Differential regulation of MAP kinase by contraction and insulin in skeletal muscle: metabolic implications

JØRGEN F. P. WOJTASZEWSKI,1,2 JAN LYNGE,1 ALLAN B. JAKOBSEN,1 LAURIE J. GOODYEAR,2 AND ERIK A. RICHTER1

1Copenhagen Muscle Research Centre, August Krogh Institute, Copenhagen University, DK-2100 Copenhagen, Denmark; and 2Research Division, Joslin Diabetes Center, and Department of Medicine, Brigham and Women’s Hospital and Harvard Medical School, Boston, Massachusetts 02215

Wojtaszewski, Jørgen F. P., J an Lynge, Allan B. Jakobsen, Laurie J. Goodyear, and Erik A. Richter. Differential regulation of MAP kinase by contraction and insulin in skeletal muscle: metabolic implications. Am. J. Physiol. 277 (Endocrinol. Metab. 40): E724–E732, 1999.—We have investigated the activation of the extracellular signal-regulated kinases (ERK1 and ERK2) by muscle contraction and insulin in perfused rat skeletal muscle. Both stimuli activated ERK1 and ERK2 by an upstream kinase MAP/ERK kinase (MEK)-dependent mechanism, as the MEK inhibitor PD-98059 inhibited ERK phosphorylation. The presence of the phosphatidylinositol (PI) 3-kinase inhibitors LY-294002 and wortmannin totally eradicated ERK1 and ERK2 phosphorylation in response to insulin but not contraction. Insulin and muscle contraction activated muscle glucose transport, glycogen synthase, and amino acid transport independently of ERK signaling, whereas the PI 3-kinase inhibitors abolished the stimulatory effects of insulin but not those of contraction on these three cellular processes. We conclude that 1) insulin and contraction activate ERK signaling in skeletal muscle, 2) ERK signaling is not necessary for activation of glucose and amino acid transport or glycogen synthase activity by contraction and insulin in skeletal muscle; and 3) insulin-induced activation of MEK, the upstream activator of ERK, is dependent on PI 3-kinase, whereas contraction utilizes a different mechanism.

glucose and amino acid transport; glycogen synthase; phosphatidylinositol 3-kinase; extracellular signal-regulated kinase; MEK; PD-98059; LY-294002; wortmannin

THE MITOGEN ACTIVATED PROTEIN (MAP) kinase signaling pathways consist of at least three parallel cascades, including the extracellular signal-regulated kinase (ERK), the c-jun NH2-terminal kinase (JNK), and the p38 kinase pathway. All of these cytosolic signaling proteins require dual phosphorylation on threonine and tyrosine residues for their activation (16, 29, 45). Muscle contraction and exercise in vivo are potent activators of these signaling cascades in both rodent and human skeletal muscle (2, 3, 18). Insulin is also an activator of the ERK signaling cascade (18, 23, 35, 59), whereas its action on JNK and the p38 kinase in rodent skeletal muscle is still unclear (18, 35). The ERK proteins are phosphorylated and thus activated by the upstream kinase MAP/ERK kinase (MEK). Insulin regulation of MEK activity is presumed to involve the activation of the insulin receptor tyrosine kinase, provoking Shc-Grb2-SOS complex formation, which, in turn, activates Ras and Raf (reviewed in Refs. 14, 51). Exercise also activates Raf and MEK in human skeletal muscle (3), and these molecules may therefore be shared upstream signaling elements for the activation of the ERK proteins by insulin and muscle contraction. In the present study, we investigated this possibility in various muscle fiber types of the perfused rat hindlimb, with the MEK-specific inhibitor PD-98059. Furthermore, the fungal toxin wortmannin inhibits the insulin activation of ERK signaling in a variety of cultured cell systems (11, 48, 53). Whether this also occurs in insulin-responsive mammalian tissues is unknown. Thus we investigated whether insulin- and contraction-induced ERK activation was sensitive to inhibition by both wortmannin and LY-294002, two structurally unrelated phosphatidylinositol (PI) 3-kinase inhibitors (37, 38, 52).

