*Original Article*

# Effects of Endurance Exercise Training on Insulin Signaling in Human Skeletal Muscle

## Interactions at the Level of Phosphatidylinositol 3-Kinase, Akt, and AS160

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**The purpose of this study was to investigate the mechanisms explaining improved insulin-stimulated glucose uptake after exercise training in human skeletal muscle. Eight healthy men performed 3 weeks of one-legged knee extensor endurance exercise training. Fifteen hours after the last exercise bout, insulin-stimulated glucose uptake was ~60% higher ($P$ < 0.01) in the trained compared with the untrained leg during a hyperinsulinemic-euglycemic clamp. Muscle biopsies were obtained before and after training as well as after 10 and 120 min of insulin stimulation in both legs. Protein content of Akt1/2 (55 ± 17%, $P$ < 0.05), AS160 (25 ± 8%, $P$ = 0.08), GLUT4 (52 ± 19%, $P$ < 0.001), hexokinase 2 (HK2) (197 ± 40%, $P$ < 0.001), and insulin-responsive aminopeptidase (65 ± 15%, $P$ < 0.001) increased in muscle in response to training. During hyperinsulinemia, activities of insulin receptor substrate-1 (IRS-1)–associated phosphatidylinositol 3-kinase (PI3-K) ($P$ < 0.005), Akt1 ($P$ < 0.05), Akt2 ($P$ < 0.005), and glycogen synthase (GS) (percent I-form, $P$ < 0.05) increased similarly in both trained and untrained muscle, consistent with increased phosphorylation of Akt $Thr^{308}$, Akt $Ser^{473}$, AS160, glycogen synthase kinase (GSK)-3α $Ser^{21}$, and GSK-3β $Ser^{9}$ and decreased phosphorylation of GS site 3a+b (all $P$ < 0.005). Interestingly, training improved insulin action on thigh blood flow, and, furthermore, in both basal and insulin-stimulated muscle tissue, activities of Akt1 and GS and phosphorylation of AS160 increased with training (all $P$ < 0.05). In contrast, training reduced IRS-1–associated PI3-K activity ($P$ < 0.05) in both basal and insulin-stimulated muscle tissue. Our findings do not support generally improved insulin signaling after endurance training; rather it seems that improved insulin-stimulated glucose uptake may result from hemodynamic adaptations as well as increased cellular protein content of individual insulin signaling components and molecules involved in glucose transport and metabolism. *Diabetes* 56: 2093–2102, 2007**

Exercise training is a potent intervention to improve insulin action on glucose homeostasis in both healthy and insulin-resistant individuals as well as in patients with type 2 diabetes (1–5). This beneficial effect of exercise training is partly due to improved insulin action on glucose uptake in skeletal muscle (3,6,7); however, the underlying mechanisms are not clear.

Insulin-stimulated glucose uptake in skeletal muscle involves a concert of events initiated by delivery of insulin and glucose to the muscle cell via the vascular system. Subsequent binding of insulin to the extracellular part of the insulin receptor leads to intracellular signaling, resulting in a shift in the steady-state distribution of glucose transport proteins (GLUT4) favoring the plasma membrane and T-tubules (8,9). This process involves translocation and docking/fusion of GLUT4-containing vesicles to the membrane (10), allowing for increased transport of glucose. Furthermore, insulin signaling stimulates nonoxidative glucose metabolism involving activation of glycogen synthase (GS), the rate-limiting enzyme in the storage of glucose in glycogen particles (11).

Skeletal muscle adaptations to training are multiple, including improved hemodynamic effects of insulin (7,12). Furthermore, exercise training results in changes in expression and/or activity of proteins involved in glucose uptake/metabolism in both rodent (13,14) and human skeletal muscle (2,15–17). Although only a few observations have been made in human muscle, insulin signaling to stimulate glucose uptake may be improved under some exercise training conditions. Thus, short-term (7 days) cycle exercise training increases insulin-stimulated phosphatidylinositol 3-kinase (PI3-K) activity in young, but not middle-aged, subjects (18,19). In the present investigation, we focused mainly on possible adaptations downstream of PI3-K, including the newly recognized signal component, AS160. This protein, containing a Rab GAP domain (20), is

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Received for publication 8 December 2006 and accepted in revised form 2 May 2007.

Published ahead of print at http://diabetes.diabetesjournals.org on 18 May 2007. DOI: 10.2337/db06-1698.

aPKC, atypical protein kinase C; GS, glycogen synthase; GSK, glycogen synthase kinase; HK2, hexose kinase 2; IRAP, insulin-responsive aminopeptidase; IR, insulin receptor; IRS-1, insulin receptor substrate-1; PI3-K, phosphatidylinositol 3-kinase; PWL, peak work load; SNAP, soluble *N*-ethylmaleimide–sensitive factor attachment protein; SNARE, soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor.

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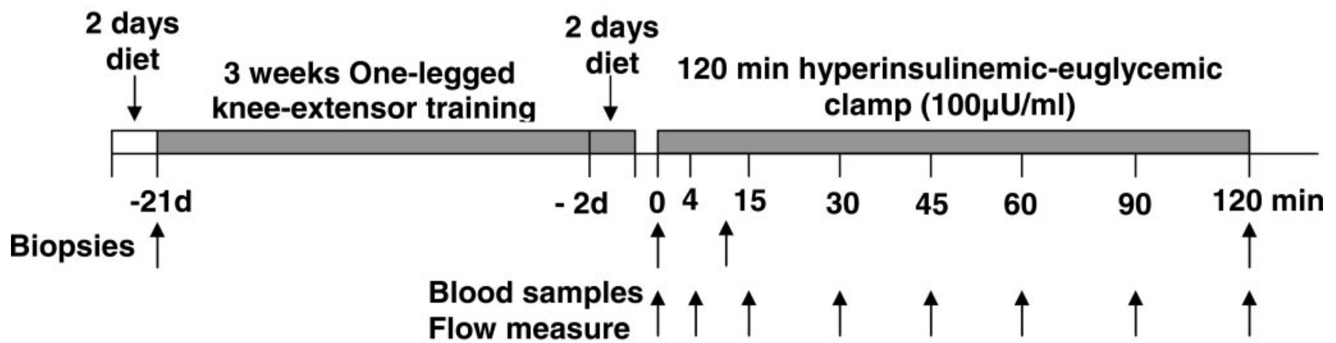


**FIG. 1. Schematic illustration of the experimental day. Left gray area indicates 3 weeks of exercise training. Right gray area indicates a hyperinsulinemic-euglycemic clamp (120 min) performed 15 h after the last exercise bout. Upward arrows indicate time point of either muscle biopsies or blood sampling/venous blood flow measures in both legs. Downward arrows indicate periods of mixed diet.**

targeted by the protein kinase Akt2 under insulin stimulation (21) and promotes translocation of GLUT4-containing vesicles to the plasma membrane (20). AS160 is important in both adipocytes and murine skeletal muscle for insulin to stimulate glucose uptake (20,22–24). In addition, we examined the expression of soluble *N*-ethylmaleimide attachment protein receptor (SNARE) proteins such as Syntaxin4 and Munc18c and of the insulin-responsive aminopeptidase (IRAP). This allowed us to evaluate whether exercise training–induced changes in GLUT4 protein content occur in coordination with changes in proteins associated with GLUT4 and/or proteins involved in docking/fusion of GLUT4-containing vesicles, which may play a direct role for skeletal muscle insulin action (25–28).

Collectively, the aim of the present study was to thoroughly investigate human skeletal muscle adaptations to endurance exercise training resulting in improved insulin-stimulated glucose uptake. Using a one-legged training regimen, we investigated primarily local contraction–induced adaptations, as systemic impact is minor under these conditions. In addition, by combining the hyperinsulinemic-euglycemic clamp technique and femoral arterial and venous catheterization, we also provide information on the delivery and clearance of insulin and glucose to the skeletal muscle.

## RESEARCH DESIGN AND METHODS

Eight healthy young men (aged 25 ± 1 years; height 181 ± 2 cm; weight 81 ± 2 kg; BMI 24.6 ± 0.5 kg/m$^2$; and $VO_{2max}$ 49 ± 1 ml $O_2 \cdot min^{-1} \cdot kg^{-1}$) gave their informed consent before participating in the study. The study was approved by the local ethics committee (identification no. KF 01-070/96) and performed in accordance with the Declaration of Helsinki. All subjects had comparable (<5% difference) peak work loads (PWLs) of the knee extensors in the two legs. PWL was determined in each leg using the one-legged dynamic knee extensor apparatus (29).

