Insulin, but Not Contraction, Activates Akt/PKB in Isolated Rat Skeletal Muscle*

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Insulin and muscle contraction potently stimulate glucose uptake in mammalian skeletal muscle. Studies in muscle and adipose tissue have shown that insulin induces its receptor-dependent phosphorylation of insulin receptor substrates 1 and 2, which leads to activation of polyphosphatidylinositol (PI) 3'-kinase. In contrast, muscle contraction stimulates glucose transport via a mechanism that is independent of insulin, but the two pathways may converge downstream at the level of stimulation of GLUT4 translocation. In the present study, we have examined the role of Akt, an insulin-activated serine threonine kinase that has previously been shown to increase glucose transport in adipocytes. Either insulin or in vitro muscle contraction significantly elevated glucose transport in isolated rat epitrochlearis and soleus muscles. However, Akt kinase activity was significantly stimulated by insulin and not contraction. Moreover, wortmannin, an inhibitor of PI 3'-kinase, completely blocked the insulin-stimulated increase in Akt activity and glucose transport but did not alter either of these parameters in contracting muscles. The increases in Akt activity were paralleled by a decrease in the electrophoretic mobility of Akt, indicative of phosphorylation of Akt by an upstream kinase. These changes in Akt mobility appeared to be at least partially because of phosphorylation of Akt on serine 473. A putative downstream target of Akt, p70 S6 kinase, showed similar changes in mobility in response to insulin but not contraction. These data support the view that Akt is a downstream target of PI 3'-kinase and is involved in the signaling pathways involved in insulin but not contraction stimulation of glucose transport in skeletal muscle. These data provide further evidence that two distinct pathways exist for the stimulation of glucose transport in mammalian skeletal muscle.

Insulin and muscle contraction are two powerful stimulators of skeletal muscle glucose transport (1, 2). Although it is known that both of these stimuli activate glucose transport by causing the translocation of GLUT4 to the plasma membrane, the exact nature of the signaling pathways that are involved remains unknown (3–5). Presently, insulin has been shown to initiate its signal through tyrosine phosphorylation of insulin receptor substrate proteins and subsequent docking of proteins that contain Src homology 2 sites (6). One such protein is PI3'-kinase, whose p85 regulatory subunit binds to insulin receptor substrates 1 and 2 and in turn activates its p110 catalytic subunit. However, this pathway does not appear to be involved in stimulation of glucose transport by muscle contraction (7, 8).

Although activation of PI 3'-kinase appears necessary for activation of glucose transport by insulin, the identity of the downstream mediators remains largely unknown (6, 9). A potential candidate for such a regulator of insulin action is Akt (also known as protein kinase B), a serine threonine kinase that is activated by insulin and other growth factors (i.e. PDGF) (10, 11). It has recently been shown that wortmannin, an inhibitor of PI 3'-kinase, also inhibits insulin-stimulated Akt activity (11). Additionally, expression of a dominant negative p85 subunit in 3T3 L1 adipocytes inhibits PDGF-stimulated Akt activity (12). Although the exact biological function of Akt is unknown, it has been hypothesized to play a role in glycolgen metabolism because it phosphorylates glycogen synthase kinase 3, which controls glycogen synthesis (10, 13).

Recently, it has been shown that expression of a constitutively active Akt mutant in 3T3 L1 adipocytes or in isolated rat adipocytes increased glucose transport by inducing the translocation of GLUT4 to the plasma membrane (10, 14, 15). Thus, it appears that Akt may represent a major PI 3'-kinase effector in insulin stimulation of glucose transport.

Although muscle contraction does not stimulate glucose transport by a PI 3'-kinase-dependent mechanism, there is some evidence that there may be a common pathway downstream of PI 3'-kinase. For example, polymyxin B, an inhibitor of protein kinase C, inhibits both insulin- and contraction-stimulated glucose transport (16). Therefore, it is possible that insulin and contraction may share some common signaling step. Because of previous evidence that constitutively active Akt induces GLUT4 translocation in adipocytes (11, 14, 15) and that Akt could be activated independently of PI 3'-kinase, we hypothesized that Akt may represent this common step. The role of Akt in contraction-stimulated glucose transport in skeletal muscle has not yet been examined. In the present study, we explored the possible role of Akt in mediating contraction-and insulin-stimulated glucose transport in skeletal muscle.

