Akt signaling in skeletal muscle: regulation by exercise and passive stretch

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First published July 1, 2003; 10.1152/ajpendo.00228.2003.—Akt signaling is a critical pathway for cell survival in multiple systems. Akt activity is increased in response to numerous stimuli, including a wide variety of growth factors and hormones (reviewed in Ref. 13). Almost all reports suggest that Akt activity is controlled by phosphatidylinositol 3-kinase (PI 3-kinase; reviewed in Refs. 36 and 45), through the production of phosphatidylinositol 3,4-bisphosphate and/or phosphatidylinositol 3,4,5-trisphosphate, which recruit Akt from the cytosol to the membrane (reviewed in Refs. 13 and 45). This interaction results in a conformational change in Akt, leading to phosphorylation at Thr308 in the activation loop of the kinase domain by 3-phosphoinositide-dependent protein kinase-1 (1, 6) and Ser473 in the hydrophobic motif at the COOH terminus by a mechanism that is still unclear (5, 19, 27, 42). Several studies have reported that Akt can be activated in some cell systems by mechanisms independent of PI 3-kinase activation, for example, in response to growth hormone treatment (34) and increases in intracellular calcium (49) or cAMP (29). The mechanism for a PI 3-kinase-independent activation of Akt is poorly understood.

We and others have recently reported that Akt activity is increased in response to skeletal muscle contractile activity in the rat (26, 33, 43). In these experiments, contractile activity was produced via direct and can alter rates of protein metabolism, both of which may be responsible for the chronic adaptations in skeletal muscle to repeated bouts of exercise. A central issue in this field is to elucidate the underlying molecular signaling mechanisms that regulate these important metabolic and transcriptional events in contracting skeletal muscle. In recent years, several studies have demonstrated that contractile activity stimulates multiple signaling molecules in skeletal muscle (reviewed in Refs. 32 and 46), but despite these advances, our understanding of the molecular regulation of contraction-stimulated signaling events in skeletal muscle is still in its infancy.

Akt/protein kinase B is a serine/threonine kinase that has emerged as a critical signaling component for mediating numerous cellular responses. Contractile activity has recently been demonstrated to stimulate Akt signaling in skeletal muscle. Whether physiological exercise in vivo activates Akt is controversial, and the initiating factors that result in the stimulation of Akt during contractile activity are unknown. In the current study, we demonstrate that treadmill running exercise of rats using two different protocols (intermediate high or high-intensity exhaustive exercise) significantly increases Akt activity and phosphorylation in skeletal muscle composed of various fiber types. To determine if Akt activation during contractile activity is triggered by mechanical forces applied to the skeletal muscle, isolated skeletal muscles were incubated and passively stretched. Passive stretch for 10 min significantly increased Akt activity (2-fold) in the fast-twitch extensor digitorum longus (EDL) muscle. However, stretch had no effect on Akt in the slow-twitch soleus muscle, although there was a robust phosphorylation of the stress-activated protein kinase p38. Similar to contraction, stretch-induced Akt activation in the EDL was fully inhibited in the presence of the phosphatidylinositol 3-kinase inhibitor wortmannin, whereas glycogen synthase kinase-3 (GSK3) phosphorylation was only partially inhibited. Stretch did not cause dephosphorylation of glycogen synthase on GSK3-targeted sites in the absence or presence of wortmannin. We conclude that physiological exercise in vivo activates Akt in multiple skeletal muscle fiber types and that mechanical tension may be a part of the mechanism by which contraction activates Akt in fast-twitch muscles.

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electrical stimulation (43), nerve stimulation to produce contractions in situ (26, 33), or in isolated skeletal muscles stimulated to contract in vitro (33). In contrast, contractile activity was reported to have no effect on Akt activity or phosphorylation in some early studies (12, 22, 23, 37, 40, 47), suggesting that contraction may regulate Akt in an intensity-, time-, and fiber type-specific manner. In addition to these discrepant findings, there have been no reports showing Akt activation in skeletal muscle exposed to exercise in vivo, raising the possibility that contraction-stimulated Akt activity is an artifact of the contraction model systems. Thus one purpose of the current study was to determine if exercise in vivo increases Akt phosphorylation and activity in skeletal muscle. To this end, rats were subjected to treadmill running exercise with two different intensities of exercise, and Akt phosphorylation and activity were assessed in several hindlimb skeletal muscles composed of different muscle fiber types.