After 2 days of a controlled diet (55.5 ± 0.4% carbohydrate, 29.4 ± 0.4% fat, and 15.1 ± 0.2% protein), the subjects arrived at the laboratory after an overnight fast (Fig. 1). After 30 min of supine rest, needle biopsies were obtained from musculus vastus lateralis in both legs under subcutaneous anesthesia (~2–3 ml Xylocaine [10 mg/ml lidocaine]; Astra, Mölndal, Sweden). Muscle tissue, still in the needle, was frozen in liquid nitrogen and stored at −80°C. This procedure was repeated after training, and muscle biopsies were obtained 15 h after the last exercise bout.

After the biopsy procedure, Teflon catheters were inserted into one femoral artery and both femoral veins. A thermistor (Edslab probe 94-030-2.5F; Baxter, Alleroed, Denmark) was inserted through both femoral catheters and advanced 8 cm proximally for blood flow determination. Subsequently, the subjects underwent a 120-min hyperinsulinemic-euglycemic clamp (1.5 $mU \cdot min^{-1} \cdot kg^{-1}$) initiated with a bolus injection of insulin (9 mU/kg) (Actrapid; Novo Nordisk, Bagsvaerd, Denmark) given over 1 min. Blood samples were drawn simultaneously from the femoral catheters before and during (4, 15, 30, 45, 60, 90, and 120 min) insulin infusion. At each time point, venous blood flow in the thighs was measured by using the constant infusion thermodilution technique (30) after occlusion of the lower leg with an inflated cuff (>220 mmHg). Needle biopsies were obtained from the vastus lateralis muscle in each thigh after 10 (C10) and 120 (C120) min of insulin infusion.

**Training.** Training consisted of four sessions during the 1st week, five sessions the 2nd week, and six sessions the 3rd week. The duration gradually increased from 1 to 2 h per session. Training intensity varied between 70 and 85% of PWL and was progressively increased in accordance with an expected increase in PWL during this type of training (31). Included in each training session was work for 5–7 min at 100% of PWL to ensure recruitment of the majority of muscle fibers in the knee extensor region (32). Because PWL in the untrained leg is unaffected by this protocol (31), evaluation of endurance capacity was only done in the trained leg as part of the last training session.

**Insulin and glucose.** Plasma insulin concentrations were determined using a radioimmunoassay kit (Insulin RIA 100; Pharmacia, Uppsala, Sweden), and glucose concentrations were measured using an glucose analyzer (YSI-2700 Select; Yellow Springs Instruments, Yellow Springs, OH)

**Muscle glycogen.** Muscle glycogen content was determined as glycosyl units after acid hydrolysis of freeze-dried muscle tissue by fluorometric methods (33).

**Activities of citrate synthase and 3-hydroxyacyl-CoA dehydrogenase.** Maximal activities of citrate synthase and 3-hydroxyacyl-CoA dehydrogenase were measured in freeze-dried muscle tissue by fluorometric methods (33).

**Muscle lysate preparation.** Freeze-dried and dissected muscle tissue was homogenized as described (34), and the homogenate was rotated end over end at 4°C for 1 h before being centrifuged for 30 min (17,500*g*, 4°C). The supernatant (lysate) was harvested, and total protein content was determined using the bicinchoninic acid method (Pierce, Rockford, IL).

**SDS-PAGE and Western blotting.** Muscle lysate proteins were separated by SDS-PAGE and Western blotting followed by immunodetection as described previously (35).

**Antibodies used.** For protein detection, the following antibodies were used. Anti-P85α (06-497), anti-Akt1/2 (07-416), anti-Akt2 (07-372), anti-glycogen synthase kinase (GSK)-3α (06-391), and anti-AS160 (07-741) were from Upstate Biotechnology (Lake Placid, NY). Anti-GLUT4 (ab1346) was from Chemicon (Temecula, CA). Anti-atypical protein kinase C (aPKC) ζ/ι (sc-216), anti-Munc18c (sc-23014), and anti-Syntaxin4 (sc-14455) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-GSK-3β (22320) was from Transduction Laboratories (Lexington, KY). Anti-HK2 (HXK21-A) was from Alpha Diagnostics (San Antonio, TX). Anti-soluble *N*-ethylmaleimide–sensitive factor attachment protein (SNAP) 23 (PA1-738) was from Affinity BioReagents (Golden, CO). Anti-GS was kindly donated by Professor Oluf Pedersen (Steno Diabetes Center, Gentofte, Denmark) (36). Total AS160 protein was measured using an antibody (ab24469) from Abcam (Cambridge, U.K.) detecting the C-terminal amino acids 1287–1299 of human TBC1D4. The anti-insulin receptor (IR) monoclonal CT3 antibody was raised against the COOH terminus of the IRβ subunit, and the anti-insulin receptor substrate-1 (IRS-1) antibody was raised against bacterially expressed glutathione *S*-transferase fusion protein using the 236 amino acids of the carboxyl-terminal part of rat IRS-1 (both kindly donated by Dr. Ken Siddle, Cambridge University, Cambridge, U.K.). Finally, the anti-IRAP antibody was raised against the N-terminal cytoplasmic domain of IRAP (kindly donated by Dr. Susanna R. Keller, University of Virginia, Charlottesville, VA).

For detection of protein phosphorylation the following antibodies were used. Anti-Akt Ser$^{473}$ (9271), anti-GSK-3 Ser$^{21}$/Ser$^{9}$ (9331), anti-aPKCζ/ι Ser$^{410}$

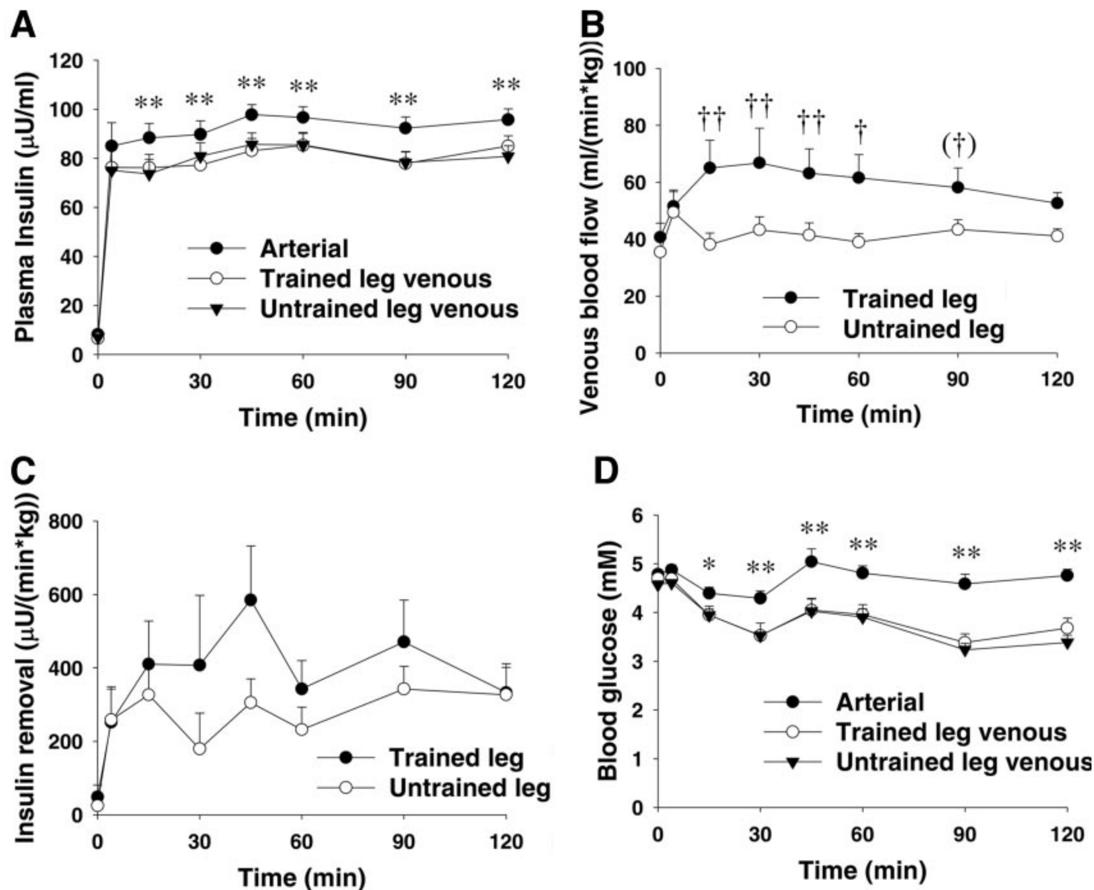


**FIG. 2.** ***A*****: Plasma insulin concentration.** ***B*****: Venous blood flow.** ***C*****: Insulin removal.** ***D*****: Blood glucose concentrations. All were measured during a 120-min hyperinsulinemic-euglycemic clamp. (†)*P* = 0.08; †*P* < 0.05, ††*P* < 0.005 versus untrained values. **P* < 0.05; ***P* < 0.005 versus venous values. Values are means ± SE; *n* = 8.**

(9378), and anti-phospho-(Ser/Thr) Akt substrate for detection of Akt phospho sites on AS160 (9611) were from Cell Signaling Technology (Beverly, MA). Anti-Akt $Thr^{308}$ (06-678) was from Upstate Biotechnology. For detection of GS site 3a+3b ($Ser^{640}$ and $Ser^{644}$) cophosphorylation, an antibody was raised against the peptide PYPRPPASpVPPSpPSLSR (residues 634–650 of human GS) as described (37). This antibody was kindly donated by Dr. Bo F. Hansen (Diabetes Biology, Novo Nordisk, Bagsvaerd, Denmark).

