Are tyrosine kinases involved in mediating contraction-stimulated muscle glucose transport?

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Wright, David C., Paige C. Geiger, Dong-Ho Han, and John O. Holloszy. Are tyrosine kinases involved in mediating contraction-stimulated muscle glucose transport? Am J Physiol Endocrinol Metab 290: E123–E128, 2006. First published September 13, 2005; doi:10.1152/ajpendo.00280.2005.—Muscle contractions and insulin stimulate glucose transport into muscle by separate pathways. The contraction-mediated increase in glucose transport is mediated by two mechanisms, one involves the activation of 5′-AMP-activated protein kinase (AMPK) and the other involves the activation of calcium/calmodulin-dependent protein kinase II (CAMKII). The steps leading from the activation of AMPK and CAMKII to the translocation of GLUT4 to the cell surface have not been identified. Studies with the use of the tyrosine kinase inhibitor genistein suggest that one or more tyrosine kinases could be involved in contraction-stimulated glucose transport. The purpose of the present study was to determine if tyrosine kinases were involved in contraction-stimulated glucose transport in rat soleus and epitrochlearis muscles. Contraction-stimulated glucose transport was completely prevented by pretreatment with genistein (100 µM) and the related compound butein (100 µM). However, the structurally distinct tyrosine kinase inhibitors 4-amino-5-(4-chlorophenyl)-7-[(t-buty]pyrazolo[3,4-d]pyridine and herbimycin did not reduce contraction-stimulated glucose transport. Furthermore, genistein and butein inhibited glucose transport even when muscles were exposed to these compounds after being stimulated to contract. Muscle contractions did not result in increases in tyrosine phosphorylation of proteins such as proline-rich tyrosine kinase and SRC. These results provide evidence that tyrosine kinases do not mediate contraction-stimulated glucose transport and that the inhibitory effects of genistein on glucose transport result from direct inhibition of the glucose transporters at the cell surface.

5-aminoimidazole-4-carboxamide ribonucleoside; exercise; glucose transporter 4; hypoxia

EXERCISE AND INSULIN STIMULATE GLUCOSE TRANSPORT BY SEPARATE PATHWAYS (17). Both 5′-AMP-activated protein kinase (AMPK; see Refs. 4, 14, 19, 22, 24) and activation of calcium/calmodulin-dependent protein kinase (CAMKII) (32) by increases in cytosolic Ca2+ (33) are involved in mediating the stimulation of muscle glucose uptake by contractions.

The signaling mechanisms linking the activation of AMPK and/or CAMK to increases in skeletal muscle glucose transport are not known. However, a number of lines of evidence raised the possibility that activation of one or more tyrosine kinases might be involved in the pathways leading from muscle contractions to an increase in muscle glucose transport. In support of this possibility, it has been reported that activation of AMPK with 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) results in activation of the proline-rich tyrosine kinase PYK2 in rat skeletal muscle and that the tyrosine kinase inhibitor genistein prevents stimulation of glucose transport by AICAR (7). These investigators concluded that the effects of exercise that are dependent on AMPK are mediated by PYK2. Additionally, stimulation of glucose transport and GLUT4 translocation by osmotic shock in adipocytes and L6 myotubes is prevented by genistein (6, 18, 28). It has also been shown that Iomoincyt, a compound that increases cytosolic Ca2+, causes an increase in the phosphorylation of PYK2 in vascular smooth muscle (11). In this context, the purpose of the present study was to determine if tyrosine kinases are involved in the signaling pathway through which muscle contractions result in increases in glucose transport.

MATERIALS AND METHODS

Materials. 2-Deoxy-[1,2-3H]glucose (2-DG) was purchased from American Radiolabeled Chemicals (St. Louis, MO). [14C]mannitol was obtained from ICN Radiochemicals (Irvine, CA). Genistein, 4-amino-5-(4-chlorophenyl)-7-[(t-buty]pyrazolo[3,4-d]pyridine (PP2), and herbimycin were obtained from Calbiochem (La Jolla, CA). An antibody that detects tyrosine-phosphorylated proteins (PY99) was purchased from Sigma (St. Louis, MO). The horseradish peroxidase-conjugated donkey anti-rabbit IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Anti-phospho-PYK2 (tyrosine 402) and anti-phospho-SRC (tyrosine 416) antibodies were purchased from Biosource International (Camarillo, CA) and Cell Signaling (Beverly, MA), respectively. SRC is a tyrosine kinase whose activation is required for increases in PYK2 activity. Enhanced chemiluminescence (ECL) reagents were obtained from Amersham (Arlington Heights, IL). All other chemicals were obtained from Sigma.