In multiple cell types, externally applied forces such as stretch can activate several signaling pathways, including the mitogen-activated protein (MAP) kinases (9, 25, 30, 48) and Akt (17, 28, 41). Stretch of striated muscle cells in vitro or in vivo results in changes in cellular growth, survival, and metabolism (29, 31, 44, 48), which are similar to the responses to contractile activity in these cells. In the current study, we hypothesized that the mechanical force applied to skeletal muscle during contraction is the initiating signal for the activation of Akt. To test this hypothesis, Akt activity was determined using isolated skeletal muscles treated with passive stretch. In addition, to understand if PI 3-kinase is involved in Akt regulation during stretch, experiments were performed in the absence and presence of the PI 3-kinase inhibitor wortmannin.

EXPERIMENTAL PROCEDURES

Experimental animals. Protocols for animal use were reviewed and approved by the Institutional Animal Care and Use Committee of the Joslin Diabetes Center and were in accordance with National Institutes of Health guidelines. Male Sprague-Dawley rats obtained from Taconic (Germantown, NY) were fed standard laboratory chow and water ad libitum.

Treadmill running exercise and insulin injections. For exercise studies, male Sprague-Dawley rats weighing ~175 g were subjected to either a steady-state or maximal-effort “ramp” treadmill exercise protocol. Steady-state exercise was performed at 20 m/min up a 12% grade for 5, 30, and 60 min, whereas during the maximal-ramp protocol running speed was held constant at 16 m/min, and the incline of the treadmill was increased 1% every 2 min until exhaustion was achieved. Rats were killed by decapitation, and several hindlimb muscles representing various muscle fiber types were quickly dissected, frozen in liquid nitrogen, and stored at ~80°C until processed. The fiber type composition of each muscle studied is as follows: extensor digitorum longus (EDL): type I, 3%; type IIA, 54%; and type IIB, 43%; soleus: type I, 83%; type IIA, 17%; and type IIB, 0%; red gastrocnemius: type I, 28%; type IIA, 63%; and type IIB, 9%; and white gastrocnemius: type I, 0%; type IIA, 13%; and type IIB, 87% (21). For insulin studies, conscious rats were injected with a supraphysiological dose of insulin to achieve maximal activation of Akt in skeletal muscle (20 U/rat ip) and were studied 10 min later.

Isolated muscle contraction and passive stretch in vitro. Rats weighing 60–80 g were killed by cervical dislocation, and EDL and soleus muscles were dissected rapidly. Tendons from both ends of the muscle were tied with suture (silk 4–0) and mounted on an incubation apparatus to maintain resting length. Isolated muscles were preincubated for 20 min in Krebs-Ringer bicarbonate (KRB) buffer (in mM: 117 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 KH₂PO₄, 1.2 MgSO₄, and 24.6 NaHCO₃, pH 7.5) containing 2 mM pyruvate. Pyruvate (2 mM) was always present in the KRB buffer for the entire incubation period. After preincubation, muscles were incubated for an additional 5- to 20-min period for basal, contraction, or stretch treatments. Muscles studied under basal conditions were incubated in KRB buffer. For isolated contractions, muscles were transferred to a supporting apparatus with electrodes (Harvard Apparatus, Holliston, MA) and were stimulated to contract for 10 min (train rate = 2/min, train duration = 10 s, pulse rate = 100 Hz, duration = 0.1 ms). Force production during the contraction protocol was monitored with an isometric force transducer (Kent Scientific, Litchfield, CT) and recorded with a chart recorder (Kipp & Zonen, Delft, Holland). Passive stretch experiments were performed as previously described (9). Briefly, the force transducer was adjusted continuously to maintain muscle length and passive tension for 0.06, 0.12, and 0.24 N within the muscle throughout the protocol. For inhibitor experiments, wortmannin (500 nM) or vehicle (0.1% DMSO) was present throughout the entire incubation period. For both contraction and stretch studies. All buffers were kept at 37°C throughout the experiment and were continuously gassed with 95% O₂–5% CO₂. Immediately after the treatments, muscles were quickly frozen in liquid nitrogen.