Before analyses, it was verified that all antibodies produced a band at the expected molecular weight. Furthermore, it was confirmed that the loaded amount of protein was within a dynamic range for each particular antibody. Representative blots are shown in Fig. 4.

**PI3-K activity.** Activity of PI3-K associated with IRS-1 was determined as described (34).

**Akt1 and Akt2 activities.** Isoform-specific Akt activity was measured after sequential immunopurification of Akt2 (07-372, Upstate Biotechnology) followed by Akt1/2 (07-416, Upstate Biotechnology) as described (38).

**GS activity.** GS activity was measured in muscle homogenates by using a Unifilter 350 microtiter plate assay (Whatman; Frisenette, Ebeltoft, Denmark) as described by Thomas et al. (39).

**Statistics.** Data were evaluated using two-way ANOVA with repeated measures for both time and leg. A Student-Newman-Keuls test was used as a post hoc test. Correlations were investigated using the Pearson product moment correlation. A significance level of $P \leq 0.05$ was chosen. Some mean values are presented as percent difference from pretraining values. These values and the corresponding standard error are calculated from the individual percent difference for each subject. All data are expressed as means ± SE.

## RESULTS

**General adaptations to training.** The general adaptations to the present training regimen have been published previously (35). In brief, 15 h after the last exercise bout, no significant effect of training was observed on glycogen content (NS). However, PWL of the knee extensors increased 16 ± 2% ($P < 0.001$) in the trained leg. Furthermore, maximal activities of citrate synthase and 3-hydroxyacyl-CoA dehydrogenase increased 42 ± 19 and 38 ± 6%, respectively (both $P < 0.005$), compared with values before training (35).

**Insulin-stimulated glucose uptake.** In response to insulin infusion, the arterial insulin concentration reached a level of ~90 μU/ml, as illustrated in Fig. 2*A*. At the whole-body level, the glucose infusion rate to maintain euglycemia was $7.0 \pm 0.3$ mg · $min^{-1}$ · $kg^{-1}$. Extraction of insulin and glucose (Figs. 2*C* and 3*A* and *B*) was calculated across each thigh using the Fick principle, by multiplication of venous blood flow (Fig. 2*B*) and insulin and glucose arteriovenous differences (Fig. 2*A* and *D*). After training, insulin-stimulated glucose uptake increased to a greater extent in the trained leg (Fig. 3*A* and *B*) mainly due to a larger insulin-induced increase in total blood flow in the trained leg (Fig. 2*B*). When blood flow was evaluated at individual time points, a significant training effect was observed after 15, 30, 45, and 60 min of insulin stimulation (Fig. 2*B*). In Fig. 3*B*, glucose uptake was calculated as an average from 0 to 120 min of insulin infusion and a ~60% greater ($P = 0.008$) insulin-stimulated glucose uptake observed in the trained leg.

**Western blot analyses.** All data on muscle protein content are expressed as relative changes in response to training. The absolute protein contents for none of the proteins investigated differed in the two legs before the training intervention (NS, data not shown).

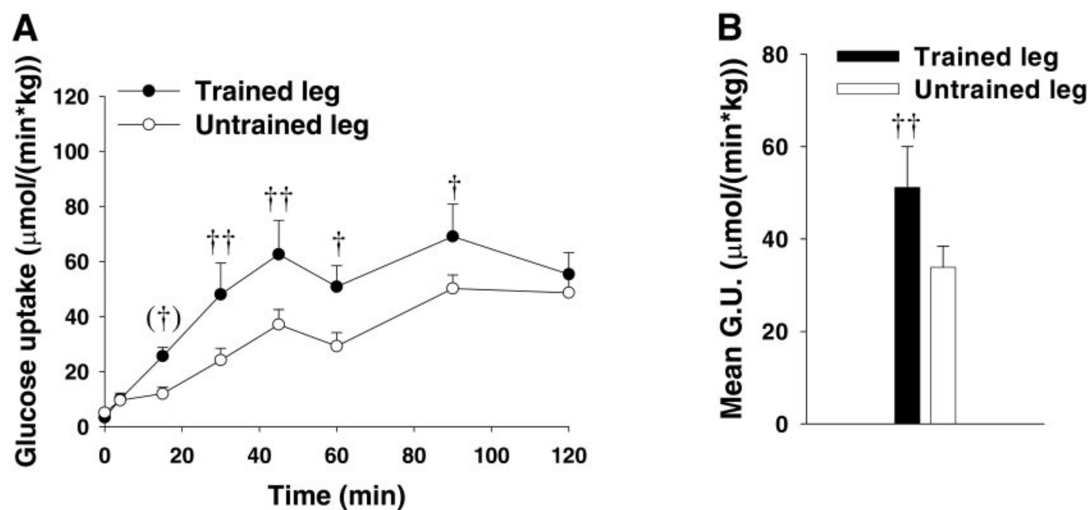


**FIG. 3.** ***A*****: Insulin-stimulated glucose uptake measured during a 120-min hyperinsulinemic-euglycemic clamp.** ***B*****: Glucose uptake (G.U.) expressed as weighted mean. (†)*P* = 0.08; †*P* < 0.05; ††*P* < 0.005 versus untrained values. Values are means ± SE; *n* = 8.**

**Protein contents of insulin signaling molecules.** Protein contents of the components of the insulin signaling pathway are illustrated in Figs. 4*A* and 5. In response to training, the protein content of Akt1/2 increased (55 ± 17%, $P < 0.05$), whereas that of AS160 (25 ± 8%, $P = 0.08$) tended to increase, with no effect of training in the untrained muscle. In contrast, no change in response to training was observed in protein content of IR, IRS-1, P85α, Akt2, aPKCζ/ι, GSK-3α, or GSK-3β.

**Protein contents of membrane fusion molecules** Protein contents of Munc18c, Syntaxin4, and SNAP-23 did not change with training as shown in Figs. 4*A* and 6*A* (all NS).

**Protein contents of HK2, GLUT4, GS, and IRAP.** In response to training, protein contents of HK2, GLUT4, and IRAP increased 197 ± 40, 52 ± 19, and 65 ± 15%, respectively (all $P < 0.001$), whereas GS protein content was unaltered (NS) (Figs. 4*A* and 6*B*). Interestingly, considering that IRAP is associated with the GLUT4 storage vesicle, the responses to training (after minus before) in protein contents of GLUT4 and IRAP were significantly correlated ($r^2 = 0.47$, $P = 0.05$) when evaluated in the trained muscle.