MATERIALS AND METHODS

Animals—Specific pathogen-free male Wistar rats weighing 100–125 g were obtained from Charles River Laboratories (Boston, MA). Upon arrival, rats were housed four to a cage in a temperature-controlled animal room maintained on a 12:12-h light-dark cycle. The rats were fed ad libitum National Institutes of Health standard chow and water.

Muscle Preparation and Incubation—Rats in the post-prandial state were anesthetized with 5 mg of sodium pentobarbital/100 g of body weight. After excision, the muscles were rinsed briefly in ice-cold Henseleit buffer (2DG, 2-deoxyglucose; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; TTB, Tween Tris-buffered saline).

1 The abbreviations used are: PI, phosphatidylinositol; PDGF, platelet-derived growth factor; BSA, bovine serum albumin; KHB, Krebs-Henseleit buffer; 2DG, 2-deoxyglucose; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; TTB, Tween Tris-buffered saline.

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weight. Epitrochlearis or soleus muscles were dissected out, blotted on gauze, and transferred to 25-ml Erlenmeyer flasks containing 2 ml of KHB containing 0.1% bovine serum albumin (BSA), 32 mM mannitol, and 8 mM glucose. The flasks were incubated in a shaking water bath maintained at 29 °C for 1 h and were continuously gassed with 95% O2/5% CO2. Soleus muscles were split prior to incubation to allow for proper diffusion of substrate in these muscles (17). Prior to incubation under basal conditions (no additions) or stimulation with insulin or contraction, all muscles were allowed to recover (preincubation) for 1 h in the absence of insulin (17). Following the preincubation, muscles were incubated under basal (no additions) or insulin-stimulated (13.3 nM) conditions for 30 min or electrically stimulated to contract as described below. Wortmannin was dissolved in Me2SO, and when present in the incubations, a similar quantity of Me2SO was added to the control muscles. The concentration of Me2SO added to the incubation medium never exceeded 0.5%. When wortmannin was present during incubation with insulin or contraction, it was also included in the preincubation medium. Following the 30-min incubation, the muscles were frozen between tongs cooled to the temperature of liquid nitrogen and used for measurement of Akt kinase activity and Western blotting. Alternatively, the muscles were transferred to flasks containing 2 ml of KHB containing 0.1% BSA, 40 mM mannitol, 2 mM pyruvate, and the same additions as in the previous incubation and used for measurement of glucose transport. The flasks were incubated for 10 min at 29°C to wash out glucose; the gas phase in the flasks was 95% O2/5% CO2.

**Muscle Contraction**—The effect of contraction on glucose transport activity and signaling molecules was investigated using a specially designed apparatus that has been described previously (18). Briefly, each muscle was pinned at approximately resting length in a contraction apparatus that is designed such that two platinum electrodes lie on either side of the muscle (Biomedical Instrumentation Group, University of Pennsylvania School of Medicine). The contraction periods consisted of 200-ms trains of 100 Hz using a model S48 square wave stimulator (Grass Instruments, Warwick, RI) attached to a Stimusplitter (MedLab Instruments, Loveland, CO). The trains were delivered at 2/s at 10–15 V. Glucose transport activity was measured using 2DG as described in detail previously (19).

**Akt Immunoprecipitation**—Isolated muscles were incubated under the experimental conditions described above. Following incubation, the muscles were trimmed of their tendons, blotted, and frozen between tongs cooled to the temperature of liquid nitrogen. Muscles were kept stored at −80°C until processed.

Lysates were prepared from the incubated muscles essentially as described previously (20). Briefly, frozen muscles were homogenized in ice-cold 50 mM HEPES, pH 7.2, 2 mM EDTA, 30 mM NaF, 1% Triton X-100, 10% glycerol, 10 mM NaF, 150 mM NaCl, 2 mM NaVO4, 5 μg/ml leupeptin, 1.5 mg/ml benzamidine, 0.5 mg/ml pepstatin A, 2 μg/ml aprotinin, 1 mM 4-(aminoethyl)-benzenesulfonyl-fluoride (Pefabloc, Boehringer Mannheim), and 10 μg/ml antipain) and mixed end over end for 45 min at 4°C. Lysates were then spun at 18,000 x g for 15 min, and protein was determined on the supernatant via the bicinchoninic acid method (Pierce) using crystalline BSA as a standard. Aliquots of the supernatant corresponding to 2 mg of protein were immunoprecipitated for 2 h with 5 μl of affinity purified anti-Akt antibody (raised against 16 amino acids of the carboxyl terminus of rat Akt 2). Akt activity was measured as described previously (11).