Activated ERK proteins mediate the phosphorylation of the p90 ribosomal S6 kinase (p90RSK). Both the ERK and p90RSK translocate to the nucleus, promoting phosphorylation of transcription factors such as c-myc, c-fos, and Elk-1 (45). Therefore, a regulatory role of gene transcription has long been assigned to this activation of the ERK signaling cascade by insulin, other growth factors, and perhaps also by muscle contractile activity. However, it is unknown whether the potent activation of ERK signaling by muscle contractile activity has a regulatory role in any of the acute metabolic changes observed in response to contractile activity. There is now substantial evidence indicating that contraction/exercise and insulin utilize different signaling mechanisms in the stimulation of glucose transport. Insulin stimulates skeletal muscle glucose transport by an insulin receptor, insulin receptor substrate, PI 3-kinase, and perhaps an Akt-dependent signaling pathway (reviewed in Refs. 9, 13, 51), whereas the mechanisms utilized by muscle contractile activity do not involve these signaling molecules (22, 33, 54). In addition, insulin activation of glycogen synthase may be P70S6K and/or glycogen synthase kinase 3 dependent but is independent of ERK (5, 8, 12, 46). The signaling mechanisms utilized by muscle contraction for activation of these metabolic processes are still unresolved. Therefore, another goal of the present study was to clarify whether contraction-induced glucose and amino acid transport and glycogen synthase activity are mediated by ERK signaling.
MATERIALS AND METHODS

All experiments were approved by the Danish Animal Experimental Inspectorate and complied with the European Convention for the Protection of Vertebrate Animals Used for Experiments and Other Scientific Purposes (Council of Europe no. 123, Strasbourg, France, 1985). Male Wistar rats weighing 200–250 g were maintained on a constant 12:12-h light-dark cycle and received normal rat chow and water ad libitum.

Surgery

The rats were anesthetized by an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body wt) and prepared surgically for hindquarter perfusion, as described by Ruderman et al. (43). In some experiments, perfusion of only one hindlimb was carried out and therefore the contralateral common iliac artery and vein were ligated.

Perfusion Medium

All perfusions were performed with a cell-free perfusate as previously described (55). The perfusate consisted of Krebs-Henseleit buffer solution and 0.1% BSA (fraction V, Sigma, St. Louis, MO) dialyzed for 24 h against 11 vol of Krebs-Henseleit buffer solution (pore size 10–15 kDa), 0.15 mM pyruvate, and 4.2 IU/ml heparin. During the hindlimb perfusion, the arterial perfusate was continuously gassed with a mixture of 95% oxygen and 5% carbon dioxide. Perfusate PO2, PCO2, and pH were determined within 20 min in perfusate samples obtained anaerobically (ABL 510 acid base analyzer, Radiometer, Copenhagen, Denmark). Measurements of arterial perfusate were performed on average a pH of ~7.4, a PCO2 of ~36.0 mmHg, and a PO2 of ~540 mmHg. The temperature of the perfusate was 35°C, which results in a muscle temperature in the calf muscles of ~32°C (55).

Hindlimb Perfusion

The first 25 ml of perfusate that passed through the hindquarter was discarded, whereupon the perfusate was recirculated at a flow of either 15 or 20 ml/min during perfusion of one or both hindlimbs, respectively. All perfusions consisted of 25 min of preperfusion before any stimulation was initiated.

Muscle contraction. Calf muscles were contracted isometrically by stimulation of the sciatic nerve with supramaximal (-25 V) trains of 200 ms delivered every 1 s for various time points as described previously (55). The impulse frequency and duration within the train were 100 Hz and 0.1 ms, respectively. Only one leg was perfused in these experiments, and during electrical stimulation flow was 20 ml/min. Tension developed by the calf muscle was measured by an isometric muscle tensiometer and recorded by a pen writer.

Insulin stimulation. Perfusion of the hindlimb in the rested nonstimulated (basal) or insulin-stimulated state was carried out with perfusions of both hindlimbs. When insulin stimulation was applied, human insulin (Actrapid, Novo Nordisk, Bagsvaerd, Denmark) was added after 25 min of preperfusion, yielding a perfusate concentration of 20,000 µU/ml. Insulin stimulation continued for 20 min before any measurements were performed.