TREATMENT OF RATS AND MUSCLE PREPARATIONS. Male Wistar rats (Charles River) weighing 80–120 g were provided with Purina Rat Chow and water ad libitum. Food was removed at 5:00 PM the evening before the experiment. Rats were anesthetized by an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body wt) followed by the removal of the epitrochlearis and soleus muscle for the determination of 2-DG uptake (16, 34). Soleus muscles were split longitudinally into strips before incubation to allow adequate diffusion of oxygen and substrates (16). All protocols were approved by the Animal Studies Committee of Washington University.

Muscle treatments. After dissection, muscles were allowed to recover for 60 min in flasks containing 2 ml of Krebs-Henseleit bicarbonate buffer (KHB) with 8 mM glucose, 32 mM mannitol, and a gas phase of 95% O2-5% CO2. For evaluation of the effect of hypoxia, muscles were incubated for 90 min in KHB that had been gassed with 95% N2-5% CO2, as this has been shown to induce the maximal effect of hypoxia on glucose transport.

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transport (5). Before the determination of glucose transport, hypoxia-treated muscles were incubated in vials containing oxygenated KHB containing 2 mM sodium pyruvate and 36 mM mannitol for 10 min at 30°C to allow for the resynthesis of ~P and to remove glucose from the extracellular space (5).

Other muscles were stimulated to contract using a Grass SII stimulator as described in detail previously (15). Tetanic contractions

Fig. 1. Effect of pretreatment with genistein or butein on the stimulation of 2-deoxy-[1,2-3H]glucose (2-DG) transport by contractions or hypoxia in rat epitrochlearis (A) and soleus (B) muscles. Muscles were exposed to genistein (100 μM) or butein (100 μM) for 60 min before and during muscle contractions or hypoxia, followed by measurement of 2-DG transport in the muscles, as described in MATERIALS AND METHODS. Values are means ± SE for 6–26 muscles/group. *P < 0.01 vs. all other groups.

Fig. 2. Herbimycin (50 μM) and 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyridine (PP2, 10 μM) inhibit insulin- but not contraction-stimulated 2-DG uptake. A: isolated rat epitrochlearis muscles were incubated with herbimycin or PP2 for 60 min before and during muscle contractions followed by measurement of 2-DG transport. Values are means ± SE for 6–15 muscles/group. *P < 0.01 vs. all other groups. B: soleus muscle strips were incubated with herbimycin (50 μM) or PP2 (10 μM) for 60 min before and during treatment with a maximally effective concentration of insulin (2 mU/ml) for 30 min. Values are means ± SE for 7–8 muscles/group. *P < 0.05 vs. basal. #P < 0.05 vs. insulin. C: PP2 inhibits basal phosphorylation of SRC. Muscles were incubated for 1 h before and during 10 in vitro tetanic muscle contractions. Values are means ± SE for 4 muscles/group. *P < 0.05 vs. all other groups.
were produced by stimulation at 100 Hz with 0.2-ms pulses for 10 s at a rate of one contraction per minute for 10 min. In some experiments, the tyrosine kinase inhibitors genistein (100 μM), butein (100 μM), PP2 (50 μM), or herbimycin (100 μM) were included in the incubation medium; in these experiments, the muscles were incubated with or without the inhibitor for 60 min before as well as during contractions or hypoxia. In experiments in which the effect of genistein on glucose transporter function was evaluated, muscles were first stimulated to contract in vitro or made hypoxic and then exposed to genistein only during the determination of glucose transport. Because these inhibitors are light sensitive, the flasks were wrapped in foil.