**Phosphorylation of insulin signaling molecules.** In response to insulin stimulation, phosphorylation of Akt $Thr^{308}$, Akt $Ser^{473}$, GSK-3α $Ser^{21}$, GSK-3β $Ser^{9}$, and Ser/Thr Akt phospho sites on AS160 significantly increased in both trained and untrained muscle tissue (all $P < 0.005$) (Fig. 7). This increase was observed already after 10 min, and for

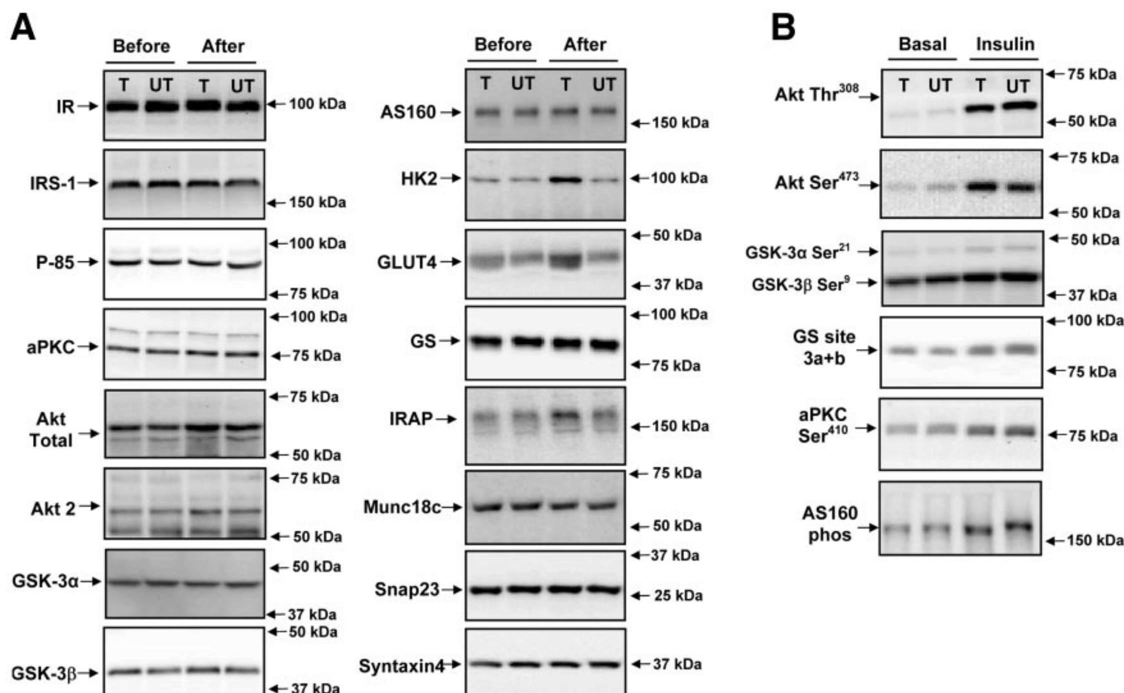


**FIG. 4. Representative immunoblots showing protein expression (*A*) and phosphorylation (*B*) of molecules involved in insulin-stimulated glucose uptake in trained (*T*) and untrained (*U*) muscle. In *A*, expression before and after 3 weeks of one-legged knee extensor endurance training is shown. In *B*, phosphorylation in response to basal (post) and elevated insulin levels (C10) is shown. Electrophoretic mobility is indicated by molecular mass markers (arrows on right).**

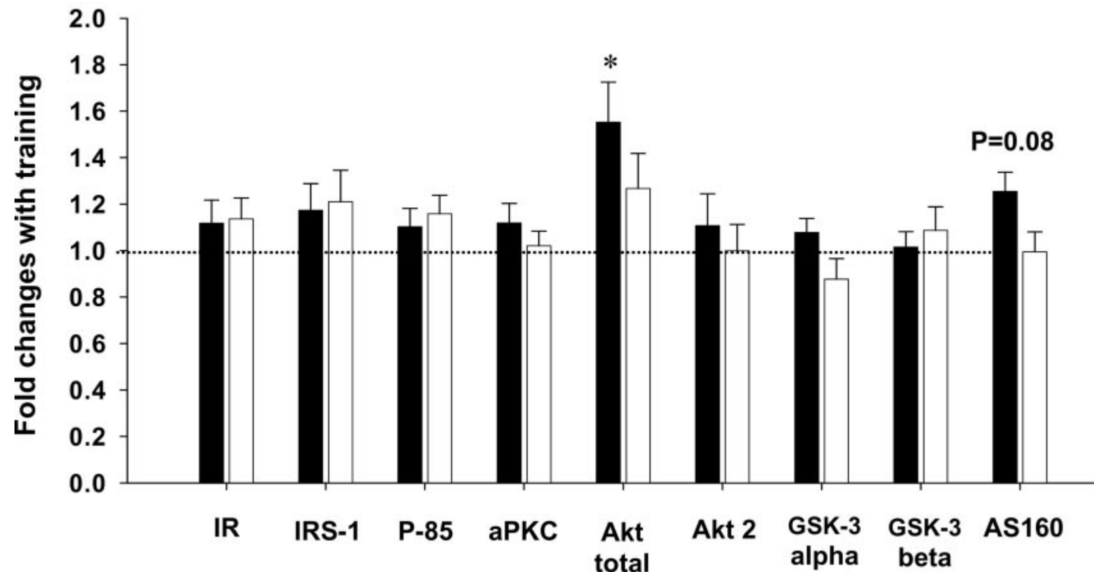


**FIG. 5. Protein content of insulin signaling molecules. Values are shown as posttraining values divided by pretraining values thereby expressing fold changes in response to training. ■, trained muscle; □, untrained muscle. Dotted line shows pretraining levels. *$P$ < 0.05 versus pretraining values. Values are means ± SE; $n$ = 8.**

Akt (Thr$^{308}$/Ser$^{473}$) phosphorylation, a small further increase was observed after 120 min of insulin stimulation. A trend ($P < 0.06$) toward increased aPKCζ/ι Thr$^{410}$ phosphorylation was observed after 10 min but not after 120 min of insulin stimulation. Phosphorylation of GS site 3a+b decreased in response to insulin stimulation ($P < 0.005$) but only after 120 min (Figs. 4$B$ and 7). Training did not influence the phosphorylation state of Akt Ser$^{473}$, Akt Thr$^{308}$, GSK-3α Ser$^{21}$, GSK-3β Ser$^{9}$, or GS site 3a+b either in the basal state or during insulin stimulation (all NS). However, phosphorylation of AS160 was significantly higher after training before ($P < 0.05$) as well as during insulin stimulation (C10, $P < 0.05$; C120, $P = 0.05$) compared with untrained muscle (Figs. 4$B$ and 7).

**Activities of insulin signaling molecules.** Insulin stimulation resulted in an increase in activity of IRS-1–associated PI3-K ($P < 0.005$), Akt1 ($P < 0.05$), Akt2 ($P < 0.005$), and GS (percent I-form) ($P < 0.05$) in both trained and untrained muscle (Fig. 8). The response to insulin was already evident after 10 min of stimulation except at the level of GS, for which a significant increase was only observed after 120 min. In response to training, GS total activity was significantly higher both in the basal state and during insulin stimulation ($P < 0.05$) and, similarly, GS activity expressed as the I-form (nanomoles per minute per milligram) was significantly higher in trained than in untrained muscle (after training +33 ± 10%, C10 +62 ± 16%, and C120 +26 ± 11%; all $P < 0.01$). In contrast,

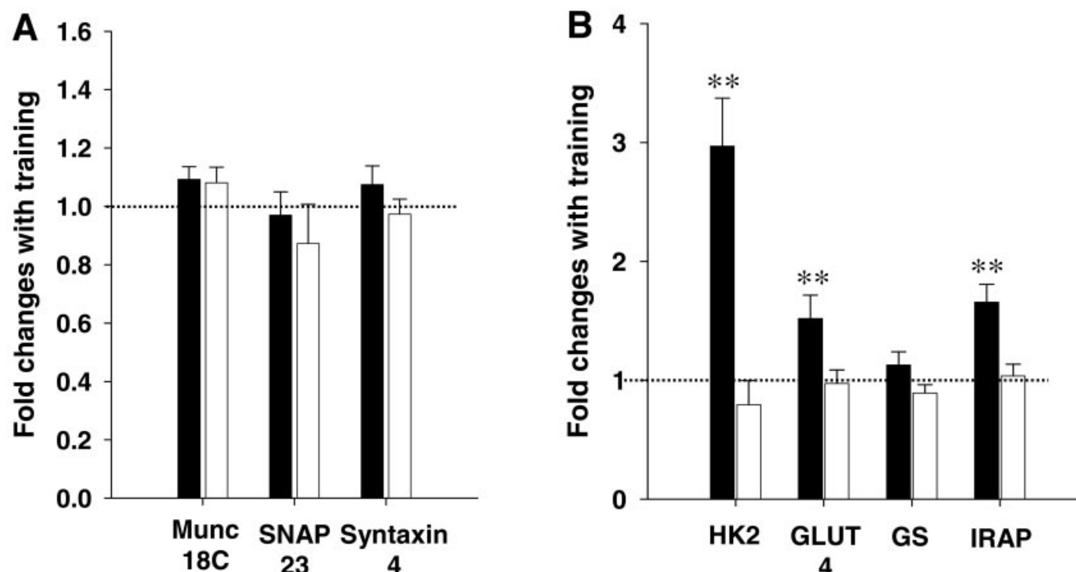


**FIG. 6. Protein content of membrane fusion proteins ($A$), HK2, GLUT4, GS, and IRAP ($B$). Values are shown as posttraining values divided by pretraining values, thereby expressing fold changes in response to training. Dotted line shows pretraining levels. **$P$ < 0.005 versus pretraining values. Values are means ± SE; $n$ = 8.**