Kinase inhibitors. The phosphorylation and therefore the activation of the ERK isoforms [ERK1 (p44) and ERK2 (p42)] was inhibited by adding the MEK inhibitor PD-98059 (2′-amino-3′-methoxyflavone; 10–50 µM). For inhibition of PI 3-kinase, two structurally unrelated compounds with different modes of action, wortmannin and LY-294002, were used (37, 38, 52). When an inhibitor was used, it was added to the perfusate at the beginning of the perfusion. Because the inhibitors were dissolved in DMSO, the perfusate of the corresponding control experiments contained the same concentration of DMSO (1:1,000 vol/vol). All inhibitors were from Alexis (Lifefingen, Switzerland). As shown previously (55), the inhibitors used did not affect force developed by the calf muscles during electrical stimulation or oxygen uptake of the hindlimb during any intervention. In addition, muscle biopsies did not display changes in total muscle water content or extracellular space due to the presence of the inhibitors (data not shown).

Muscle Glucose Transport

To estimate muscle membrane glucose transport, the muscle uptake of 8 mM 2-deoxy-D-[2,6-3H]glucose (2-DG; 57 Ci/mmol) in relation to the uptake of 1 mM D-[1-14C]mannitol (58 mCi/mmol; New England Nuclear, Boston, MA) was measured over a period of 30 (basal) or 5 min (electrical stimulation and insulin) as previously described (56). At the end of perfusion, muscle biopsies were taken from three different portions of the calf muscle: the superficial medial portion of the gastrocnemius [white gastrocnemius (WG), consisting mainly of fast-twitch glycolytic fibers], the soleus muscle (SOL; consisting mainly of slow-twitch oxidative fibers), and the deep medial portion of the gastrocnemius muscle [red gastrocnemius (RG), consisting mainly of fast-twitch oxidative fibers; Ref. 1]. The biopsies were cut out, trimmed of connective tissue, blotted, freeze-clamped with aluminum clamps cooled in liquid N2, and stored at −80°C until analyzed. Radioactivity was measured in perchloric acid extracts with a liquid scintillation counter (Packard Instruments, Downers Grove, IL). Muscle 2-DG uptake was calculated as described previously (56).

Muscle Amino Acid Transport

Muscle amino acid transport was estimated with the amino acid transporter system A specific substrate α-[1-14C](methyl amino)isobutyric acid (MeAIB, 56 mCi/mmol) and the extracellular marker D-[1-1H(N)]mannitol (22.5 Ci/mmol; American International, Arlington Heights, IL). The amino acid transport measurement was initiated by the addition of a mixture of cold and radiolabeled MeAIB and D-mannitol to the perfusate, yielding a final concentration of 0.5 mM of both MeAIB (0.08 mCi/ml) and D-mannitol (0.08 mCi/ml), and transport was measured over a period of 10 min.

Muscle Glycogen Synthase

Glycogen synthase activity was determined in soleus and red and white gastrocnemius muscle by a modification of the method by Thomas et al. (50) as described by Richter et al. (42). The activity is given as fractional velocity determined as the ratio (in %) of the glycogen synthase activity in the presence of 0.17 mM glucose 6-phosphate compared with the activity in the presence of 8 mM glucose 6-phosphate.

ERK Proteins and ERK Phosphorylation

Portions of soleus and red and white gastrocnemius muscle, weighing 30–40 mg, were homogenized in a HEPES buffer (pH 7.4) containing 1% Nonidet P-40, and insoluble material was removed by centrifugation as previously described (55). Protein concentration in the supernatant was measured with bicinchoninic acid protein assay reagent, by use of a microtiter plate protocol at 37°C for 30 min (Pierce Chemical, Rockford, IL). Muscle protein was resolved by 8 or 10%
SDS-PAGE and transferred to an Immobilon-P membrane with a semidy transfer apparatus (Pharmacia, Piscataway, NJ). The membrane was blocked with 5% fat-free milk in a 10 mM Tris-base buffer containing 0.9% NaCl (pH 7.4) (TSM buffer) and then incubated overnight at 4°C with primary antibody in TSM buffer. The primary antibody was either a rabbit anti-ACTIVE MAPK pAb (250 ng/ml; Promega, Madison, WI), which only recognizes the dually phosphorylated (or active) form of the ERK1 and ERK2, a rabbit anti-MAP-kinase (AB-2; Calbiochem, Cambridge, MA), which recognizes both ERK1 and ERK2 isoforms, or a phosphospecific Akt antibody that recognizes Akt only when phosphorylated on SER473 (New England Biolabs, Boston, MA). The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies in TSM buffer and immunocomplexes were visualized by enhanced chemiluminescence (Amersham International). Multiple exposures were obtained on Hyperfilm-enhanced chemiluminescence film, and densitometric scanning was performed by a Phosphor mager (Molecular Dynamics, Sunnyvale, CA). To account for gel-to-gel variation, control samples were included on all gels and data are expressed relative to these.

