Leucine modulates contraction- and insulin-stimulated glucose transport and upstream signaling events in rat skeletal muscle

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Leucine modulates contraction- and insulin-stimulated glucose transport and upstream signaling events in rat skeletal muscle. J Appl Physiol 108: 274–282, 2010. First published November 25, 2009; doi:10.1152/japplphysiol.00420.2009.—Leucine has profound effects on glucose metabolism in muscle; however, the effects of leucine on glucose transport in muscle have not been well documented. We investigated the effects of leucine on contraction- and insulin-stimulated glucose transport in isolated rat epitrochlealis muscle in vitro. In the absence of insulin, tetanic contraction increased 3-O-methyl-d-glucose (3-MG) transport and Thr\(^{172}\) phosphorylation of the catalytic \(\alpha\)-subunit of 5'-AMP-activated protein kinase (AMPK), a signaling intermediary leading to insulin-independent glucose transport. Leucine (2 mM, 30 min) significantly enhanced contraction-stimulated 3-MG transport and AMPK phosphorylation, accompanied by increased phosphorylation of p70 S6 kinase (p70S6K) Thr\(^{389}\). The stimulatory effects of leucine on 3-MG transport and AMPK phosphorylation were canceled by STO-609 blockade of Ca\(^{2+}\)/calmodulin-dependent protein kinase kinase (CaMKK) or rapamycin blockade of p70S6K. On the other hand, leucine blunted insulin-stimulated 3-MG transport and reduced insulin-stimulated Akt Thr\(^{473}\) phosphorylation. Insulin increased insulin-stimulated p70S6K Thr\(^{389}\) phosphorylation and enhanced the inhibitory phosphorylation of the insulin receptor substrate 1 (IRS1) Ser\(^{636/639}\). Furthermore, the effects of leucine on insulin-stimulated 3-MG transport and IRS phosphorylation were abolished by rapamycin. These results indicate that leucine activates contraction-stimulated glucose transport and inhibits insulin-stimulated glucose transport in skeletal muscle by activating mammalian target of rapamycin (mTOR)/p70S6K signaling. Increased contractions-stimulated AMPK Thr\(^{172}\) phosphorylation and insulin-stimulated IRS1 Ser\(^{636/639}\) phosphorylation might be responsible for these opposing effects of leucine, respectively.

5'-AMP-activated protein kinase; p70 S6 kinase; insulin receptor substrate-1; Akt; glucose metabolism

Under most physiological conditions, glucose transport across the cell membrane is the rate-limiting step for glucose utilization by skeletal muscle. Exercise (i.e., muscle contraction) and insulin are the most physiologically relevant stimulators of glucose transport in skeletal muscle. Glucose transporter 4 (GLUT4) is the main glucose transporter isoform expressed in skeletal muscle, and the translocation of GLUT4 from an intracellular location to the plasma membrane and T tubules is the main mechanism through which insulin and exercise increase skeletal muscle glucose transport (reviewed in 10, 14).

Accumulating evidence strongly supports a key role for the branched-chain amino acid (BCAA) leucine as a signaling molecule in the regulation of muscle metabolism. In particular, it plays a role in stimulating protein synthesis via rapid and potent activation of the mammalian target of rapamycin (mTOR) signaling pathway (reviewed in 48). However, few studies have examined the physiological relevance of leucine on glucose transport in skeletal muscle. Doi et al. (8) found that oral administration of 1.35 g/kg body wt of leucine, which produced a plasma concentration of 1.844 mM, did not increase glucose transport in the gastrocnemius muscle of normal rats 1 h after its administration. Baum et al. (3) reported that insulin-stimulated glucose uptake was unaltered even though insulin-stimulated phosphatidylinositol (PI) 3-kinase activity was blunted significantly in the rat gastrocnemius muscle after oral administration of 1.35 g/kg leucine. In contrast, Nishitani et al. found that oral administration of 1.5 g/kg BCAA produced total plasma BCAA concentrations of ~2 mM, and that stimulation with 2 mM leucine for 20–30 min increased glucose transport by 50% under insulin-free conditions in isolated soleus muscle from normal rats (29) and in rats with CCl\(_4\)-induced cirrhosis (30). Thus, whether basal and insulin-stimulated glucose transport is affected by leucine is controversial. Furthermore, no studies have been conducted on the effects of leucine on contraction-stimulated glucose transport.

