phosphorylated AS160 is evident as 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 as 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, 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 if 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.

Both endurance- (16-18) and strength-oriented (19, 20) exercise training can 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, non-diabetic (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 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 present 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 if strength- and/or endurance-oriented exercise training could rescue these impairments.
RESEARCH DESIGN AND METHODS

Experimental Design

Study 1 utilized a cross-sectional design encompassing young 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

Seventy-three participants (41 women, 32 men) comprising a wide age range (18 to 84 y), were recruited specifically for this study. Physical characteristics of subjects are provided in Table 1. All participants were non-smokers and participated in less than 1 hour/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 their decade of age (32). Individuals with heart disease, diabetes, endocrine and/or metabolic disorders and those on lipid altering medication were excluded. 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 VO$_2$peak. Body composition was measured by dual X-ray absorptiometry (DEXA) 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 07:00 after a 12-h overnight fast. A 2-hr euglycemic-hyperinsulinemic clamp was utilized 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 mU·m$^2$·min), followed by a continuous infusion of insulin at a submaximal dosage of 100 mU·m$^2$·min. 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 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 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 as we have previously reported that components of insulin signal transduction (PI3-kinase activation and
Akt Ser-473 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 of cellular protein) was separated by SDS-PAGE and electrotransferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA), and probed overnight with Cell Signaling (Beverly, MA) antibodies for phospho-Akt substrate (PAS), p-Akt-Ser473 (recognizes Akt-Ser472/473/474), AS160, Akt2, and COXIV. Membranes were also probed for p-AS160-Thr642 (Millipore), p-AS160-Ser666 (Millipore), p-AS160-Ser588 (Symansis NZ Ltd, Timaru, New Zealand), GLUT4 (Affinity BioReagents, Golden, CO), RIP140 (Santa Cruz, 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 immunoglobulin G 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 3 hours with protein A sepharose beads (GE Healthcare Biosciences Corp., Piscataway, NJ). Supernatant portions from sample were removed and immunocomplexes subjected to Western blot analyses.

**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 either ≤ 35 years of age (young) or ≥ 55 years of age (aged). These individuals were then randomized into either a 12 week endurance (n=12 young, n=11 aged) or strength (n=11 young, n=11 aged) training program. Two individuals were subsequently excluded from the young, endurance group due to non-compliance (Figure 1).

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 (Table 2 and Table 3). The euglycemic-hyperinsulinemic clamp with muscle biopsies was performed prior to exercise training and approximately 40 hours after the final exercise training session (Figure 1). All subjects completed a 24 hour diet record the day prior to their pre-training euglycemic-hyperinsulinemic clamp and then asked to duplicate this diet the day prior to 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% VO₂ peak for a total of 180 minutes/week (3-4 sessions/week). To ensure the appropriate workload during training, a VO₂ peak test was performed after 6 weeks of training and workload adjusted appropriately.
**Strength training**

Strength training consisted of both 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 seconds 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 both 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 step-wise 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 analysis of variance (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 ± S.E.M. Statistical significance was defined as $P \leq 0.05$. 
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, VO\textsubscript{2}\text{peak}, and elevated body and trunk fat percentage compared to the 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 VO\textsubscript{2}\text{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 ± 0.4 µIU/mL) to 160 ± 6 µIU/mL. Whole-body insulin action (M-value) declined with age (r = -0.52, \(P<0.001\), Supplemental Figure 1). Gender 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, percent body fat, percent trunk fat, waist to hip ratio, VO\textsubscript{2}\text{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 phospho-Akt substrate (PAS) (approximately 2- to 8–fold) and of Akt2 Ser-473 by approximately 30-fold. As presented in Figure 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, percent body fat, percent trunk fat, waist-to-hip ratio, VO$_{2}$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 both 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, $P<0.05$) (Supplemental Figure 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, $P=0.06$) (Supplemental Figure 2).

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

**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, VO$_2$peak, and fasting blood chemistries were evident between the young and aged groups prior to initiating the 12 weeks of exercise training (Table 2 & Table 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), VO$_2$peak, and 1-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 VO$_2$peak (Table 3). Insulin action also increased irrespective of group, while 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 (Supplemental Figure 3). Analysis of 3-day diet records revealed
no significant changes in caloric intake (total, protein, carbohydrate, and fat) during the training period.