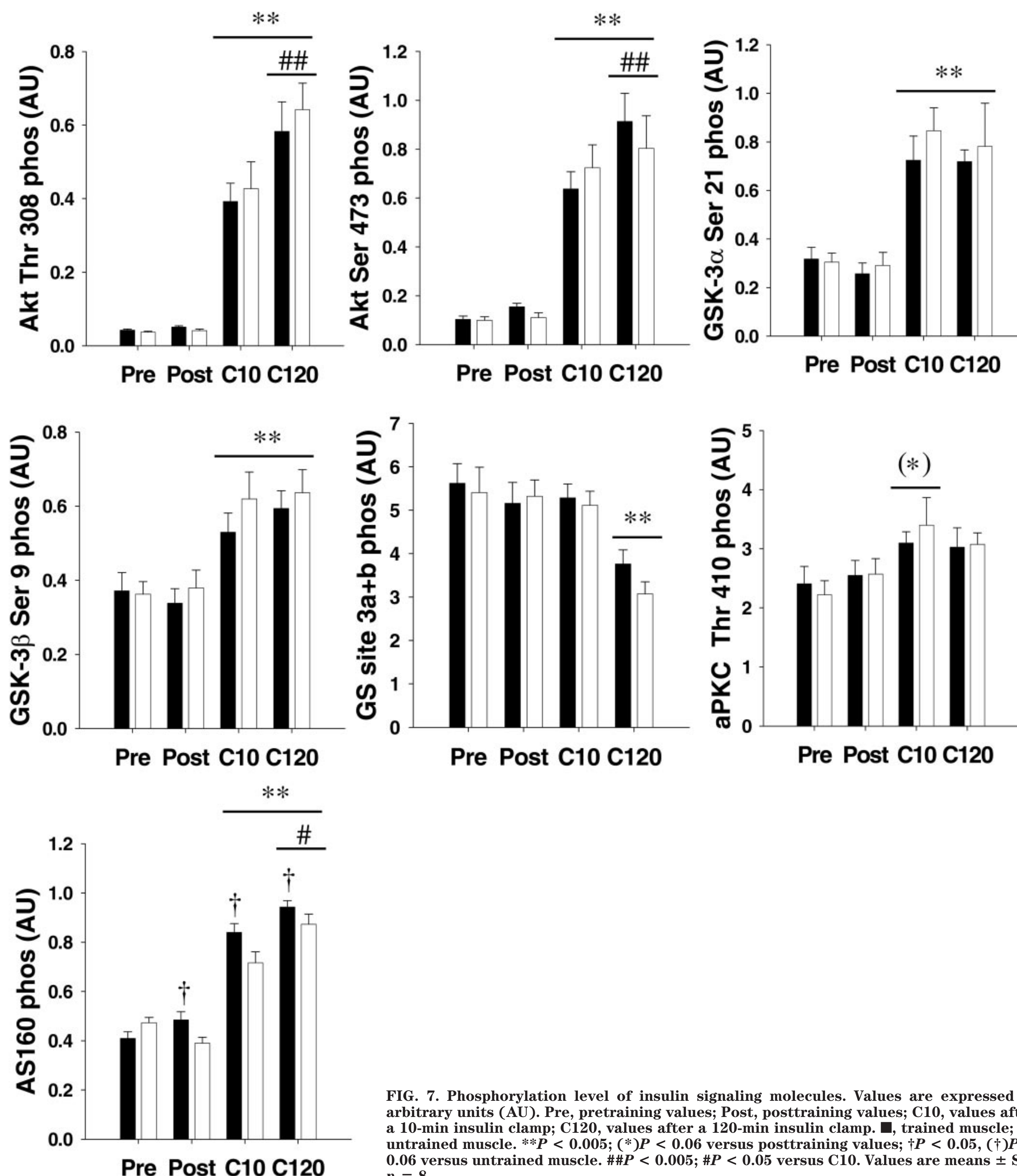


**FIG. 7. Phosphorylation level of insulin signaling molecules. Values are expressed in arbitrary units (AU). Pre, pretraining values; Post, posttraining values; C10, values after a 10-min insulin clamp; C120, values after a 120-min insulin clamp. ■, trained muscle; □, untrained muscle. **$P$ < 0.005; (*)$P$ < 0.06 versus posttraining values; †$P$ < 0.05, (†)$P$ < 0.06 versus untrained muscle. ##$P$ < 0.005; #$P$ < 0.05 versus C10. Values are means ± SE; $n$ = 8.**

activity of IRS-1–associated PI3-K was significantly reduced ($P < 0.05$) at these time points in trained compared with untrained muscle (Fig. 8). Finally, a positive main effect of training was observed for Akt1 activity ($P < 0.05$), although in mean values a higher Akt1 activity in the trained muscle was only observed after training and during insulin stimulation (Fig. 8).

## DISCUSSION

In response to 3 weeks of one-legged endurance exercise training, insulin-stimulated glucose uptake markedly increased in trained compared with untrained muscle. This increase coincided with an increase in protein expression of GLUT4, IRAP, and HK2, as well as increased GS total activity in skeletal muscle. In addition, increased expres-

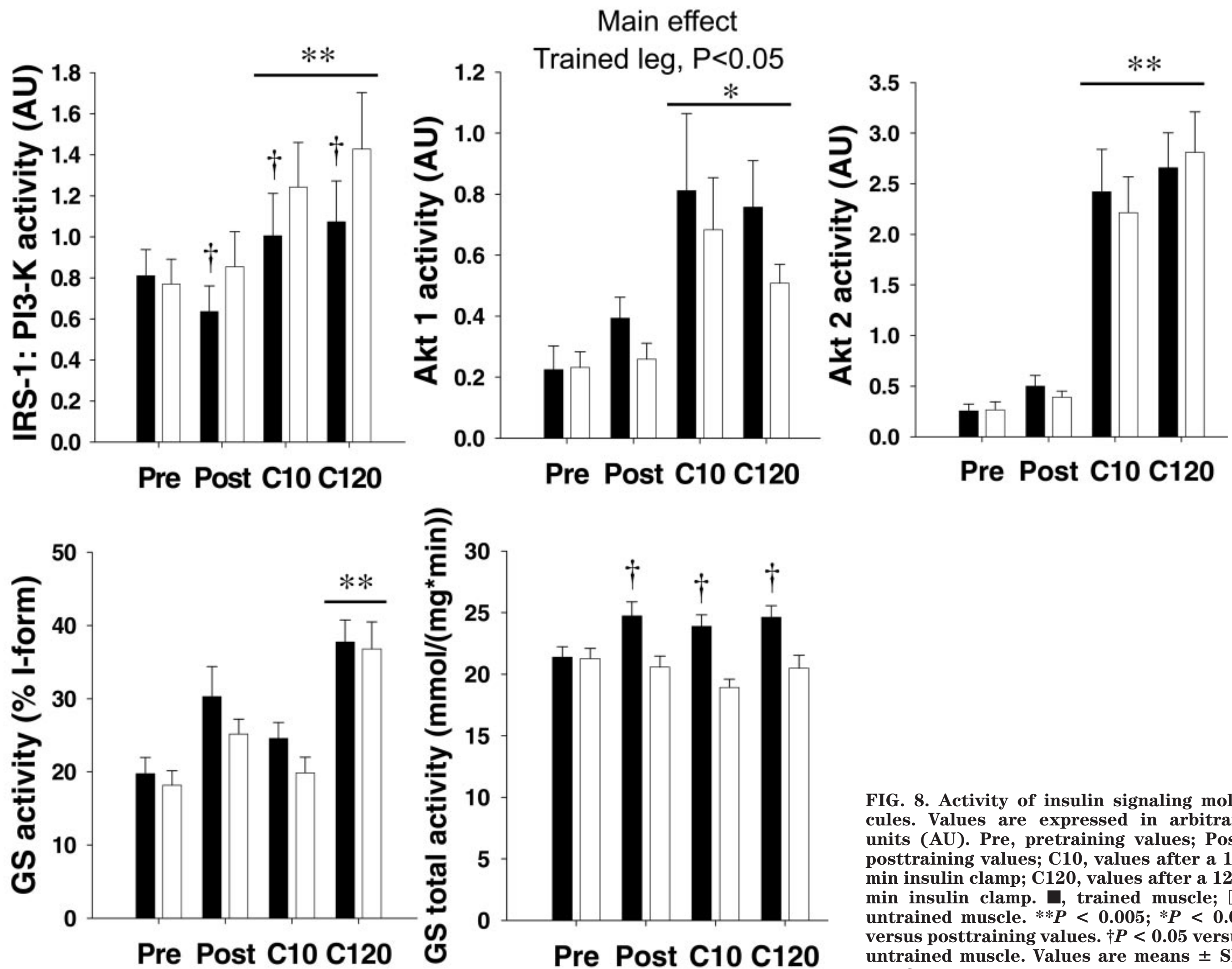


**FIG. 8. Activity of insulin signaling molecules. Values are expressed in arbitrary units (AU). Pre, pretraining values; Post, posttraining values; C10, values after a 10-min insulin clamp; C120, values after a 120-min insulin clamp. ■, trained muscle; □, untrained muscle. **$P$ < 0.005; *$P$ < 0.05 versus posttraining values. †$P$ < 0.05 versus untrained muscle. Values are means ± SE; $n$ = 8.**

sion and activity/phosphorylation of Akt and AS160 were evident after training. These adaptations are likely to improve the intracellular conditions for uptake and metabolism of glucose. Interestingly, training resulted in reduced basal and insulin-stimulated IRS-1–associated PI3-K activity.