We conducted this study to determine whether leucine modulates contraction- and insulin-stimulated glucose transport in skeletal muscle. For this purpose, we used an isolated rat skeletal muscle preparation to eliminate the effects of systemic confounders such as circulatory, humoral, and neural factors and intestinal absorption of leucine. We used rat epitrochlealis muscle, a mixture of ~65% of fast-twitch white, ~20% fast-twitch red, and ~15% slow-twitch red fibers (27, 28). Because the epitrochlealis is a thin, flat muscle capable of absorbing oxygen and nutrients from a buffered solution in vitro (27, 28), it is useful for examining the direct effects of pharmacological manipulation and contraction on skeletal muscle metabolism.

Materials and Methods

Animals. Male Sprague-Dawley rats aged 6 wk were obtained from Shimizu Breeding Laboratories (Kyoto, Japan). Animals were housed in an animal room maintained at 23°C with a 12:12-h light-dark cycle and fed a standard laboratory diet and water ad libitum. After an overnight fast, the rats weighing ~150 g were randomly assigned to experimental groups. All protocols for animal use and euthanasia were reviewed and approved by the Kyoto University Graduate School of Human and Environmental Studies, Kyoto University Graduate School of Medicine, and Kyoto University Radioisotope Research Center.

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**Muscle incubation.** Rats were killed by cervical dislocation without anesthesia, and the epimysial and superficial epimysial muscles were rapidly isolated, trimmed of connective tissue, and incubated as previously described (12, 13, 40) with some modifications. Both ends of each muscle were tied with sutures (silk 3-0; Nittyo Kogyo, Tokyo, Japan), and each muscle was mounted on an incubation apparatus with the resting tension set to 0.5 g. Muscles were preincubated in 7 ml of Krebs-Ringer bicarbonate buffer (KRB) (117 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 24.6 mM NaHCO₃) containing 2 mM pyruvate (KRBp) for 40 min. For tetanic contraction, muscles were then incubated in 7 ml of fresh buffer for 40 min in the absence or presence of 2 mM leucine and were stimulated during the last 10 min of the incubation period (1/min train rate, 10-s train duration, 100-Hz pulse rate, 0.1-ms pulse duration, 100 V) using an electric stimulator (SEN-3301; Nihon Koden, Tokyo, Japan). Force production was monitored using an isometric force transducer with amplifier (TRN001; Kent Scientific, Torrington, CT) and recorded with Pantos U-228-2P-500 recorder (Pantos, Kyoto, Japan). Basal muscle samples for contraction were identically preincubated and incubated without contraction. For insulin treatment, muscles were incubated in 7 ml of fresh buffer for 30 min in the absence or presence of 2 mM leucine and were then incubated in 7 ml of fresh buffer with 1 μM of insulin in the absence or presence of 2 mM leucine for 30 min. Basal muscle samples for insulin stimulation were identically preincubated and incubated without insulin stimulation. The buffers were continuously gassed with 95% O₂-5% CO₂ and maintained at 37°C. When present, 1 μM rapamycin (Sigma, St. Louis, MO) or 5 μM STO-609 (CalBiochem/EMD Biosciences, Gibbstown, NJ) was added continuously gassed with 95% O₂-5% CO₂ and maintained at 37°C. When present, 1 μM rapamycin (Sigma, St. Louis, MO) or 5 μM STO-609 (CalBiochem/EMD Biosciences, Gibbstown, NJ) was added during the preincubation and incubation periods. The maximal concentration of vehicle (DMSO) for rapamycin and STO-609 was 0.1%, which did not affect any assay. Force generation was not affected by reagents. The muscles were then used for 3-O-methyl-D-glucose (3-MG) transport measurement or immediately frozen in liquid nitrogen and subsequently analyzed for ATP and phosphocreatine (PCr) (see ATP and PCr assay) or used for Western blot analysis.