Measurement of glucose transport activity. Glucose transport activity was measured using 2-DG (13). After the various treatments, the muscles were rinsed for 10 min at 29°C in 2 ml of oxygenated KHB containing 40 mM mannitol to remove glucose and treatment agents. After the rinse step, muscles were incubated for 20 min at 29°C in flasks containing 2 ml of KHB with 4 mM 2-DG (1.5 μCi/ml) and 36 mM [14C]mannitol (0.2 μCi/ml) and in some experiments 100 μM genistein or butein, with a gas phase of 95% O2-5% CO2, in a shaking incubator. The muscles were then blotted, clamp-frozen, and processed for determination of intracellular 2-DG accumulation and extracellular space (34).

Western blot analysis. Isolated rat epitrochlearis muscles were stimulated to contract in vitro as described above and freeze-clamped immediately after the last contraction. Muscles were homogenized 10:1 (volume-to-weight ratio) in modified RIPA buffer, and protein concentration was determined by the method of Lowry et al. (20). Western blot analysis, as described previously (32), was used for the determination of contraction-induced changes in the phosphorylation status of PYK2 and SRC. Anti-phospho-PYK2 (tyrosine 402), anti-phospho-SRC (tyrosine 416), and PY99, an antibody that detects tyrosine-phosphorylated proteins, primary antibodies were used at a concentration of 1:1,000. Bands were visualized via ECL.

Statistical analysis. Data are presented as means ± SE. Comparisons between the means of multiple groups were made using one-way ANOVA followed by a post hoc comparison using Fisher’s least significant difference method.

RESULTS

Genistein and butein inhibit stimulation of glucose transport by contractions and hypoxia. Genistein is a tyrosine kinase inhibitor that has been shown to inhibit the phosphorylation of receptor and SRC tyrosine kinases with similar effectiveness (2, 10). The inhibition is competitive with respect to ATP and noncompetitive with respect to the phosphate acceptor (2). Butein, an isoflavone related to genistein, inhibits receptor tyrosine kinase autophosphorylation and the activation of SRC tyrosine kinases through a similar mechanism as genistein (34). The inhibitory effects of genistein and butein appear to be specific to tyrosine kinases, since these compounds, at concentrations that inhibit tyrosine kinase activity, have no effect on protein kinase C (2, 34), cAMP-dependent protein kinase (2, 34), or AMPK activity (Wright, Han, and Holloszy, unpublished observation).

As shown in Fig. 1, contraction stimulated increases in glucose transport in rat fast-twitch epitrochlearis, and slow-twitch soleus muscles were completely prevented by the tyrosine kinase inhibitor genistein (100 μM) and the related inhibitor butein (100 μM). We were unable to detect any changes in tension development in the presence of these inhibitors (data not shown). Hypoxia is thought to stimulate glucose transport through a similar signaling mechanism as muscle contractions (5). Genistein and butein also blocked hypoxia-induced increases in 2-DG uptake in both rat epitrochlearis and soleus muscles (Fig. 1). AICAR induced an approximately threefold increase in 2-DG uptake in rat epitrochlearis muscles (basal 0.49 ± 0.05 μmol·ml⁻¹·20 min⁻¹, AICAR 1.44 ± 0.10 μmol·ml⁻¹·20 min⁻¹). Treatment with genistein (0.30 ± 0.06 μmol·ml⁻¹·20 min⁻¹) and butein (0.37 ± 0.05 μmol·ml⁻¹·20 min⁻¹) prevented the AICAR-induced increase in 2-DG uptake. Similarly, caffeine (3.5 mM)-stimulated (basal

![Fig. 3. Muscle contractions do not cause detectable increases in the tyrosine phosphorylation of proteins when probing with PY99, an antibody that detects tyrosine-phosphorylated proteins (A), or SRC and proline-rich tyrosine kinase (PYK2) phosphorylation (B). Rat epitrochlearis muscles were stimulated to contract in vitro and clamp-frozen immediately after 2, 4, or 8 contractions. Quantified data are presented as means ± SE for 4 muscles/group.](image-url)
0.35 ± 0.06 μmol·ml⁻¹·20 min⁻¹, caffeine 1.01 ± 0.14 μmol·ml⁻¹·20 min⁻¹) 2-DG uptake was blocked by both genistein (0.43 ± 0.13 μmol·ml⁻¹·20 min⁻¹) and butein (0.27 ± 0.06 μmol·ml⁻¹·20 min⁻¹) in isolated rat epitrochlearis muscle.