In the present study, glucose uptake was calculated using the Fick principle, based on measurements of total venous blood flow and glucose arteriovenous differences across the thigh region. This method does not allow for a distinction between nutritive and non-nutritive flow, and thus it cannot be directly evaluated if delivery of blood-borne substrates at the cellular level is improved by training as demonstrated in rodents (12). We observe that total venous blood flow is higher in the trained thigh (Fig. 2*B*), at least during the early part of insulin stimulation, showing that part of the hemodynamic response to insulin has been improved. Furthermore, there appeared to be increased ($P$ = 0.07) removal of insulin (Fig. 2*C*) from the bloodstream in the trained thigh, perhaps suggesting that delivery of insulin is improved with training. These observations support the fact that hemodynamic adaptations are an important part of the muscular response to training. Curiously, we did not observe improved insulin signaling evaluated by IRS-1–associated PI3-K activity in trained muscle. Thus, although potentially important for insulin-stimulated glucose uptake, the functional implications of vascular adaptations to training in the present study remain elusive.

In response to exercise training several interactions with the insulin signaling cascade were observed. Notably, a significant reduction (~20%) in IRS-1–associated PI3-K activity in both basal and insulin-stimulated muscle was detected (Fig. 8). This reduction could not be attributed to changes in protein content of either IRS-1 or the p85 regulatory subunit of PI3-K and seems to be contradictory to the finding of improved PI3-K signaling after training in rodent muscle (13,14,40,41). However, the existing data in human skeletal muscle are not equivocal. Previously, one cross-sectional study reported a greater fold increase in IRS-1–associated PI3-K activity in response to insulin stimulation in trained compared with untrained subjects (42). Furthermore, it has been shown that insulin-stimulated phosphotyrosine-associated PI3-K activity is greater after 7 days of endurance training in young (18) but not in middle-aged obese (19,43) or type 2 diabetic subjects (43). In contrast, in response to 8 weeks of endurance training, IRS-1–associated PI3-K activity was ~20% lower in basal and insulin-stimulated trained muscle of elderly type 2 diabetic patients and corresponding control subjects (44), although this was only significant in basal samples of the control subjects. Thus, the adaptations at the level of PI3-K

may depend on the length of the exercise training regimen and subject characteristics. In this regard, it should be emphasized that changes in phosphotyrosine-associated PI3-K activity with training might not involve regulation of IRS-1 and thus might not lead to changes in glucose uptake. Clearly more research is needed to fully understand regulation of PI3-K in response to training in human muscle. However, on the basis of the present study and earlier observations (44), it does not appear that improved insulin action on glucose uptake after training requires improved insulin signaling at the level of IRS-1–associated PI3-K in human skeletal muscle. In fact, training for 3 weeks (this study) or 8 weeks (44) can lead to reduced IRS-1–associated PI3-K signaling.

Despite reduced IRS-1–associated PI3-K activity after training, downstream signaling through Akt2 was normal, and Akt1 activity appeared to be increased in basal and insulin-stimulated muscle after training. The latter findings can probably be ascribed to increased protein content of Akt1 in response to training, as indicated by a ~50% increase in total Akt protein but unaltered Akt2 content (Fig. 5). The observation of increased total Akt protein with training was confirmed in elderly subjects with and without type 2 diabetes (44). Curiously, no effect of training was observed in regard to Akt phosphorylation, which indicates that phosphorylation at $Thr^{308}$ and $Ser^{473}$ may not always reflect changes in activity. However, it should be emphasized that the antibodies used for detection of Akt phosphorylation are not isoform specific, and thus differences in Akt1 phosphorylation might not be detectable because of "dilution" by phosphorylation of Akt2, the primary Akt isoform for regulation of glucose metabolism in skeletal muscle (21,45).

Activation of Akt in skeletal muscle leads to phosphorylation of $GSK\text{-}3^{Ser21/9}$, which deactivates the enzymes (46). This subsequently leads to dephosphorylation (site 3a+b) and activation of GS (47). On the basis of the present study, this phosphorylation sequence is not affected by training (Fig. 7). This finding indicates that Akt1 is not a major kinase regulating GS in human skeletal muscle in response to insulin stimulation.

Interestingly, activation of GS (I-form, nanomoles per minute per milligram) and total GS activity (Fig. 8) increased with training as observed in both basal and insulin-stimulated muscle. This adaptation has previously been observed in response to both strength and endurance training regimens (2,44). However, in contrast to the study by Holten et al. (2), no detectable significant change in protein expression of GS was observed in the present study. Collectively, increased GS activity is a consistent finding in trained human muscle. However, this increase should not be ascribed to changes in insulin signaling to GS activation.

Insulin signaling to GLUT4 translocation is believed to involve activation of both Akt and aPKC in skeletal muscle. Previously in our group, it has been demonstrated that trained subjects compared with untrained subjects have similar protein contents of aPKC but higher basal aPKC activity in skeletal muscle (48). Because of tissue limitations, aPKC activity could not be evaluated in the present study. However, it was shown that training per se does not influence protein content or phosphorylation of aPKC on $Thr^{410}$ when measured in whole tissue lysates. Furthermore, it was observed that physiological insulin stimulation at best caused only a transient increase in phosphorylation at this site (C10, $P < 0.06$; C120, $P > 0.1$). Considering that increased aPKC phosphorylation has previously been observed in rodent skeletal muscle in response to supraphysiological insulin stimulation (49), a transient increase in response to physiological insulin stimulation in human muscle seems plausible.

Interestingly, phosphorylation of AS160 downstream of Akt was increased in both basal and insulin-stimulated muscle after training. This novel observation is probably not related to upstream signaling through Akt1 (21). In contrast, it may be caused by changes in activity of 5′AMP-activated protein kinase acting upstream of AS160 (24,50) as this activity has previously been shown to increase in response to the present training regimen (35). Alternatively, the adaptive response in AS160 phosphorylation may partly be explained by increased protein content of AS160 after training, although this increase did not reach statistical significance ($P = 0.08$). In support of the latter interpretation, if AS160 phosphorylation was expressed relative to protein content after training, the effect of training on AS160 phosphorylation disappears ($P = 0.7$; data not shown). Several reports indicate a role of AS160 in stimulation of GLUT4 translocation (20,22,51). In the present study, the effect of improved AS160 signaling on glucose uptake is not easily addressed. This is partly due to the problem of defining the underlying mechanisms. Furthermore, increased AS160 phosphorylation in trained muscle is observed in response to basal insulin levels at which no detectable difference in glucose uptake was observed compared with untrained muscle.

Insulin signaling leads to translocation of GLUT4-containing vesicles to the plasma membrane and T-tubules, and, through formation of the SNARE core complex, vesicles fuse with the membrane, allowing for GLUT4 enrichment (52). In the present study, it is shown that protein contents of SNARE proteins Syntaxin4 and SNAP23 as well as the SNARE adaptor protein Munc18c do not change with training. These observations seems to indicate that exercise training does not influence the available sites for docking of GLUT4-containing vesicles. In contrast, a large increase in GLUT4 protein expression was observed. This latter observation is consistently found in response to both endurance and strength training and has been closely associated with improved insulin responsiveness after training (2,15,17,53,54). We furthermore provide correlative evidence ($r^2 = 0.47$, $P = 0.05$) that training-induced changes in GLUT4 protein occur in coordination with changes in protein expression of IRAP. This finding is supported by a previous cross-sectional human study (55) and suggests that also in human muscle IRAP is not only a marker of GLUT4-containing vesicles but also may be directly involved in maintaining GLUT4 expression and vesicle trafficking (28,56). In the present study, an ~200% increase in the protein content of HK2 was observed. Previously, increased HK2 activity has been observed in response to training in humans (57), and increased protein expression and activity of HK2 have been observed after electrically stimulated cycle exercise training in tetraplegic subjects (17). Furthermore, overexpression of HK2 and in particular combined overexpression of HK2 and GLUT4 in mouse muscle resulted in a marked increase in glucose uptake in skeletal muscle in response to insulin stimulation in vivo (54). Thus, the adaptive response to training seen in the present study in terms of protein expression of GLUT4, IRAP, and HK2 may be of major importance explaining the observed increased glucose uptake.