**AS160 phosphorylation**

In agreement with the correlation analyses (Supplemental Figure 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 pre-training, sedentary state (Figure 4). Endurance training increased insulin-stimulated AS160 phosphorylation of PAS by \(~60\%\) in young and \(~75\%\) in insulin-resistant aged individuals \( (P<0.05, \text{Figure } 5A) \) while AS160 Ser-588 phosphorylation increased \(~25\%\) in both groups \( (P<0.05, \text{Figure } 5B) \). 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 \(~57\%\) \( (P<0.05, \text{Figure } 5C) \), 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 (Figure 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 \(~75\%\) \( (\text{Figure } 6A, P<0.01) \) in both groups, whereas, insulin stimulated Thr-642 phosphorylation increased by \(~33\%\) and \(~73\%\) in young and aged individuals \( (\text{Figure } 6C, P<0.05) \), respectively. In addition, strength training increased insulin-stimulated Ser-666 phosphorylation of AS160 by \(~100\%\) \( (\text{Figure } 6D, P<0.05) \) in the insulin-resistant, aged group. There were no changes in Ser-588 \( (\text{Figure } 6B) \), Ser-751 \( (\text{data not shown}) \), or Ser-318 \( (\text{data not shown}) \) with strength training.
Exercise training had no effect on AS160 protein content (data not shown). Basal phosphorylation levels of PAS were significantly reduced (~50%, \( P < 0.01 \)) in response to strength training, which contributed to the significant training effect (Fig 5A). Exercise training had no other effect on basal AS160 phosphorylation levels. Endurance training increased GLUT4 (~10% for young and ~15% for aged, \( P < 0.05 \), Supplemental Figure 4) while 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 \( (P < 0.05) \).

Prior to exercise training, Akt2 protein levels did not differ between the groups (data not shown). Endurance training increased Akt2 protein levels by ~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 (Supplemental Figure 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 (Figure 2, Figure 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 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 type 2 diabetics (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 (Figures 2 and 4) in combination with others (4, 13) provides 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 (Figure 3B), provide novel evidence that the impairment in AS160 phosphorylation may be linked to the association of AS160 with RIP140, which in turn induces insulin resistance (Figure 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 predictor 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 both phosphorylation of Akt Ser-473 and Thr-308 are 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 present study, however, Sharma et al., (44) recently reported reduced
insulin-stimulated Akt2-Thr308 phosphorylation in the soleus of aged (25 month) compared to 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 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). Additionally, O’Gorman et al. (22) reported that short-term endurance training (7 days) was not sufficient to increase insulin-stimulated AS160 phosphorylation in obese, non-diabetic, or diabetic individuals. However, conclusions from these studies (21, 22) are limited based on the use of the phospho-Akt substrate (PAS) antibody.

A key finding in the present study was that decrements in specific insulin-stimulated AS160 phosphorylation sites were improved with exercise training (Figure 5, Figure 6), with the exception of AS160 Ser-588 (Figure 6B), which did not appear to be responsive to strength training (Figure 6B). Vind et al. (13) previously reported increased insulin-induced AS160 phosphorylation on Ser-588 in type 2 diabetic patients, but not non-diabetic controls 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 cross-sectional data we determined that percent body fat was the best predictor of AS160 Ser-588 phosphorylation. Percent body fat 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. It has
been hypothesized that PKCζ activity regulates AS160 Ser-588 phosphorylation (42) and it has been reported that endurance training increases skeletal muscle PKCζ activity (45). While speculative, it is plausible to suggest that our endurance training program improved PKCζ 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 lifespan (1, 24, 26, 30, 46), our data demonstrated that whole-body insulin action declined with age (Supplemental Figure 1). The nature of this age-related insulin resistance have 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
to 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, it is possible that the age or exercise related differences in AS160 phosphorylation in our current study could have been influenced by changes in muscle fiber type. In addition, despite previous research suggesting maximal AS160 phosphorylation occurs at 60 minutes of a 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 present 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, twelve weeks of either 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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FIGURES LEGEND

**Figure 1.** Overview of experimental design. Young ≤ 35 y (range: 18-35 y); Aged ≥ 55 y (range: 55-84 y).

**Figure 2.** Relationships between 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), Ser-318 (n=60) (F) with whole-body insulin action. For PAS (A), AS160 was immunoprecipitated and then blotted with the PAS antibody. All other blots used phospho-specific antibodies (B-F). Values, in arbitrary units (AU) were normalized to a control sample run on each blot and then presented relative to total AS160 protein.