PP2 and herbimycin do not inhibit contraction-stimulated glucose transport. PP2 and herbimycin are tyrosine kinase inhibitors that inhibit SRC and receptor tyrosine kinase activity (9, 27, 29). The pyrazolopyrimidine compound PP2 inhibits tyrosine kinase activity by binding to a cleft between the NH₂- and COOH-terminal lobes in an area normally occupied by ATP (10). Herbimycin inhibits kinase function through an irreversible binding to sulfhydryl groups of the kinase (29).

Treatment of isolated rat epitrochlearis muscles before and during in vitro tetanic muscle contractions with the tyrosine kinase inhibitors PP2 or herbimycin did not inhibit contraction-stimulated glucose transport in epitrochlearis muscles (Fig. 2). Similarly, herbimycin had no effect on hypoxia or AICAR-stimulated glucose transport in rat epitrochlearis muscles (data not shown). To determine if the lack of an inhibitory effect on glucose transport by PP2 and herbimycin was the result of the compounds not entering the muscle, we tested the effects of these agents on insulin-stimulated 2-DG uptake. As seen in Fig. 2, both PP2 and herbimycin significantly attenuated insulin-stimulated 2-DG uptake. To further determine if the tyrosine kinase inhibitors were entering the muscle fibers, epitrochlearis muscles were treated with PP2 and stimulated to contract. Although muscle contractions did not result in increases in SRC phosphorylation, basal levels of phosphorylation were significantly reduced by PP2 treatment.

Contractions do not increase protein tyrosine phosphorylation. To determine if muscle contractions induce increases in the phosphorylation of SRC or PYK2, rat epitrochlearis muscles were stimulated to contract in vitro, and muscles were clamp-frozen after two, four, or eight in vitro tetanic muscle contractions. At all time points tested, we were unable to detect contraction-induced increases in the phosphorylation of PYK2 or of the nonreceptor tyrosine kinase SRC in rat epitrochlearis or a consistent pattern of contraction-induced increases in tyrosine-phosphorylated proteins when probing with PY99, an antibody that detects tyrosine-phosphorylated proteins (Fig. 3).

Genistein and butein inhibit glucose transport after activation by contractions or hypoxia. Because we did not detect contraction-induced increases in the phosphorylation of tyrosine kinases, or the inhibition of glucose transport with the tyrosine kinase inhibitors herbimycin A and PP2, we evaluated the possibility that the inhibitory effect of genistein and butein was due to a direct inhibition of GLUT4 transporter activity. Isolated rat soleus and epitrochlearis muscles were stimulated to contract, or made hypoxic, and then exposed to genistein or butein for 20 min during the determination of 2-DG uptake. As shown in Fig. 4, genistein inhibited (i.e., reversed) hypoxia and contraction-induced increases in 2-DG uptake in both epitrochlearis and soleus muscles when present only during the mea-
sured of 2-DG uptake. A similar, but slightly less robust, inhibitory effect was seen with butein.

Genistein and butein inhibit basal glucose transport. Isolated rat soleus and epitrochlearis muscles were treated with genistein and butein for 1 h before the determination of 2-DG uptake. As seen in Fig. 5, both compounds significantly inhibited basal levels of glucose transport.

DISCUSSION

Muscle contractions stimulate glucose transport through a mechanism that is distinct from that of insulin (17). Accumulating evidence suggests that both the activation of AMPK and increases in cytosolic Ca\(^{2+}\) followed by the activation of CAMKII mediate contraction-stimulated increases in glucose transport (14, 22, 24, 32, 33). In support of this possibility, we found that the increases in glucose transport induced by a subcontraction concentration of caffeine, a sarcoplasmic reticulum Ca\(^{2+}\)-releasing agent, and by AICAR, a compound that activates AMPK, are additive and not different from that induced by maximally effective contractile activity in rat fast-twitch epitrochlearis muscle (32). These results are consistent with the finding by Mu et al. (23) that expression of a dominant inhibitory mutant of AMPK in mouse muscle resulted in only an ~30 to 40% decrease in contraction-stimulated glucose transport.