In summary, this study provides an extensive evaluation of the adaptive response of skeletal muscle to 3 weeks of endurance exercise training by tracking alterations in protein expression, kinase activation, and the phosphorylation level of the insulin signaling cascade. At the extracellular level, training improves the hemodynamic effects of insulin, possibly allowing for improved delivery of insulin and glucose. Several interactions between training and insulin signaling were observed, including decreased IRS-1–associated PI3-K activity but increased AS160 phosphorylation. This dissociation does not support generally improved insulin signaling to glucose uptake; instead it appears that downstream of PI3-K, the observed training effects are at least partly a result of increased cellular protein content of individual insulin signaling components and increased expression of GLUT4, IRAP, and HK2. In concert with increased GS activity, it is suggested that these adaptations allow for more efficient transport and intracellular metabolism of glucose in the myocyte.

## ACKNOWLEDGMENTS

This work was supported by the Commission of the European Communities (contract LSHM-CT-2004-005272 EXGENESIS), the Danish Medical Research Council, the Novo Nordisk Foundation, the Danish Diabetes Association, and the Copenhagen Muscle Research Centre and by grants from the Ministry of Food, Agriculture and Fisheries and the Ministry of Family and Consumer Affairs. A.R. was supported by a fellowship from the Carlsberg Foundation. J.F.P.W. was supported by a Hallas Møller fellowship from the Novo Nordisk Foundation.

We thank present and former members of the laboratory, Betina Bolmgren, Jesper B. Birk, Kristian Elbo, and Jens R. Daugaard for helpful discussions, suggestions, and expert technical assistance. Furthermore, we thank Dr. Ken Siddle for kindly donating antibodies against IR and IRS-1 as well as Dr. Bo F. Hansen, Dr. Susanna R. Keller, and Professor Oluf Pedersen for kindly donating the antibodies against GS site 3a+b, IRAP, and GS, respectively.

## REFERENCES

1. Saltin B, Lindgarde F, Houston M, Horlin R, Nygaard E, Gad P: Physical training and glucose tolerance in middle-aged men with chemical diabetes. *Diabetes* 28 (Suppl. 1):30–32, 1979
2. Holten MK, Zacho M, Gaster M, Juel C, Wojtaszewski JF, Dela F: Strength training increases insulin-mediated glucose uptake, GLUT4 content, and insulin signaling in skeletal muscle in patients with type 2 diabetes. *Diabetes* 53:294–305, 2004
3. Dela F, Larsen JJ, Mikines KJ, Ploug T, Petersen LN, Galbo H: Insulin-stimulated muscle glucose clearance in patients with NIDDM: effects of one-legged physical training. *Diabetes* 44:1010–1020, 1995
4. Hughes VA, Fiatarone MA, Fielding RA, Kahn BB, Ferrara CM, Shepherd P, Fisher EC, Wolfe RR, Elahi D, Evans WJ: Exercise increases muscle GLUT-4 levels and insulin action in subjects with impaired glucose tolerance. *Am J Physiol* 264:E855–E862, 1993
5. Ruderman NB, Ganda OP, Johansen K: The effect of physical training on glucose tolerance and plasma lipids in maturity-onset diabetes. *Diabetes* 28 (Suppl. 1):89–92, 1979
6. James DE, Kraegen EW, Chisholm DJ: Effects of exercise training on in vivo insulin action in individual tissues of the rat. *J Clin Invest* 76:657–666, 1985
7. Dela F, Mikines KJ, Von Linstow M, Secher NH, Galbo H: Effect of training on insulin-mediated glucose uptake in human muscle. *Am J Physiol* 263:E1134–E1143, 1992
8. Marette A, Burdett E, Douen A, Vranic M, Klip A: Insulin induces the translocation of GLUT4 from a unique intracellular organelle to transverse tubules in rat skeletal muscle. *Diabetes* 41:1562–1569, 1992
9. Tong P, Khayat ZA, Huang C, Patel N, Ueyama A, Klip A: Insulin-induced cortical actin remodeling promotes GLUT4 insertion at muscle cell membrane ruffles. *J Clin Invest* 108:371–381, 2001
10. Thurmond DC, Pessin JE: Molecular machinery involved in the insulin-regulated fusion of GLUT4-containing vesicles with the plasma membrane (Review). *Mol Membr Biol* 18:237–245, 2001
11. Roach PJ: Glycogen and its metabolism. *Curr Mol Med* 2:101–120, 2002
12. Rattigan S, Wallis MG, Youd JM, Clark MG: Exercise training improves insulin-mediated capillary recruitment in association with glucose uptake in rat hindlimb. *Diabetes* 50:2659–2665, 2001
13. Chibalin AV, Yu M, Ryder JW, Song XM, Galuska D, Krook A, Wallberg-Henriksson H, Zierath JR: Exercise-induced changes in expression and activity of proteins involved in insulin signal transduction in skeletal muscle: differential effects on insulin-receptor substrates 1 and 2. *Proc Natl Acad Sci USA* 97:38–43, 2000
14. Luciano E, Carneiro EM, Carvalho CR, Carvalheira JB, Peres SB, Reis MA, Saad MJ, Boschero AC, Velloso LA: Endurance training improves responsiveness to insulin and modulates insulin signal transduction through the phosphatidylinositol 3-kinase/Akt-1 pathway. *Eur J Endocrinol* 147:149–157, 2002
15. Dela F, Handberg A, Mikines KJ, Vinten J, Galbo H: GLUT 4 and insulin receptor binding and kinase activity in trained human muscle. *J Physiol* 469:615–624, 1993
16. Langfort J, Viese M, Ploug T, Dela F: Time course of GLUT4 and AMPK protein expression in human skeletal muscle during one month of physical training. *Scand J Med Sci Sports* 13:169–174, 2003
17. Hjeltnes N, Galuska D, Bjornholm M, Aksnes AK, Lannem A, Zierath JR, Wallberg-Henriksson H: Exercise-induced overexpression of key regulatory proteins involved in glucose uptake and metabolism in tetraplegic persons: molecular mechanism for improved glucose homeostasis. *FASEB J* 12:1701–1712, 1998
18. Houmard JA, Shaw CD, Hickey MS, Tanner CJ: Effect of short-term exercise training on insulin-stimulated PI 3-kinase activity in human skeletal muscle. *Am J Physiol* 277:E1055–E1060, 1999
19. Tanner CJ, Koves TR, Cortright RL, Pories WJ, Kim YB, Kahn BB, Dohm GL, Houmard JA: Effect of short-term exercise training on insulin-stimulated PI 3-kinase activity in middle-aged men. *Am J Physiol* 282:E147–E153, 2002
20. Sano H, Kane S, Sano E, Miinea CP, Asara JM, Lane WS, Garner CW, Lienhard GE: Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation. *J Biol Chem* 278:14599–14602, 2003
21. Bouzakri K, Zachrisson A, Al-Khalili L, Zhang BB, Koistinen HA, Krook A, Zierath JR: siRNA-based gene silencing reveals specialized roles of IRS-1/Akt2 and IRS-2/Akt1 in glucose and lipid metabolism in human skeletal muscle. *Cell Metab* 4:89–96, 2006
22. Gonzalez E, McGraw TE: Insulin signaling diverges into Akt-dependent and -independent signals to regulate the recruitment/docking and the fusion of GLUT4 vesicles to the plasma membrane. *Mol Biol Cell* 17:4484–4493, 2006
23. Zeigerer A, McBrayer MK, McGraw TE: Insulin stimulation of GLUT4 exocytosis, but not its inhibition of endocytosis, is dependent on RabGAP AS160. *Mol Biol Cell* 15:4406–4415, 2004
24. Kramer HF, Witczak CA, Fujii N, Jessen N, Taylor EB, Arnolds DE, Sakamoto K, Hirshman MF, Goodyear LJ: Distinct signals regulate AS160 phosphorylation in response to insulin AICAR, and contraction in mouse skeletal muscle. *Diabetes* 55:2067–2076, 2006
25. Spurlin BA, Thomas RM, Nevins AK, Kim HJ, Kim YJ, Noh HL, Shulman GI, Kim JK, Thurmond DC: Insulin resistance in tetracycline-repressible Munc18c transgenic mice. *Diabetes* 52:1910–1917, 2003
26. Khan AH, Thurmond DC, Yang C, Ceresa BP, Sigmund CD, Pessin JE: Munc18c regulates insulin-stimulated GLUT4 translocation to the transverse tubules in skeletal muscle. *J Biol Chem* 276:4063–4069, 2001
27. Yang C, Coker KJ, Kim JK, Mora S, Thurmond DC, Davis AC, Yang B, Williamson RA, Shulman GI, Pessin JE: Syntaxin 4 heterozygous knockout mice develop muscle insulin resistance. *J Clin Invest* 107:1311–1318, 2001
28. Yeh TY, Sbodio JI, Tsun ZY, Luo B, Chi NW: Insulin-stimulated exocytosis of GLUT4 is enhanced by IRAP and its partner tankyrase. *Biochem J* 2006
29. Andersen P, Adams RP, Sjogaard G, Thorboe A, Saltin B: Dynamic knee extension as model for study of isolated exercising muscle in humans. *J Appl Physiol* 59:1647–1653, 1985
30. Richter EA, Mikines KJ, Galbo H, Kiens B: Effect of exercise on insulin action in human skeletal muscle. *J Appl Physiol* 66:876–885, 1989
31. Richter EA, Jensen P, Kiens B, Kristiansen S: Sarcolemmal glucose transport and GLUT-4 translocation during exercise are diminished by endurance training. *Am J Physiol* 274:E89–E95, 1998
32. Gollnick PD, Piehl K, Saltin B: Selective glycogen depletion pattern in human muscle fibres after exercise of varying intensity and at varying pedalling rates. *J Physiol* 241:45–57, 1974