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

**Figure 4.** Fold change (insulin stimulated over basal) in AS160 PAS (A), Ser-588 (B), Thr-642 (C), Ser-666 (D) in young (n=18-21, age range: 18-35 y, white bars) and aged (n=19-22, age range: 55-84 y, black bars) individuals. Data are means ± SEM. *p<0.05.

**Figure 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), 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 y, range: 18-34 y, white bars) and aged (age: 69.0 ± 2.2 y, range: 57-84 y, black bars) individuals. Data are presented as fold change in phosphorylation levels normalized to total AS160 protein levels. Line above bars represents main effect for age (short bar) or training (long bar). Data are means ± SEM. *p<0.05 vs pre-training; † p<0.05 vs young at that comparable time. (E) Representative blots using AS160 phospho-specific antibodies and total protein in young and aged individuals under non-insulin (B) and insulin-stimulated (I) conditions. For PAS, AS160 was initially immunoprecipitated and then blotted with the PAS antibody.
Figure 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), Ser-666 (n=10, young, n=10, aged) (D) in response to insulin before and after 12 weeks of strength training in young (age: 23.6 ± 1.5 y, range: 20-35 y, white bars) and aged (69.3 ± 2.7 y, range: 55-82 y, black bars) individuals. Data are presented as fold change in phosphorylation levels normalized to total AS160 protein levels. Line above bars represents main effect for age (short bar) or training (long bar). Data are means ± SEM. *p<0.05 vs pre-training; † p<0.05 vs young at that comparable time. (E) Representative blots using AS160 phospho-specific antibodies and total protein in young and aged individuals under non-insulin (B) and insulin-stimulated (I) conditions. For PAS, AS160 was initially immunoprecipitated and then blotted with the PAS antibody.
Table 1. Participant characteristics for cross-sectional study

<table>
<thead>
<tr>
<th></th>
<th>Men</th>
<th>Women</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>32</td>
<td>41</td>
</tr>
<tr>
<td>Age (years)</td>
<td>43.0 ± 4.3 (19-84)</td>
<td>45.0 ± 3.2 (18-76)</td>
</tr>
<tr>
<td>Mass (kg)</td>
<td>80.5 ± 2.1 (62.7-105.9)</td>
<td>68.4 ± 1.8 (52.3-97.7)*</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.6 ± 0.7 (19-34)</td>
<td>25.2 ± 0.6 (18-35)</td>
</tr>
<tr>
<td>Body Fat (%)</td>
<td>23.0 ± 1.7 (8-39)</td>
<td>38.1 ± 1.3 (16-53)*</td>
</tr>
<tr>
<td>Trunk Fat (%)</td>
<td>27.2 ± 2.1 (7.0-45.1)</td>
<td>39.1 ± 1.5 (14.3-54.8)*</td>
</tr>
<tr>
<td>FFM (kg)</td>
<td>57.6 ± 1.0 (48.3-72.4)</td>
<td>38.9 ± 0.8 (30.5-51.8)*</td>
</tr>
<tr>
<td>Waist:Hip ratio</td>
<td>0.86 ± 0.02 (0.76-1.04)</td>
<td>0.76 ± 0.01 (0.66-0.92)*</td>
</tr>
<tr>
<td>VO₂peak (ml/kg/min)</td>
<td>34.8 ± 2.1 (12.6-58.7)</td>
<td>26.0 ± 1.2 (12.6-42.2)*</td>
</tr>
<tr>
<td>Fasting Plasma Glucose (mg/dL)</td>
<td>89.2 ± 1.4 (73-105)</td>
<td>88.1 ± 1.5 (71-118)</td>
</tr>
<tr>
<td>Fasting Plasma Insulin (µU/mL)</td>
<td>5.5 ± 0.6 (1.5-14.4)</td>
<td>5.9 ± 0.8 (1.5-29.5)</td>
</tr>
</tbody>
</table>

Data are presented mean ± SEM (range). BMI, body mass index; FFM, fat-free mass; VO₂peak, peak oxygen uptake.