Chemicals

Unless stated otherwise, chemicals were all of analytical grade from Sigma.

Statistics

Data are expressed as means ± SE. Statistical evaluation was done by ANOVA. When ANOVA revealed significant differences, the groups were identified with a post hoc test corrected for multiple comparisons (Student-Newman-Keuls test). Differences between groups were considered statistically significant when \( P < 0.05 \).

RESULTS

The ERK signaling pathway is potently activated by different environmental and physical stressors (4, 7, 15, 45, 57), and the ERK proteins are therefore likely to be activated if muscle dissection is performed vigorously or if the perfusion per se elicits an environmental stress on the muscle fibers. However, the dual phosphorylation (Thr183 and Tyr185) of both the ERK1 and ERK2, which reflects activity, was not different in muscle obtained from perfused hindlimb in the basal state compared with muscle freeze-clamped before dissection in anesthetized rats (Fig. 1). Thus the perfused hindlimb muscle is a suitable model for investigation of the ERK signaling pathway in skeletal muscle, when combined with careful muscle dissection. Accordingly, muscles preperfused with the MEK-inhibitor PD-98059 (50 µM) in the resting nonstimulated state displayed only a minor decrease in dual phosphorylation of ERK1 and ERK2 compared with control (data not shown). The content of immunoreactive ERK1 (4.0 ± 1.0, 3.6 ± 0.08, 3.5 ± 0.08, means ± SE, n = 3, arbitrary units) and ERK2 (18 ± 1, 21 ± 1, 19 ± 1, means ± SE, n = 3, arbitrary units) was not different between soleus and white and red gastrocnemius muscles, respectively.

Both muscle contraction and insulin (Figs. 2 and 3) induced dual phosphorylation of the ERK proteins. Muscle contractions for 5 or 10 min induced a similar increase in ERK phosphorylation (data not shown). This activation was inhibited in muscles preperfused with PD-98059 in a dose-dependent manner at concentrations ranging from 10 to 50 µM and was totally inhibited by 50 µM in the perfused hindlimb muscles (Fig. 2). Insulin stimulation of ERK1 and ERK2 was maximal at 15–20 min (data not shown). The stimulatory effect of insulin was variable among the different muscle fiber types, with red gastrocnemius being the most responsive muscle. In addition, insulin was a less potent stimulator for ERK activation compared with muscle contraction, especially in white gastrocnemius and soleus muscles (Fig. 3). The ERK phosphorylation by insulin was totally inhibited by 50 µM PD-98059 and interestingly also by 1 µM wortmannin, a concentration necessary to inhibit muscle PI 3-kinase totally in this model (55). In contrast, wortmannin (1 µM) had no effect on the contraction-induced ERK1 and ERK2 dual phosphorylation in any of the fiber types investigated (Fig. 3). To further support that insulin utilizes a PI 3-kinase-dependent regulation of ERK signaling, we applied the LY-294002 PI 3-kinase inhibitor to our model. We measured Akt phosphorylation on Ser473 as an endogenous reporter of PI 3-kinase activity in addition to 2-DG uptake, a known PI 3-kinase-dependent insulin-stimulated process in skeletal muscle. As shown in Fig. 4, LY-294002 inhibits both insulin-induced Akt Ser473 phosphorylation and glucose transport in perfused skeletal muscle, with highly similar dose dependency (IC50 = ~10–30 µM) in all three fiber types. Near total inhibition (85–95%) was obtained by the highest concentration used (60 µM). In strong agreement with the data obtained with wortmannin, LY-294002 totally inhibited ERK phosphorylation by insulin in all three muscle types (Fig. 5). Thus MEK activation mediates ERK phosphorylation in response
to both insulin and contraction. In addition, these data strongly suggest that ERK phosphorylation by insulin is PI 3-kinase dependent, whereas contraction utilizes another mechanism.