Preparation of skeletal muscle tissue lysates. Frozen muscles were pulverized and homogenized with a polytron (Brinkmann Instruments, Westbury, NY) on ice in lysis buffer (20 mM Tris, pH 7.5, 5 mM EDTA, 10 mM Na₂PO₄, 100 mM NaF, 2 mM Na₃VO₄, 1% Nonidet P-40, 10 μM leupeptin, 3 mM benzamidine, 10 μg/ml aprotinin, and 1 mM PMSF). Homogenates were rotated end over end for 10 min at 4°C. Membranes were probed with horseradish peroxidase-conjugated secondary antibodies (1:2,000) in TBST containing 10% BSA (Fro schen Biosciences, CA) or 5% milk or 5% BSA at room temperature. The membranes were incubated in TBST containing either 3% nonfat dry milk or 5% BSA with the indicated antibodies overnight at 4°C. Membranes were probed with horseradish peroxidase-conjugated secondary antibodies (1:2,000) in TBST contain-
ing 5% nonfat dry milk for 1 h at room temperature, and antibody-bound proteins were visualized using an enhanced chemiluminescence system (Perkin-Elmer Life Sciences, Boston, MA). Bands were scanned and quantitated by densitometry (Molecular Dynamics, Sunnyvale, CA).

Akt activity assay. For Akt activity assays, muscle lysates (300–500 μg) were subjected to immunoprecipitation for 4 h at 4°C with 3 μg of anti-Akt antibody raised against residues 1–149 of Akt1, which cross-reacts with both Akt1 and Akt2, or anti-Akt3 antibody coupled to protein G-Sepharose (Amersham Pharmacia Biotechnology). The immune pellets were washed extensively, and in vitro kinase assays were performed as described previously (37) using Akt/SGK peptide (RPRAATF, obtained from Upstate Biotechnology) as substrate.

Glycogen assay. A portion of pulverized muscle was hydrolyzed in 2 N HCl for 2 h at 95°C, neutralized with 2 N NaOH, and assayed spectrophotometrically for glucose content as described previously using a hexokinase-dependent assay kit (8).

Statistics. Data are expressed as means ± SE. Statistical analysis was undertaken using one-way ANOVA. When ANOVA revealed significant differences, further analysis was performed using Tukey’s post hoc test for multiple comparisons. Differences between groups were considered statistically significant at P < 0.05.

RESULTS

Treadmill running exercise activates Akt in rat skeletal muscles. We first determined if Akt activity is increased in response to treadmill running exercise in rat skeletal muscle. Figure 1A shows the time course of the activation of Akt in white gastrocnemius muscle in response to steady-state treadmill running exercise (20 m/min, 12% grade). Akt activity tended to increase with 5 min of exercise and was significantly increased by approximately twofold at 30 min and remained elevated at 60 min. Total Akt protein expression did not change with exercise (data not shown). To compare physiological exercise with insulin, a known potent activator of Akt, a subset of rats was given a supramaximal dose of insulin (20 U/rat ip), and skeletal muscles were obtained 10 min afterward. Compared with basal conditions, insulin treatment resulted in a robust ~12-fold increase in Akt activity (n = 3, data not shown). We also measured Akt3 activity (described under EXPERIMENTAL PROCEDURES) with 30 min of exercise in white gastrocnemius muscle, and no significant activation was observed, whereas insulin injection increased Akt3 activity by 1.5-fold over basal (P < 0.05, n = 3, data not shown).