The activation of CAMKII, the CAMK isofrom found in skeletal muscle (26), appears to mediate the effect of increases in cytosolic Ca\(^{2+}\) concentration on glucose transport. This is evidenced by the findings that muscle contractions and subcontraction concentrations of caffeine cause increases in CAMKII phosphorylation and that the specific CAMK inhibitors 1-[\(N\)-bis-(5-isoquinolinesulfonyl)-\(N\)-methyl-l-tyrosyl]-4-phenylpiperazine (KN62) and 2-\(N\)-(2-hydroxyethyl)-\(N\)-(4-methoxybenzenesulfonyl)amino-\(N\)-cholorocinnamyl]-\(N\)-methylbenzylamine (KN93; see Ref. 8) prevent caffeine-induced increases in 2-DG uptake (32).

To date, the steps in the signaling pathways located downstream of AMPK and CAMK that mediate contraction-stimulated glucose transport have not been identified. However, a number of studies raise the possibility that a tyrosine kinase might be involved. Chen et al. (7) reported that activation of AMPK with AICAR results in activation of PYK2 in rat skeletal muscle and that genistein prevents AICAR stimulation of glucose transport. Also GLUT4 translocation and stimulation of glucose transport by osmotic shock in adipocytes and L6 myotubes is inhibited by genistein (6, 18, 28).

These findings led us to test the hypothesis that tyrosine kinases are involved in mediating contraction-induced glucose transport. Our initial results suggested that this hypothesis was correct. However, our subsequent findings argue against this idea. Although genistein and butein blocked contraction- and hypoxia-induced increases in glucose transport in both fast- and slow-twitch rat skeletal muscle, a similar inhibitory effect was seen when muscles were exposed to these agents after the activation of the glucose transport process. These results are compatible with the previous finding in 3T3-L1 adipocytes that genistein treatment after insulin stimulation resulted in a complete inhibition of 2-DG uptake (3). Interestingly, a similar nonspecific inhibition of GLUT4 has recently been reported with the p38 mitogen-activated protein kinase inhibitor SB-203580 (2, 25). In agreement with previous studies that found an inhibitory effect of genistein on GLUT1, the glucose transporter isoform that primarily mediates basal glucose transport (1, 21, 30), we found that genistein and butein partially inhibited basal 2-DG uptake. Taken in concert, these findings suggest that the inhibitory effect of isoflavone compounds on glucose transport is the result of a direct binding to, and inhibition of, the activity of the glucose transporters at the cell surface. Because both genistein and butein inhibited basal and contraction/ hypoxia-stimulated glucose transport, it appears that both GLUT4 and GLUT1 are affected by these compounds.

Further evidence arguing against the involvement of tyrosine kinases in contraction-stimulated glucose transport comes from the finding that PP2 and herbimycin, tyrosine kinase inhibitors that are not structurally or mechanistically similar to butein and genistein, had no inhibitory effect on contraction- or hypoxia-stimulated glucose transport. Finally, in vitro tetanic muscle contractions of the rat epitrochlearis did not result in increases in the phosphorylation of PYK2 or SRC, a tyrosine kinase in which activation is required for increases in PYK2 activity. This finding is consistent with a report by Goodyear et al. (12) that contractions do not increase tyrosine phosphorylation in rat skeletal muscle and with a study by Widegren et al. (31) in which cycle ergometer exercise did not result in increases in the phosphorylation of PYK2.

In conclusion, the results of the present study demonstrate that, although the isoflavone compounds genistein and butein inhibit contraction-stimulated glucose transport, this effect appears to be mediated through an inhibition of glucose transporter function. Our results also argue against the hypothesis that tyrosine kinase activation is involved in contraction-stimulated glucose transport.

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