We next investigated whether the potent activation of ERK signaling was necessary for the stimulatory effects on glucose and amino acid transport as well as glycogen synthase activity by muscle contraction. Muscle contraction increased 2-DG uptake significantly above basal by more than 12-fold in all three muscle fiber types (Fig. 6). This increase was not significantly affected by the presence of PD-98059, although at the highest concentration (50 µM) a tendency for a blunted glucose transport was observed in white and red gastrocnemius muscle (Fig. 6). Glycogen synthase was potently activated in all three fiber types, whereas amino acid transport was increased in red gastrocnemius and soleus, but not in white gastrocnemius, in response to muscle contraction. When stimulated by contraction, neither of these processes was affected by total inhibition of ERK or PI 3-kinase signaling (Table 1; Fig. 7). A maximally effective dose of insulin also stimulated glycogen synthase activity and amino acid (MeAIB) transport in perfused skeletal muscle (Table 1; Fig. 7). Whereas MEK inhibition had no effect, both glycogen synthase activity and MeAIB transport activation by insulin were virtually prevented in the presence of the PI 3-kinase inhibitors (Table 1; Fig. 7). Thus acute metabolic responses to insulin and contraction are not mediated by ERK signaling. However, insulin, but not contraction, activates amino acid and glucose transport as well as glycogen synthase by PI 3-kinase-dependent mechanisms in rat skeletal muscle.

**DISCUSSION**

Exercise has been shown to activate ERK signaling in skeletal muscle (2, 3, 18, 54). In the present study, we show that muscle contraction induces dual phosphorylation of skeletal muscle ERK1 and ERK2, which in accordance with previous studies, closely reflects ERK activity (34). Despite an apparently similar expression of ERK1 and ERK2 protein among the different muscle fiber types, insulin, and to a lesser extent muscle contraction, induced a lower ERK1 and ERK2 phosphorylation in soleus muscles compared with white and red gastrocnemius. We have previously observed that the soleus muscle is relatively resistant to stimulation of various metabolic processes by contraction compared with the gastrocnemius muscles. For example, maximal effects of contraction on glucose transport are only seen during very intense electrical stimulation (40). Such a phenomenon could also apply to the activation of signaling molecules such as the ERK proteins. In contrast, slow-twitch oxidative fibers are in general considered to be highly sensitive to...
stimulation by insulin, and it is somewhat surprising to see the poor insulin effect on ERK phosphorylation in the soleus muscle. We cannot attribute this to differences in the activation time course between the three muscle types, as they all display maximal phosphorylation after 15–20 min of insulin stimulation. Furthermore, soleus and white gastrocnemius muscles have the ability to increase ERK phosphorylation by contraction to a much higher extent than seen in response to insulin. Thus it remains unclear why insulin is such a moderate activator of ERK in these two muscle fiber types.

In all three muscle types, contraction-induced ERK phosphorylation was inhibited by PD-98059, suggesting that the activation is dependent on phosphorylation by MEK. Activation of ERK proteins by insulin was also dependent on MEK, in agreement with studies in cultured cells (11, 48, 53) and isolated muscle in vitro (5, 46). Interestingly, insulin-induced but not contraction-induced ERK phosphorylation was inhibited by both wortmannin and LY-294002. This provides evidence for the existence of at least two upstream stimulatory pathways of MEK in skeletal muscle that must be differently activated by insulin and muscle contraction. Wortmannin-insensitive activation of ERK signaling has previously been reported in parietal and Chinese hamster ovary cells when stimulated with epidermal growth factor and phorbol esters (36, 53). Nakamura et al. (36) reported that the effect of phorbol esters may be protein kinase C (PKC) dependent, as the effect was inhibited by a PKC inhibitor (Ro-31-8220). In addition, in vitro cell studies indicate that PKC has a role in Raf activation by phorbol esters (39, 44). Together, these studies suggest that PKC could be a wortmannin-insensitive (37) upstream element of MEK. Interestingly, muscle contraction causes translocation of PKC to muscle membranes, thereby presumably activating PKC (41). Thus members of the PKC family may activate MEK, through Raf, in response to contraction in skeletal muscle.