33. Lowry OH, Passonneau JV: *A Flexible System of Enzymatic Analysis*. New York, Academic Press, 1972
34. Wojtaszewski JF, Hansen BF, Kiens B, Richter EA: Insulin signaling in human skeletal muscle: time course and effect of exercise. *Diabetes* 46:1775–1781, 1997
35. Frosig C, Jorgensen SB, Hardie DG, Richter EA, Wojtaszewski JF: 5′-AMP-activated protein kinase activity and protein expression are regulated by endurance training in human skeletal muscle. *Am J Physiol* 286:E411–E417, 2004
36. Vestergaard H, Andersen PH, Lund S, Schmitz O, Junker S, Pedersen O: Pre- and posttranslational upregulation of muscle-specific glycogen synthase in athletes. *Am J Physiol* 266:E92–E101, 1994
37. Hojlund K, Staehr P, Hansen BF, Green KA, Hardie DG, Richter EA, Beck-Nielsen H, Wojtaszewski JF: Increased phosphorylation of skeletal muscle glycogen synthase at $NH_2$-terminal sites during physiological hyperinsulinemia in type 2 diabetes. *Diabetes* 52:1393–1402, 2003
38. Hojlund K, Wojtaszewski JF, Birk J, Hansen BF, Vestergaard H, Beck-Nielsen H: Partial rescue of in vivo insulin signalling in skeletal muscle by impaired insulin clearance in heterozygous carriers of a mutation in the insulin receptor gene. *Diabetologia* 49:1827–1837, 2006
39. Thomas JA, Schlender KK, Larner J: A rapid filter paper assay for UDPglucose-glycogen glucosyltransferase, including an improved biosynthesis of UDP-14C-glucose. *Anal Biochem* 25:486–499, 1968
40. Saengsirisuwan V, Perez FR, Sloniger JA, Maier T, Henriksen EJ: Interactions of exercise training and alpha-lipoic acid on insulin signaling in skeletal muscle of obese Zucker rats. *Am J Physiol* 287:E529–E536, 2004
41. Krisan AD, Collins DE, Crain AM, Kwong CC, Singh MK, Bernard JR, Yaspelkis BB III: Resistance training enhances components of the insulin signaling cascade in normal and high-fat-fed rodent skeletal muscle. *J Appl Physiol* 96:1691–1700, 2004
42. Kirwan JP, Del Aguila LF, Hernandez JM, Williamson DL, O'Gorman DJ, Lewis R, Krishnan RK: Regular exercise enhances insulin activation of IRS-1-associated PI3-kinase in human skeletal muscle. *J Appl Physiol* 88:797–803, 2000
43. O'Gorman DJ, Karlsson HK, McQuaid S, Yousif O, Rahman Y, Gasparro D, Glund S, Chibalin AV, Zierath JR, Nolan JJ: Exercise training increases insulin-stimulated glucose disposal and GLUT4 (SLC2A4) protein content in patients with type 2 diabetes. *Diabetologia* 49:2983–2992, 2006
44. Christ-Roberts CY, Pratipanawatr T, Pratipanawatr W, Berria R, Belfort R, Kashyap S, Mandarino LJ: Exercise training increases glycogen synthase activity and GLUT4 expression but not insulin signaling in overweight nondiabetic and type 2 diabetic subjects. *Metabolism* 53:1233–1242, 2004
45. Cho H, Mu J, Kim JK, Thorvaldsen JL, Chu Q, Crenshaw EB III, Kaestner KH, Bartolomei MS, Shulman GI, Birnbaum MJ: Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKBβ). *Science* 292:1728–1731, 2001
46. Welsh GI, Proud CG: Glycogen synthase kinase-3 is rapidly inactivated in response to insulin and phosphorylates eukaryotic initiation factor eIF-2B. *Biochem J* 294 (Pt 3):625–629, 1993
47. McManus EJ, Sakamoto K, Armit LJ, Ronaldson L, Shapiro N, Marquez R, Alessi DR: Role that phosphorylation of GSK3 plays in insulin and Wnt signalling defined by knockin analysis. *EMBO J* 24:1571–1583, 2005
48. Nielsen JN, Frosig C, Sajan MP, Miura A, Standaert ML, Graham DA, Wojtaszewski JF, Farese RV, Richter EA: Increased atypical PKC activity in endurance-trained human skeletal muscle. *Biochem Biophys Res Commun* 312:1147–1153, 2003
49. Kanoh Y, Sajan MP, Bandyopadhyay G, Miura A, Standaert ML, Farese RV: Defective activation of atypical protein kinase C ζ and λ by insulin and phosphatidylinositol-3,4,5-$(PO_4)_3$ in skeletal muscle of rats following high-fat feeding and streptozotocin-induced diabetes. *Endocrinology* 144:947–954, 2003
50. Treebak JT, Glund S, Deshmukh A, Klein DK, Long YC, Jensen TE, Jorgensen SB, Viollet B, Andersson L, Neumann D, Wallimann T, Richter EA, Chibalin AV, Zierath JR, Wojtaszewski JF: AMPK-mediated AS160 phosphorylation in skeletal muscle is dependent on AMPK catalytic and regulatory subunits. *Diabetes* 55:2051–2058, 2006
51. Watson RT, Pessin JE: Bridging the GAP between insulin signaling and GLUT4 translocation. *Trends Biochem Sci* 31:215–222, 2006
52. Ishiki M, Klip A: Minireview: Recent developments in the regulation of glucose transporter-4 traffic: new signals, locations, and partners. *Endocrinology* 146:5071–5078, 2005
53. Hardin DS, Azzarelli B, Edwards J, Wigglesworth J, Maianu L, Brechtel G, Johnson A, Baron A, Garvey WT: Mechanisms of enhanced insulin sensitivity in endurance-trained athletes: effects on blood flow and differential expression of GLUT 4 in skeletal muscles. *J Clin Endocrinol Metab* 80:2437–2446, 1995
54. Fueger PT, Shearer J, Bracy DP, Posey KA, Pencek RR, McGuinness OP, Wasserman DH: Control of muscle glucose uptake: test of the rate-limiting step paradigm in conscious, unrestrained mice. *J Physiol* 562:925–935, 2005
55. Yu M, Blomstrand E, Chibalin AV, Wallberg-Henriksson H, Zierath JR, Krook A: Exercise-associated differences in an array of proteins involved in signal transduction and glucose transport. *J Appl Physiol* 90:29–34, 2001
56. Keller SR, Davis AC, Clairmont KB: Mice deficient in the insulin-regulated membrane aminopeptidase show substantial decreases in glucose transporter GLUT4 levels but maintain normal glucose homeostasis. *J Biol Chem* 277:17677–17686, 2002
57. Phillips SM, Han XX, Green HJ, Bonen A: Increments in skeletal muscle GLUT-1 and GLUT-4 after endurance training in humans. *Am J Physiol* 270:E456–E462, 1996