**Western Blotting**—Akt-immunoprecipitated samples or 200 μg of crude lysate protein were prepared for SDS-PAGE by the addition of 2× Laemmli sample buffer and were boiled for 2 min. The samples, along with molecular weight markers (Sigma) were loaded on a 7.5% SDS-PAGE gel. Resolved samples were transferred to PVDF membrane (Bio-Rad). The membranes were rinsed in water and blocked in 5% nonfat dry milk in Tween Tris-buffered saline (TTBS), pH 7.5, for 1 h. The membranes were rinsed in TTBS and incubated overnight in anti-Akt, anti-p70 S6 kinase (courtesy of Dr. Margaret Chou, University of Pennsylvania), or anti-phospho-Akt antibodies (Ser-473 Antibody kit, New England Biols, Beverly, MA). The membranes were rinsed in TTBS and incubated in horseradish peroxidase-coupled goat anti-rabbit antibody (Cappel, Durham, NC) for 2 h. The membranes were then rinsed in TTBS, and the resolved bands were detected via ECL (Amer sham Pharmacia Biotech).

**RESULTS**

**Muscle Glucose Transport**—Insulin stimulation increased the rate of glucose transport 4- and 5-fold over basal for epitrochlearis and soleus muscles, respectively (Fig. 1). Wortmannin (1 μM) completely inhibited the insulin-stimulated increase in transport. Electrically stimulated contraction increased the rate of glucose transport 3-fold in epitrochlearis and 2-fold in soleus muscles. In contrast to insulin stimulation, 1 μM wortmannin did not significantly decrease the rate of contraction-stimulated transport in either muscle. These results are in agreement with previously published data (7, 8).

**Akt Kinase Activity**—Stimulation of muscles by insulin resulted in a significant increase in Akt kinase activity in both epitrochlearis (5-fold over basal) and soleus (2-fold over basal) muscles (Fig. 2). Wortmannin completely inhibited the insulin-stimulated increase in both muscles. Muscle contraction did not significantly alter Akt kinase activity in either muscle type.

**Western Blotting of Akt and p70 S6 Kinases**—In response to insulin stimulation, Akt becomes phosphorylated, resulting in a change in its mobility on SDS-PAGE gels (15, 21–23). Insulin stimulation resulted in a shift in mobility of the Akt band to a higher molecular weight in both epitrochlearis and soleus muscles (Fig. 3). In muscles that were stimulated by insulin in the presence of 1 μM wortmannin, this band is absent. Contraction had no effect on the mobility of Akt on a SDS-PAGE gel.
Akt activity was measured in immunoprecipitates from epitrochlearis (B) and soleus (C) muscles that had been incubated in the presence or the absence of 133 nM insulin for 20 min with or without pretreatment for 1 h with 1 μM wortmannin. Additional muscles were contracted in vitro for 20 min as described under “Materials and Methods” with or without pretreatment with 1 μM wortmannin for 1 h. Muscles were frozen in liquid nitrogen at the end of the stimulation period and stored at −80 °C until they were homogenized and centrifuged, and the supernatant was used for immunoprecipitation of Akt with a polyclonal antibody as described under “Materials and Methods.” Kinase assays were performed on the immunocomplexes using histone 2B as a substrate as described under “Materials and Methods.” Values are in relative units with insulin set at 100% and are presented as means ± S.E. The number in parentheses above each bar indicates the number of observations. *, significantly different from basal. †, significantly different from insulin alone. Panel A depicts two representative autoradiograms for epitrochlearis and soleus muscles.

Additionally, Western blotting of soleus muscles showed that the shifted Akt band was recognized by an anti-phospho Akt antibody (Fig. 4). Blotting of muscles for p70 S6 kinase, a putative downstream effector of Akt that is also activated by phosphorylation, showed a pattern of mobility shift in response to insulin similar to that observed for Akt (Fig. 5).

**DISCUSSION**

Insulin transmits its intracellular signals through activation of PI 3-kinase, which is shown to be necessary for insulin activation of glucose transport (6, 24). It remains largely unknown, however, what downstream effector is responsible for the activation of glucose transport by insulin. Several targets have been proposed to mediate this effect of insulin, and most recently attention has focused on the serine-threonine kinase Akt (11, 14, 15, 21, 22). Activity of this kinase has been shown to be regulated by PI 3-kinase through binding of Akt to lipid products of PI 3-kinase and/or increased phosphorylation of Akt on serine residue 473 or threonine residue 308 or through a combination of these events (10, 25, 26).