Skeletal muscles are composed of a heterogeneous population of fiber types that vary according to their contractile and metabolic properties. To test the effects of Akt activity on exercise in different muscle fiber types, we assessed the effects of 30 min of treadmill exercise on Akt activity in several hindlimb skeletal muscles. For this purpose, we compared white gastrocnemius (a predominantly fast-twitch muscle), red gastrocnemius (a mixture of fast- and slow-twitch fibers), and soleus (predominantly slow twitch) muscles. As shown in Fig. 1B, exercise resulted in a significant elevation of Akt activity in all skeletal muscles, with the greatest change occurring in the soleus (2.5-fold above basal), followed by white gastrocnemius (2-fold) and red gastrocnemius (1.7-fold). Skeletal muscle glycogen depletion with exercise was assessed to monitor the recruitment of the different muscle fibers and the severity of exercise. Glycogen content was significantly reduced in all three skeletal muscles in response to 30 min of exercise, and the magnitude of decrease was largest in soleus (65% reduction), intermediate in red gastrocnemius (47% reduction), and minimal in white gastrocnemius (23% reduction).

To determine if the magnitude of Akt activity is dependent on the severity of exercise, rats were subjected to either a 60-min steady-state exercise protocol (20 m/min, 12% grade) or a maximal-effort protocol as described in EXPERIMENTAL PROCEDURES. With the latter protocol, rats exercised for an average of 40 ± 3 min. As shown in Fig. 2, A and B, in the red gastrocnemius muscle, Akt activity and phosphorylation on Thr308 were progressively increased with exercise intensity. Interestingly, although there was a progressive increase in Akt activity in the red gastrocnemius muscle, glycogen depletion was comparable between the two exercise intensities, suggesting that the degree of metabolic stress to the skeletal muscle does not necessarily correlate with the degree of Akt activation. In contrast to the red gastrocnemius muscle, in the fast-twitch glycolytic white gastrocnemius, Akt activity and phosphorylation were similarly increased in response to steady-state exercise and maximal exercise, despite
the fact that glycogen was reduced in an intensity-dependent manner (Fig. 2C). These data demonstrate that, similar to skeletal muscle contractile activity in situ and in vitro, treadmill running exercise increases Akt phosphorylation and activity in multiple muscle types. Exercise intensity and glycogen depletion do not necessarily correlate with Akt activation.

Passive stretch increases Akt activity in isolated EDL muscle. The initiating stimulus for the increase in Akt activity with contractile activity is not known but could be a function of forces applied to the skeletal muscle cell membrane. To determine if external forces applied to skeletal muscle increase Akt activity, we examined the effect of tension generated by passive stretch on Akt activity in isolated skeletal muscles. Figure 3A shows the time course of Akt activation in response to stretch at 0.24 N in the fast-twitch EDL muscle. This force of stretch was chosen because contractions of isolated skeletal muscles typically generate peak forces of ~0.2–0.3 N in a 30-mg muscle and because our previous studies have shown a significant increase in JNK and p38 MAP kinase signaling in skeletal muscles using this system (9). Akt activity was increased by ~1.5-fold with 5 min of stretch and ~2-fold with 10–20 min of stretch. Akt phosphorylation at Thr308 with 10 min of stretch was also increased significantly (data not shown). These results demonstrate that mechanical forces applied to the skeletal muscle in the form of passive stretch can increase Akt activity in EDL muscles and that this occurs in the absence of systemic factors.

We next determined if stretch-induced Akt activation was dose dependent and if this response occurs in a fiber type-specific manner. In the EDL muscles, all three tensions studied (0.06, 0.12, and 0.24 N) significantly increased Akt activity to the same extent (Fig. 3B). Surprisingly, and in contrast to the EDL muscle, passive stretch at all three tensions had no effect on Akt activity in the slow-twitch soleus muscles. To ensure that the stretch stimulus was adequate for both muscles, we measured phosphorylation in p38 Thr180/Tyr182, a molecule previously shown to be robustly activated by passive stretch (9). p38 phosphorylation occurred in both EDL and soleus and, in fact, was much greater in the soleus (Fig. 3C). Thus passive stretch increases Akt activity in EDL but not soleus muscles, demonstrating clear differences in the regulation of Akt activation with fiber types having different metabolic and contractile properties.