*P< 0.005 vs. men
Table 2. Changes in characteristics of young and aged individuals before and after endurance training

<table>
<thead>
<tr>
<th>Variable</th>
<th>Young Pre-training</th>
<th>Young Post-training</th>
<th>Aged Pre-training</th>
<th>Aged Post-training</th>
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<tr>
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<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (range)</td>
<td>24.4 ± 1.6 (18-34)</td>
<td>69.0 ± 2.2† (57-84)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>f/m</td>
<td>5/5</td>
<td>5/6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin Action</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M-Value (mg/kg/min)</td>
<td>7.9 ± 0.8</td>
<td>9.8 ± 0.7*</td>
<td>5.7 ± 0.5†</td>
<td>6.3 ± 0.6†</td>
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<tr>
<td>Body Composition</td>
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<tr>
<td>Mass (kg)</td>
<td>69.8 ± 2.2</td>
<td>69.2 ± 2.5</td>
<td>80.3 ± 4.2†</td>
<td>80.4 ± 4.4†</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>23.8 ± 0.9</td>
<td>23.5 ± 0.7</td>
<td>27.9 ± 1.1†</td>
<td>27.9 ± 1.2†</td>
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<tr>
<td>Body Fat (%)</td>
<td>25.5 ± 3.7</td>
<td>23.6 ± 3.5*</td>
<td>38.4 ± 2.4†</td>
<td>37.3 ± 2.6†</td>
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<tr>
<td>LBM (kg)</td>
<td>47.4 ± 2.8</td>
<td>48.8 ± 3.1*</td>
<td>47.3 ± 3.3</td>
<td>47.8 ± 3.3*</td>
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<tr>
<td>Thigh circumference (cm)</td>
<td>48.7 ± 1.6</td>
<td>48.3 ± 1.5</td>
<td>50.1 ± 1.5</td>
<td>50.3 ± 1.8</td>
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<td>Waist:Hip Ratio</td>
<td>0.78 ± 0.02</td>
<td>0.77 ± 0.02</td>
<td>0.88 ± 0.04†</td>
<td>0.87 ± 0.03†</td>
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<tr>
<td>Performance</td>
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<tr>
<td>VO₂peak (ml/kg/min)</td>
<td>35.0 ± 1.6</td>
<td>41.7 ± 1.3*</td>
<td>19.9 ± 1.2†</td>
<td>22.3 ± 1.5†</td>
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<td>KINCOM Peak Extension (N)</td>
<td>599 ± 72</td>
<td>612 ± 57</td>
<td>449 ± 47</td>
<td>468 ± 35</td>
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<tr>
<td>KINCOM Peak Flexion (N)</td>
<td>396 ± 49</td>
<td>429 ± 50</td>
<td>252 ± 32†</td>
<td>272 ± 41†</td>
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<tr>
<td>1-RM Leg Press (kg)</td>
<td>119 ± 9</td>
<td>121 ± 10</td>
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<td>1-RM Chest Press (kg)</td>
<td>49 ± 6</td>
<td>54 ± 8*</td>
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<td>37 ± 8*</td>
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<td>Fasting Blood Chemistry</td>
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<tr>
<td>Glucose (mg/dL)</td>
<td>84.3 ± 2.4</td>
<td>78.6 ± 2.8*</td>
<td>91.6 ± 3.1</td>
<td>89.1 ± 4.0*</td>
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<tr>
<td>Insulin (IU/mL)</td>
<td>4.6 ± 1.0</td>
<td>3.7 ± 1.1*</td>
<td>7.3 ± 1.2</td>
<td>4.3 ± 1.1*</td>
</tr>
</tbody>
</table>

Data are presented mean ± SEM (range). BMI, body mass index; LBM, lean body mass; VO₂peak, peak oxygen uptake. Statistics performed on absolute values.

*P< 0.05 training main effect
†P<0.05 age main effect
Table 3. Changes in characteristics of young and aged individuals before and after strength training