Our observation of LY-294002- and wortmannin-insensitive activation of ERK signaling by insulin strongly suggests that the upstream signaling mechanism is PI 3-kinase dependent in skeletal muscle tissue. Our data
are in agreement with findings obtained in L6 myoblasts, primary human myoblasts, and 3T3-L1 adipocytes in which wortmannin also abolished the activation of the ERK signaling pathway by insulin (11, 25, 48). In addition, several reports have shown that ERK activation by ligands working through G protein-coupled receptors are inhibited by wortmannin and LY-294002 (17, 21, 24, 27). Although still unresolved, some of these studies suggest that the action of these PI 3-kinase inhibitors takes place between Ras and Raf-1 kinase (11, 48). Interestingly, Ferby et al. (17) reported that calcium-independent ERK activation by platelet-activated factor in neutrophils was wortmannin sensitive but at the same time independent of p85/p110 PI 3-kinase (17). Therefore, stimulation of ERK by insulin could theoretically also be mediated by p85-independent PI 3-kinase activity, e.g., by p110* (47). The use of pharmacological inhibitors always raises concerns about specificity, questioning the validity of the data interpretation. However, in the present study, similar results were obtained with two structurally unrelated PI 3-kinase inhibitors with different modes of inhibitory action (37, 38, 52). This markedly strengthens the basis for our conclusion. Nevertheless, we cannot totally exclude the possibility that actions unrelated to PI 3-kinase by the two compounds are involved. In the present study, we used a supraphysiological concentration.

Table 1. Glycogen synthase activity

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>Insulin</th>
<th>Contraction</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WG</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>35 ± 3 (14)</td>
<td>57 ± 2* (11)</td>
<td>80 ± 4† (5)</td>
</tr>
<tr>
<td>PD-98059 (50 µM)</td>
<td>37 ± 1 (8)</td>
<td>51 ± 1* (7)</td>
<td>85 ± 3† (5)</td>
</tr>
<tr>
<td>Wortmannin (1 µM)</td>
<td>36 ± 5 (6)</td>
<td>40 ± 2 (4)</td>
<td>91 ± 2† (4)</td>
</tr>
<tr>
<td>LY-294002 (60 µM)</td>
<td>ND</td>
<td>41 ± 2 (7)</td>
<td>ND</td>
</tr>
<tr>
<td><strong>RG</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>36 ± 1 (14)</td>
<td>52 ± 3* (11)</td>
<td>80 ± 2† (5)</td>
</tr>
<tr>
<td>PD-98059 (50 µM)</td>
<td>32 ± 2 (8)</td>
<td>47 ± 3* (7)</td>
<td>80 ± 2† (5)</td>
</tr>
<tr>
<td>Wortmannin (1 µM)</td>
<td>36 ± 3 (6)</td>
<td>39 ± 1 (4)</td>
<td>82 ± 5† (4)</td>
</tr>
<tr>
<td>LY-294002 (60 µM)</td>
<td>ND</td>
<td>40 ± 1 (7)</td>
<td>ND</td>
</tr>
<tr>
<td><strong>SOL</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>37 ± 3 (14)</td>
<td>49 ± 2* (11)</td>
<td>71 ± 2† (5)</td>
</tr>
<tr>
<td>PD-98059 (50 µM)</td>
<td>33 ± 3 (8)</td>
<td>48 ± 3* (7)</td>
<td>66 ± 2† (5)</td>
</tr>
<tr>
<td>Wortmannin (1 µM)</td>
<td>39 ± 2 (6)</td>
<td>38 ± 5 (4)</td>
<td>89 ± 4† (4)</td>
</tr>
<tr>
<td>LY-294002 (60 µM)</td>
<td>ND</td>
<td>40 ± 3 (7)</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are means ± SE; nos. in parentheses, no. of experiments. Glycogen synthase activity in muscle perfused in resting nonstimulated state (basal) and during stimulation with either insulin (insulin) or contraction (contraction). Synthase activity was measured in absence or presence of MAP/ERK kinase (MEK) inhibitor (PD-98059) or phosphatidylinositol 3-kinase inhibitors (LY-294002 and wortmannin), in white (WG) and red (RG) gastrocnemius and soleus (SOL) muscle. Glycogen synthase activity expressed as fractional velocity (activity ratio in %) of synthase activity measured in presence of 0.17 mM compared to 8 mM glucose 6-phosphate. ND, not determined. * and †Significant difference (P < 0.001) from resting nonstimulated and insulin-stimulated states, respectively. PD-98059, wortmannin, and LY-294002 had no effect on total glycogen synthase activity (measured in presence of 8 mM glucose 6-phosphate) nor was total glycogen synthase activity different among 3 interventions (data not shown).

Fig. 6. 2-DG uptake rate in muscle perfused in absence or presence of MEK inhibitor PD-98059 either in resting nonstimulated state or during muscle contraction. Uptake was measured in white and red gastrocnemius and soleus muscles. Data are means ± SE of 8 independent experiments. No significant effect of PD-98059 was found in either fiber type by 1-way ANOVA.