**Western blot analysis.** Muscle samples were homogenized in ice-cold lysis buffer (1-40 wt/vol) containing 20 mM Tris · HCl (pH 7.4), 1% Triton X, 50 mM NaCl, 250 mM sucrose, 50 mM NaF, 5 mM sodium pyrophosphate, 4 mg/ml leupeptin, 50 mg/ml trypsin inhibitor, 0.1 mM benzamidine, and 0.5 mM phenylmethylsulfonyl fluoride and were centrifuged at 20,000 g for 30 min at 4°C. The protein concentration of the supernatant was determined by the Bradford assay with BSA as a standard (Nacalai Tesque, Kyoto, Japan). The protein concentration of the supernatant was determined by the Bradford assay with BSA as a standard (Nacalai Tesque, Kyoto, Japan). The protein concentration of the supernatant was determined by the Bradford assay with BSA as a standard (Nacalai Tesque, Kyoto, Japan). The protein concentration of the supernatant was determined by the Bradford assay with BSA as a standard (Nacalai Tesque, Kyoto, Japan). The protein concentration of the supernatant was determined by the Bradford assay with BSA as a standard (Nacalai Tesque, Kyoto, Japan). The protein concentration of the supernatant was determined by the Bradford assay with BSA as a standard (Nacalai Tesque, Kyoto, Japan). The protein concentration of the supernatant was determined by the Bradford assay with BSA as a standard (Nacalai Tesque, Kyoto, Japan).

**Glycogen content assay.** Glycogen content was assayed as described previously (26). Each frozen muscle was digested in 1 M NaOH at 85°C for 10 min, and the digestates were neutralized with HCl. The glycogen in the digestates was hydrolyzed by incubation in 2 M HCl for 2 h at 85°C. The digestates were neutralized with NaOH, and the concentration of hydrolyzed glucose residues was measured enzymatically using Glucose CII Test (Wako, Osaka, Japan). Glycogen content was expressed as nanomoles of glucose per milligram wet weight of muscle.

**3-MG transport.** We evaluated 3-MG transport as an index of glucose transport activity as described previously (12, 13, 40) with modifications. After the incubation period, muscles were further incubated in 2 ml of KRB containing 1 mM 3-14C-HMG (1.5 μCi/ml) (American Radiolabeled Chemicals, St. Louis, MO) and 7 mM D-[1-14C]mannitol (0.3 μCi/ml) (American Radiolabeled Chemicals) at 30°C for 10 min. Leucine, insulin, rapamycin, or STO-609 was absent during the incubation with 3-MG. The muscles were then blotted onto filter paper and then weighed and processed by incubating them in 300 μl of 1 M NaOH at 80°C for 15 min, and the digestates were neutralized with HCl. The radioactivity in aliquots of the digestates was determined by liquid scintillation counting of dual labels. The rate of 3-MG transport was expressed as micromoles of 3-MG per gram wet weight of muscle per hour.

**ATP and PCr assay.** Frozen muscles were homogenized in 0.2 M HClO₄ (3:25 wt/vol) in an ethanol-dry ice bath (−20 ~ −30°C) and centrifuged at 16,000 g for 2 min at −9°C. The supernatant of the homogenate was neutralized with a solution of 2 M KOH, 0.4 M KCl, and 0.4 M imidazole and then centrifuged at 16,000 g for 2 min at −9°C, and then subjected to enzymatic analysis (21). ATP and PCr contents were expressed as nanomoles per milligram wet weight of muscle.

**Statistical analysis.** Results are presented as means ± SE. Multiple means were compared by ANOVA followed by post hoc comparison with Tukey's test. Two means were compared with Student's t-test. Differences between groups were considered statistically significant at P < 0.05.

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**Fig. 1.** Contraction-stimulated 3-O-methyl-D-glucose (3-MG) transport activity in excess of basal 3-MG transport activity in rat epimysial muscles. Isolated muscle was preincubated for 40 min and incubated for 40 min in the absence (−) or presence (+) of rapamycin or STO-609. Leucine was added as required during the incubation period. Muscles were electrically stimulated during the last 10 min of the incubation period, after which 3-MG transport activity was measured. Values are expressed as the mean ± SE (n = 7–18 per group). Means with different letters are statistically different from each other (P < 0.01).
RESULTS