<table>
<thead>
<tr>
<th>Variable</th>
<th>Young Pre-training</th>
<th>Young Post-training</th>
<th>Aged Pre-training</th>
<th>Aged Post-training</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Age (range)</td>
<td>23.6 ± 1.5 (20-35)</td>
<td>69.3 ± 2.7 (55-82)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>f/m</td>
<td>4/7</td>
<td>6/5</td>
<td></td>
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<tr>
<td><strong>Insulin Action</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M-Value (mg/kg/min)</td>
<td>9.4 ± 0.8</td>
<td>10.8 ± 1.0*</td>
<td>6.1 ± 0.7†</td>
<td>6.9 ± 0.7†</td>
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<tr>
<td><strong>Body Composition</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mass (kg)</td>
<td>71.8 ± 3.5</td>
<td>73.3 ± 3.3*</td>
<td>76.4 ± 3.7</td>
<td>76.7 ± 3.7*</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>23.5 ± 0.7</td>
<td>25.3 ± 1.2</td>
<td>26.8 ± 1.1</td>
<td>26.9 ± 1.1</td>
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<td>Body Fat (%)</td>
<td>22.9 ± 3.7</td>
<td>22.1 ± 3.7</td>
<td>36.9 ± 2.6†</td>
<td>36.2 ± 2.7†</td>
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<tr>
<td>LBM (kg)</td>
<td>53.0 ± 3.6</td>
<td>53.9 ± 3.4*</td>
<td>45.2 ± 3.3</td>
<td>46.2 ± 3.3*</td>
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<tr>
<td>Thigh circumference (cm)</td>
<td>50.9 ± 1.1</td>
<td>51.4 ± 1.0</td>
<td>49.0 ± 1.1</td>
<td>48.5 ± 1.5</td>
</tr>
<tr>
<td>Waist:Hip Ratio</td>
<td>0.76 ± 0.02</td>
<td>0.75 ± 0.02</td>
<td>0.87 ± 0.03†</td>
<td>0.87 ± 0.03†</td>
</tr>
<tr>
<td><strong>Performance</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VO₂ peak (ml/kg/min)</td>
<td>38.2 ± 2.1</td>
<td>41.5 ± 2.8*</td>
<td>20.6 ± 1.8†</td>
<td>21.7 ± 1.9†*</td>
</tr>
<tr>
<td>KINCOM Peak Extension (N)</td>
<td>718 ± 70</td>
<td>874 ± 73</td>
<td>500 ± 43†</td>
<td>534 ± 36†</td>
</tr>
<tr>
<td>KINCOM Peak Flexion (N)</td>
<td>413 ± 38</td>
<td>530 ± 41†</td>
<td>292 ± 24†</td>
<td>331 ± 29†*</td>
</tr>
<tr>
<td>1-RM Leg Press (kg)</td>
<td>121 ± 11</td>
<td>148 ± 17†</td>
<td>81 ± 12†</td>
<td>103 ± 9†*</td>
</tr>
<tr>
<td>1-RM Chest Press (kg)</td>
<td>54 ± 10</td>
<td>58 ± 9†</td>
<td>31 ± 5</td>
<td>37 ± 5*</td>
</tr>
<tr>
<td><strong>Fasting Blood Chemistry</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>84.1 ± 2.5</td>
<td>80.1 ± 2.8*</td>
<td>92.3 ± 2.8</td>
<td>89.5 ± 2.3*</td>
</tr>
<tr>
<td>Insulin (IU/mL)</td>
<td>4.8 ± 1.1</td>
<td>3.7 ± 0.9†</td>
<td>5.1 ± 1.1</td>
<td>3.7 ± 1.0†</td>
</tr>
</tbody>
</table>

Data are presented mean ± SEM (range). BMI, body mass index; LBM, lean body mass; VO₂ peak, peak oxygen uptake. Statistics performed on absolute values.

*P < 0.05 training main effect

†P < 0.05 age main effect
**STUDY 1**
Determine if site-specific phosphorylation of AS160 is altered across a range of the adult lifespan.

**STUDY 2**
Determine if endurance- and/or strength-oriented exercise training could rescue decrements in AS160 phosphorylation.