Contraction- and stretch-induced Akt activations are wortmannin sensitive. To determine if stretch-stimulated Akt activation is inhibited by wortmannin, isolated EDL muscles were contracted or stretched (0.24 N) for 10 min in the absence or presence of wortmannin (500 nM). Wortmannin decreased basal Akt activity by ~30%. Contraction increased Akt activity by ~80%, and this activation was completely inhibited in the presence of wortmannin. Stretch-stimulated Akt activity was also fully inhibited to basal levels with wortmannin (Fig. 4A). The ability of wortmannin to inhibit Akt activity was not the result of nonspecific effects of the compound, since stretch-stimulated p38 Thr180/
Tyr182 phosphorylation was not inhibited in the presence of wortmannin (Fig. 4B). These results demonstrate that Akt activation by both contraction and passive stretch is inhibitable in the presence of wortmannin.

Effects of wortmannin on stretch-induced GSK3 phosphorylation and glycogen synthase phosphorylation. Glycogen synthase kinase-3 (GSK3) is a well-established substrate of Akt, and Akt-GSK3 signaling through PI 3-kinase has been proposed to be involved in the regulation of glycogen synthase activity in response to insulin (16). We have reported that contraction-induced increases in GSK3 phosphorylation are partially mediated through Akt but that contraction-stimulated Akt activation is not involved in the activation of glycogen synthase (33). Therefore, we examined if stretch has similar effects on GSK3 and glycogen synthase. Similar to contraction, stretch increased GSK3\H9251Ser21 and GSK3\H9252Ser9 phosphorylation (Fig. 5A). Furthermore, both the contraction- and stretch-stimulated increases in GSK3 phosphorylation were partially blunted by wortmannin, which is partially the result of inhibition of basal phosphorylation with wortmannin (Fig. 5, A and B).

We next examined whether the full inhibition of stretch-induced Akt activity and partial inhibition of GSK3 phosphorylation by wortmannin affected the regulation of glycogen synthase. Glycogen synthase phosphorylation was assessed using a phosphospecific

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**Fig. 4.** Effects of wortmannin on in vitro contraction- and stretch-stimulated Akt activity in EDL muscles. Isolated EDL muscles were incubated in the absence (−) or presence (+) of wortmannin (500 nM) for 20 min, and thereafter muscles were rested (Basal), contracted (Contraction), or stretched (Stretch, 0.24 N tension) for 10 min. Wortmannin was present throughout the entire incubation period. A: Akt activity was assayed by in vitro immune-complex assay. B: proteins (60 μg) in muscle tissue lysates were resolved by SDS-PAGE (8%) and immunoblotted with anti-phospho-p38 Thr\H180/Tyr\H182 antibody. Data are means ± SE; n = 4–5/group. *P < 0.05 vs. basal.
GSK3 appear to be critical regulators of glycogen synthase activity.

**DISCUSSION**

Physical exercise causes numerous alterations in cellular metabolism and gene expression, and identification of the intracellular signaling mechanisms responsible for these phenomena has been the focus of intensive research. In the current study, we demonstrate for the first time that the physiological stimulus of treadmill running exercise of rats increases Akt activity and phosphorylation. Interestingly, the time course of activation with treadmill exercise is different compared with in situ contractions elicited by nerve stimulation (33). In situ contractions cause a rapid and transient activation of Akt (33), whereas exercise results in a delayed and prolonged increase in Akt activity. These differences are likely the result of the fact that the extent and pattern of contractile activity are markedly different between the two models of exercise. In the in situ contraction protocol, square-wave electrical stimulation results in an instantaneous, synchronous, and maximal activation of all fibers within a given skeletal muscle, and dramatic metabolic changes can occur within 1 min (2). In contrast, skeletal muscle fiber recruitment during in vivo exercise follows a progressive pattern of activation, with longer periods of exercise necessary to elicit metabolic changes (3, 11, 15). Therefore, the sustained upregulation of Akt activity with physiological exercise may reflect the continuous recruitment of skeletal muscle fibers.