Fig. 7. α-(Methylamino)isobutyric acid (MeAIB) uptake in muscle perfused in resting nonstimulated state (open bars) and during stimulation with either muscle contraction (gray bars) or insulin (filled bars). Uptake was measured in absence or presence of MEK inhibitor PD-98059 or PI 3-kinase inhibitor wortmannin in white (A) and red gastrocnemius (B) and soleus (C) muscle. Data are means ± SE of 4–8 independent experiments. * and #Significant difference (P < 0.05) compared with corresponding resting nonstimulated state and insulin-stimulated state in absence of inhibitor, respectively. PD-294002 (17, 21, 24, 27). Although still unresolved, some of these studies suggest that the action of these PI 3-kinase inhibitors takes place between Ras and Raf-1 kinase (11, 48). Interestingly, Ferby et al. (17) reported that calcium-independent ERK activation by platelet-activated factor in neutrophils was wortmannin sensitive but at the same time independent of p85/p110 PI 3-kinase (17). Therefore, stimulation of ERK by insulin could theoretically also be mediated by p85-independent PI 3-kinase activity, e.g., by p110* (47). The use of pharmacological inhibitors always raises concerns about specificity, questioning the validity of the data interpretation. However, in the present study, similar results were obtained with two structurally unrelated PI 3-kinase inhibitors with different modes of inhibitory action (37, 38, 52). This markedly strengthens the basis for our conclusion. Nevertheless, we cannot totally exclude the possibility that actions unrelated to PI 3-kinase by the two compounds are involved. In the present study, we used a supraphysiological concentra-
tion of insulin (20,000 µU/ml) to ensure maximal effects of insulin. Therefore, we cannot exclude the possibility that insulin also activated noninsulin receptor-mediated signaling, most likely through the insulin-like growth factor I (IGF-I) receptor. Activation of IGF-I receptors also leads to activation of PI 3-kinase and ERK, apparently through a series of cellular events very similar to those activated by the insulin receptor (6). Thus, if in fact IGF-I receptors were activated during insulin stimulation in the present study, we would predict that this activation did not have any significant influence on our findings.

In contrast to the findings in the present study, Shepherd et al. (46) concluded that insulin stimulation of ERK signaling was not inhibited by wortmannin and LY-294002 in isolated human muscle strips. In that study, insulin, in the presence of wortmannin, induced an intermediary ERK2 phosphorylation that was neither significantly different from basal nor from the phosphorylation level seen in response to insulin alone. On the other hand, LY-294002 was found not to interfere with insulin-induced ERK phosphorylation. However, there was only a small increase in ERK2 phosphorylation (<30%, n = 25) in response to a maximally effective dose of insulin. It is well known that physical stretch (57) and trauma to skeletal muscle (4) are potent activators for ERK signaling. Because the human muscle strips are produced by tearing fibers from a larger biopsy, “basal” ERK phosphorylation may have been markedly elevated, explaining the small effect of insulin. Such effects of stretch might also explain the lack of effect of the PI 3-kinase inhibitors because these have no effect on contraction-stimulated ERK activation, as shown in the present study.

To the best of our knowledge the present data are the first to show the inhibitory effect of LY-294002 on insulin signaling in skeletal muscle tissue. We measured Akt Ser^473 phosphorylation as an endogenous reporter of PI 3-kinase activity. In rodent skeletal muscle, insulin-induced Akt Ser^473 phosphorylation correlates well with Akt activity (\(r^2 = 0.89, n = 42, P < 0.001\)) as well as PI 3-kinase activity (\(r^2 = 0.45, n = 14, P < 0.01\)) (Wojtaszewski and Goodyear, unpublished observations). In all three muscle types, LY-294002 inhibited insulin-induced Akt Ser^473 phosphorylation. In agreement with several studies with wortmannin (32, 58), including our own obtained in perfused hindlimb (55), intact skeletal muscle displays lower sensitivity to LY-294002 compared with cell systems grown in single layers. Thus, in the present study, we found an IC_{50} of 10–30 µM for inhibition of insulin-induced Akt Ser^473 phosphorylation by LY-294002, whereas in 3T3-L1 adipocytes IC_{50} values around 1 µM for inhibition of PI 3 kinase have been reported (10). The structural complexity of skeletal muscle tissue is likely to interfere with the uptake and distribution of these compounds within the tissue itself. Thus the average drug concentration inside the muscle fibers may not be at equilibrium with the surrounding media, creating the basis for the apparent lower sensitivity in intact muscle.