- **Endurance Training**
  - N = 10 Young, 11 Aged
  - 12 weeks
  - 120 min

- **Strength Training**
  - N = 11 Young, 11 Aged
  - ~40 h
  - 120 min

**Muscle biopsy**
Young ≤ 35 yrs; Aged ≥ 55 yrs
Figure 2. Relationships between 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), Ser-318 (n=60) (F) with whole-body insulin action (M-value). For PAS (A), AS160 was immunoprecipitated and then blotted with the PAS antibody. All other blots used phospho-specific antibodies (B-F). Values, in arbitrary units (AU) were normalized to a control sample run on each blot and then presented relative to total AS160 protein.
Figure 3. Quantification of total RIP140 (A) in the skeletal muscle of young (n=19, age: 18-35 y) vs. aged (n=20, age: 57-84 y) individuals and the quantification of RIP140/AS160 complex (B) in the skeletal muscle of young (n=11, age: 18-26) vs. aged (n=9, age: 57-82) individuals. Relationship of RIP140/AS160 complex with insulin action (n=20) (C). For determination of RIP140/AS160 complex (B and C), whole-cell muscle lysates were immunoprecipitated with AS160 and immunoblotted with RIP140 (complex formation) and AS160 (total AS160 immunoprecipitated). Values are in arbitrary units (AU) and data expressed as mean ± SEM. N.S., non-significant; *P<0.05.
Figure 4. Fold change (insulin stimulated over basal) in AS160 PAS (A), Ser-588 (B), Thr-642 (C), Ser-666 (D) in young (n=18-21, age range: 18-35 y, white bars) and aged (n=19-22, age range: 55-84 y, black bars) individuals. Data are means ± SEM. *p<0.05.
Figure 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), 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 y, range: 18-34 y, white bars) and aged (age: 69.0 ± 2.2 y, range: 57-84 y, black bars) individuals. Data are presented as fold change in phosphorylation levels normalized to total AS160 protein levels. Line above bars represents main effect for age (short bar) or training (long bar). Data are means ± SEM. *p<0.05 vs pre-training; † p<0.05 vs young at that comparable time. (E) Representative blots using AS160 phospho-specific antibodies and total protein in young and aged individuals under non-insulin (B) and insulin-stimulated (I) conditions. For PAS, AS160 was initially immunoprecipitated and then blotted with the PAS antibody.

269x332mm (300 x 300 DPI)
Figure 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), Ser-666 (n=10, young, n=10, aged) (D) in response to insulin before and after 12 weeks of strength training in young (age: 23.6 ± 1.5 y, range: 20-35 y, white bars) and aged (69.3 ± 2.7 y, range: 55-82 y, black bars) individuals. Data are presented as fold change in phosphorylation levels normalized to total AS160 protein levels. Line above bars represents main effect for age (short bar) or training (long bar). Data are means ± SEM. *p<0.05 vs pre-training; † p<0.05 vs young at that comparable time. (E) Representative blots using AS160 phospho-specific antibodies and total protein in young and aged individuals under non-insulin (B) and insulin-stimulated (I) conditions. For PAS, AS160 was initially immunoprecipitated and then blotted with the PAS antibody.

264x320mm (300 x 300 DPI)
Supplemental Figure 1. Relationship between whole-body insulin action obtained during a euglycemic-hyperinsulinemic clamp (M-value) and age.
Supplemental Figure 2. Relationships between 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) and GLUT4 protein content (n=58) (E) with age. Values are expressed in arbitrary units (AU).
Supplemental Figure 3. Skeletal muscle COX IV protein content before and after 12 weeks of endurance training in young (n=8, age: 24.4 ± 1.6 y, range: 18-34 y, white bars) and aged (n=8, age: 69.0 ± 2.2 y, range: 57-84 y, black bars) individuals (A) or strength training (B) in young (n=9, age: 23.6 ± 1.5 y, range: 20-35 y, white bars) and aged (n=9, 69.3 ± 2.7 y, range: 55-82 y, black bars) individuals. Values are in arbitrary units (AU). Data are means ± SEM. *p<0.01 vs pre-training.
Supplemental Figure 4. Skeletal muscle GLUT4 protein content before and after 12 weeks of endurance training in young (n=9, age: 24.4 ± 1.6 y, range: 18-34 y, white bars) and aged (n=11, age: 69.0 ± 2.2 y, range: 57-84 y, black bars) individuals (A) or strength training (B) in young (n=10, age: 23.6 ± 1.5 y, range: 20-35 y, white bars) and aged (n=11, 69.3 ± 2.7 y, range: 55-82 y, black bars) individuals. Values are in arbitrary units (AU). Line above bars represents main effect for training. Data are means ± SEM. *p<0.01 vs pre-training.
Supplemental Figure 5. Phosphorylation levels of skeletal muscle Akt2 Ser-473 in response to a euglycemic-hyperinsulinemic clamp before and after 12 weeks of endurance training in young (n=8, age: 24.4 ± 1.6 y, range: 18-34 y, white bars) and aged (n=9, age: 69.0 ± 2.2 y, range: 57-84 y, black bars) individuals (A) or strength training (B) in young (n=8, age: 23.6 ± 1.5 y, range: 20-35 y, white bars) and aged (n=10, 69.3 ± 2.7 y, range: 55-82 y, black bars) individuals. (C) Representative blots using immunoprecipitated Akt2 which was probed for Akt-Ser473 or total Akt2 protein in young and aged individuals under non-insulin (B) and insulin-stimulated (I) conditions. All samples were normalized to total Akt2 protein and data expressed as mean ± SEM.