Muscle contraction, a second potent stimulator of muscle glucose transport, activates transport through a PI 3-kinase-independent mechanism (7, 8, 27, 28). Indeed, much previous evidence has shown that the combined effects of contraction and insulin on glucose transport are additive to one another, which suggests that the two stimuli utilize separate pathways (2, 7, 9, 29, 30). Although muscle contraction and insulin apparently act through different proximal signaling pathways, both affect transport by promoting the translocation of GLUT4 glucose transporters to the plasma membrane, and the combined effects of the two stimuli also produce additive effects on plasma membrane GLUT4 content (3, 5, 8, 31). Moreover, there is some evidence that the two pathways may converge. Henricksen et al. (16) has shown that polymyxin B inhibits stimulation of glucose transport by both insulin and muscle contraction. Additionally, Cartee et al. (32) has shown that verapamil, a calcium channel blocker, inhibited both insulin- and hypoxia-(which mimics the effects of contraction) stimulated skeletal muscle glucose transport. Because previous studies have
shown that activation of Akt elevates glucose transport concomitant with an elevation in surface GLUT4, we hypothesized that Akt may be at this convergence point (11, 14, 15, 21, 22). This model was particularly appealing, because it has been reported that Akt can be stimulated by both PI 3'-kinase-dependent and independent pathways in the same cell type (33–36). Konishi et al. (36) were first to demonstrate that heat shock and hyperosmolarity could activate Akt by a mechanism that, unlike that for PDGF or insulin, was not inhibitable by wortmannin. Interestingly, hyperosmolarity stimulates the translocation of GLUT4 in adipocytes, although it is unlikely that this is mediated by Akt (35, 37). Agents that raise cyclic AMP also stimulate Akt independently of PI 3'-kinase (33, 34).

In contrast to this hypothesis, however, in vitro contractions did not significantly alter Akt kinase activity or phosphorylation state (as ascertained by electrophoretic mobility and reactivity with a phospho-specific antibody) in either soleus or epitrochlearis muscles (Figs. 3 and 4). This occurred despite the fact that contraction significantly elevated the rate of glucose transport in both muscle types (Fig. 1). Further, contraction did not alter phosphorylation of the putative Akt downstream effector, p70 S6 kinase, again measured by electrophoretic mobility (Fig. 5). Thus, these data suggest that activation of Akt is not necessary for stimulation of glucose transport by muscle contraction. Furthermore, they also indicate that if the pathways for stimulation of glucose transport by contraction and insulin converge, they must do so at some step downstream of Akt.

Similar to previous results, insulin increased Akt kinase activity and Akt phosphorylation (21, 22, 38, 39). These increases in Akt activity and phosphorylation state correlated with significant increases in glucose transport in both epitrochlearis and soleus muscles. The fact that wortmannin, an inhibitor of PI 3'-kinase, blocked both the elevations in glucose transport and Akt activity suggests that Akt is a downstream of PI 3'-kinase and may play a role in insulin-stimulated glucose transport in skeletal muscle. Although the present data do not conclusively prove this point, Krook et al. (22) have shown that Akt activity is depressed in muscle from diabetic Goto-Kakizaki rats and that this activity is restored by normalizing the blood glucose of the rat with phlorizin. Taken together, these data suggest that Akt may play a major role in insulin- but not contraction-stimulated glucose uptake in skeletal muscle.

In summary the present study has shown that Akt is activated by insulin but not muscle contraction in isolated skeletal muscle. Further, the increase in Akt activity because of insulin requires activation of PI 3'-kinase and appears to be correlated with phosphorylation at serine 473. Finally, these data together with previous studies support the notion that Akt is involved in the stimulation of skeletal muscle glucose transport by insulin. However, the glucose-stimulatory pathways originating at the insulin receptor and at muscle contraction must converge downstream of the Akt protein kinase.

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FIG. 5. Activation of p70 S6 kinase by insulin in isolated rat skeletal muscle. p70 S6 Western blotting was performed on epitrochlearis (A) and soleus (B) muscles that had been treated as described in the legend to Fig. 2. Muscles were processed as described in Fig. 2 except that 200 µg of crude lysate protein was loaded on a 7.5% SDS-PAGE gels and transferred to PVDF membrane. The membranes were blotted with ptop-p70 S6 kinase antibody, and the p70 S6 kinase bands were visualized with ECL reagents as described under “Materials and Methods.” The blot shown is representative of one experiment that was repeated three times.