The similar LY-294002 dose dependency of insulin-induced Akt Ser^473 phosphorylation and glucose transport seen in the present study supports the concept that PI 3-kinase is involved in insulin-stimulated glucose transport. Because Akt Ser^473 phosphorylation strongly reflects Akt activity, our data also suggest that Akt may be involved in this process as well, although this is still controversial (20, 26, 28, 49). In skeletal muscle, the role of PI 3-kinase in insulin-stimulated glucose transport has previously been based on studies with wortmannin (31, 55, 58). Amino acid transport and glycogen synthase activation by insulin were also inhibited by wortmannin in all three muscle types, and these results are in agreement with findings in murine soleus muscle (31). Our observation that LY-294002 also inhibited glycogen synthase activity by insulin in skeletal muscle is novel. Thus two different PI 3-kinase inhibitors elicit similar effects on insulin-stimulated glucose transport, glycogen synthase activity, and amino acid transport. This strongly suggests that insulin regulates these metabolic processes by a PI 3-kinase-dependent mechanism in skeletal muscle.

The downstream signaling molecules leading to metabolic effects of insulin in skeletal muscle are still unresolved. From the present data and other studies (8, 46), it appears that the ERK molecules are not involved. Recently, a new signaling link between Akt and glycogen synthase activation by insulin has emerged. Thus, in response to insulin, Akt may promote phosphorylation of glycogen synthase kinase 3, leading to deactivation of the kinase. This, in turn, leads to dephosphorylation and activation of glycogen synthase (12, 30). This pathway may only be one of the mechanisms utilized by insulin and other signaling pathways, as p70^S6K may also play an important role. However, because previous studies do not consistently show that insulin stimulation of glycogen synthase is blocked by rapamycin, a p70^S6K inhibitor, this remains unclear (5, 8, 12).

In the present study, muscle contraction potently activated glucose transport, glycogen synthase, and amino acid transport. None of these effects were sensitive to inhibition by wortmannin. This is in agreement with the observation that muscle contraction in vitro or in vivo does not activate PI 3-kinase (19, 55, 58). These data suggest that the signaling mechanisms utilized by muscle contraction and insulin for regulation of glucose and amino acid transport as well as glycogen synthase activity are different. In addition, we now provide evidence that signaling through ERK proteins is also not involved in any of these contraction-induced processes. The activation of the ERK signaling pathway may therefore not be crucial for any acute metabolic regulation in skeletal muscle, and the physiological role of this signaling cascade remains uncertain. The ERK proteins and the more distally positioned p90^rsk are assumed to translocate to the cell nucleus, causing phosphorylation of transcription factors and thereby increasing the rate of gene transcription (45). Thus it is possible that ERK activation is involved in adaptive cellular changes that occur at the level of mRNA and protein expression in response to changes in muscle...
contractile activity. Still, it must be emphasized that other pathways of the MAP kinase signaling cascade, which we have not investigated (p38 kinase and JNK), may mediate metabolic effects of muscle contractile activity (2, 18).

In summary, our data support the concept that the activation of glucose and amino acid transport and glycogen synthase activity in response to contraction and insulin are independent of the ERK signaling pathway in skeletal muscle. In addition, different upstream activators of the MEK/ERK signaling pathway are activated by insulin and muscle contraction. Finally, insulin activates ERK signaling, amino acid transport, glucose transport, and glycogen synthase activity in skeletal muscle by a PI 3-kinase-dependent mechanism, whereas these processes are activated by muscle contraction in a PI 3-kinase-independent manner.

We thank Betina Balmgren for skilled technical assistance. This study was supported by the Danish National Research Foundation Grant no. 504–14 and National Institute of Arthritis and Musculoskeletal and Skin Diseases Grant no. RO1–AR-42238. (To L. J. Goodyear.) J. F. P. Wojtaszewski was supported by a postdoctoral fellowship from Alfred Benzon’s Foundation, Denmark.

Address for reprint requests and other correspondence: J. F. P. Wojtaszewski, Copenhagen Muscle Research Centre, August Krogh Institute, 13 Universitetsparken, DK-2100 Copenhagen, Denmark (E-mail: jwotjaszewski@aki.ku.dk).

Received 22 July 1998; accepted in final form 24 May 1999.

REFERENCES