Although Akt activation followed an expected graded response to increasing exercise intensity in the red gastrocnemius, we were somewhat surprised that the increase in Akt activity did not correlate with glycogen depletion during exercise. This suggests that the magnitude of skeletal muscle fiber recruitment is not a major determinant of Akt activity in contracting skeletal muscle. This paradigm is further supported by our finding that Akt was activated to a similar extent by both steady-state and maximal exercise in the white gastrocnemius muscle. Because recruitment of white, fast-twitch muscle fiber recruitment occurs to the greatest extent during maximal and near-maximal exercise (3, 11, 15), taken together, these data suggest that factors other than exercise intensity are primarily responsible for Akt activation during exercise.

The mechanism by which exercise and contractile activity increase Akt activity in skeletal muscle is not known. Because our isolated muscle studies have shown that neural and systemic factors are not necessary to activate Akt in response to contraction in the EDL, it is likely that factors intrinsic to the muscle mediate the activation of Akt during the contraction process. In cultured cells, externally applied forces such as stretch can activate multiple signaling pathways, including MAP kinases (9, 25, 30, 48) and Akt (17, 28, 41). Interestingly, stretch of skeletal muscle cells in vitro or in vivo mimics the effects of contractile activity on cellular growth and metabolism, including...
hypertrophy (44) and enhanced glucose transport (20). Taking these observations into account, in the current study we hypothesized that the mechanical forces applied to skeletal muscle during contractions may be an initiating signal for activation of Akt. In EDL muscles, we observed that Akt activity was increased by passive stretch at the lowest force studied (0.06 N) and that the degree of activation was similar to that elicited by in vitro contractions. Also similar to contraction was the finding that stretch-induced Akt activity is wortmannin sensitive. Stretch-stimulated GSK3 phosphorylation was also increased to a similar extent to that with contraction in the EDL, and this phosphorylation was partially inhibited with wortmannin. These data raise the possibility that contraction and stretch regulate Akt and GSK3 by a common mechanism in the fast-twitch EDL muscle. Although we clearly demonstrated that passive stretch can increase Akt activity and GSK3 phosphorylation, one question not resolved is how the mechanical stress is converted to chemical signaling within the skeletal muscle. Some studies have shown that stretch increases intracellular calcium through stretch-activated calcium channels (28). Another possibility is that stretch activates a receptor tyrosine kinase, which in turn increases Akt activity by a PI 3-kinase-dependent mechanism (41). However, it seems unlikely that receptor tyrosine kinases are involved in the activation of contraction-stimulated Akt activation, since PI 3-kinase activity associated with tyrosine-phosphorylated proteins does not increase with exercise or contractile activity (18, 33, 50).

In contrast to our findings in the fast-twitch EDL muscle, in the slow-twitch soleus there was no detectable change in Akt activity in response to stretch. Interestingly, contraction of isolated soleus muscles is also ineffective in increasing Akt activity (33). These data provide evidence that the different contractile and/or metabolic properties of slow-twitch and fast-twitch fibers confer distinct signaling properties to various skeletal muscles. The lack of effect of contraction and stretch on Akt activity in isolated soleus muscles is also important in understanding mechanisms of exercise-regulated Akt activity. Because exercise in vivo increases Akt activity in the soleus, it is likely that systemic factors and/or oxidative stress play a role in exercise-induced Akt activity. Although the specific factor for this regulation is not known, H2O2 is known to be a potent activator of Akt (7, 35), and physical exercise increases the production of H2O2 from mitochondria (14).

In summary, we demonstrate that physiological treadmill running exercise in vivo activates Akt in multiple skeletal muscle fiber types. We also demonstrate that passive stretch in isolated EDL muscles in vitro activates Akt to a similar extent as muscle contractions, suggesting that mechanical forces applied to fast-twitch muscles during exercise may explain, at least in part, the activation of Akt. On the other hand, there are clear differences in the mechanisms for exercise-induced Akt activation in slow-twitch soleus muscles, since contractile activity does not increase Akt activity in the absence of the normal physiological milieu. A growing body of evidence now suggests that Akt is a critical signaling mediator for cellular growth and metabolism in skeletal muscle, and the level of contractile activity in the skeletal muscle is undoubtedly fundamental to this regulation.

**DISCLOSURES**

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