Leucine increases contraction-stimulated glucose transport in skeletal muscle. We examined whether leucine affects basal and contraction-stimulated glucose transport in skeletal muscle. In our previous studies (12, 13), we obtained maximally stimulated 3-MG transport using the contraction protocol described in MATERIALS AND METHODS. The basal activity of 3-MG transport was not affected by leucine [basal 0.10 ± 0.05 μmol·g muscle$^{-1}$·h$^{-1}$ ($n = 16$) vs. leucine 0.09 ± 0.03 μmol·g muscle$^{-1}$·h$^{-1}$ ($n = 8$); not significant (NS)], but contraction-stimulated activity of 3-MG transport (above basal) was significantly increased by 24% by leucine (Fig. 1). The stimulatory effect of leucine on contraction-stimulated 3-MG transport was blocked in the presence of rapamycin, a specific inhibitor of the mTOR/p70S6K pathway (7) (Fig. 1). Basal activity of 3-MG transport was not affected by rapamycin [basal 0.10 ± 0.05 μmol·g muscle$^{-1}$·h$^{-1}$ ($n = 16$) vs. rapamycin 0.10 ± 0.02 μmol·g muscle$^{-1}$·h$^{-1}$ ($n = 8$); NS]. Moreover, contraction-stimulated 3-MG transport activity (above basal) was also unaffected by rapamycin [contraction 0.52 ± 0.02 μmol·g muscle$^{-1}$·h$^{-1}$ ($n = 18$) vs. contraction with rapamycin 0.54 ± 0.03 μmol·g muscle$^{-1}$·h$^{-1}$ ($n = 8$); NS]. Similar results were obtained with STO-609, a specific inhibitor of Ca$^{2+}$/calmodulin-dependent protein kinase (CaMKK). The stimulatory effect of leucine on contraction-stimulated 3-MG transport was blocked in the presence of STO-609 (Fig. 1).

Leucine increases contraction-stimulated AMPK$\alpha$ Thr$^{172}$ phosphorylation. AMPK is a signaling intermediary involved in contraction-stimulated glucose transport in skeletal muscle (reviewed in 11, 16, 23). To determine the activation state of AMPK, we used Western blot analysis with a phosphospecific AMPK antibody that recognizes AMPK$\alpha$ isoforms only when AMPK$\alpha$ Thr$^{172}$ is phosphorylated. Basal phosphorylation was not affected by leucine, but leucine significantly increased contraction-stimulated phosphorylation by 27% (Fig. 2A). In parallel with 3-MG transport, the stimulatory effect of leucine on contraction-stimulated AMPK phosphorylation was abolished in the presence of rapamycin (Fig. 2B).

Leucine increases contraction-stimulated Ca$^{2+}$/calmodulin-dependent protein kinase II (CaMKII) Thr$^{287}$ phosphorylation. The phosphorylation of CaMKII isoforms migrated between 50 and 75 kDa was summed (32). Values are expressed as means ± SE ($n = 6–7$ per group). Means with different letters are statistically different from each other ($P < 0.01$).

A

![Graph showing basal and contraction-stimulated AMPK$\alpha$ Thr$^{172}$ phosphorylation](image)

B

![Graph showing basal and contraction-stimulated CaMKII Thr$^{287}$ phosphorylation](image)
Similar results were observed with STO-609. STO-609 did not affect basal and contraction-stimulated AMPK phosphorylation but clearly canceled the stimulatory effect of leucine on AMPK phosphorylation (Fig. 2B).

Leucine does not change contraction-stimulated phosphorylation of CaMKII Thr^{287}. CaMKII has been implicated to be involved in contraction-stimulated glucose transport in skeletal muscle (46). Thr^{287} phosphorylation of CaMKII is robustly increased in response to contraction in rat skeletal muscle (32, 46), and it has also been used as an indicator of elevated cytosolic Ca^{2+} (4). We found that muscle contraction significantly increased CaMKII Thr^{287} phosphorylation, but leucine did not affect the phosphorylation status of CaMKII (Fig. 3).

Leucine does not change the energy status of skeletal muscle during contraction. Skeletal muscle AMPK is activated in response to energy-depriving stresses such as muscle contraction, hypoxia, hyperosmolarity, and inhibition of oxidative phosphorylation (12). AMPK is also activated in the absence of energy deprivation by 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside (AICAR), a pharmacological stimulator of AMPK (12). To determine whether leucine increases AMPK activity during energy deprivation, we measured basal and electrically stimulated levels of ATP, PCr, and glycogen. ATP (Fig. 4A), PCr (Fig. 4B), and glycogen (Fig. 4C) levels were significantly decreased in response to contraction, but leucine had no effect on ATP, PCr, or glycogen contents. Consistent with these findings, leucine had no effect on force production during contraction (Fig. 4D).

Leucine increases basal and contraction-stimulated p70S6K Thr^{389} phosphorylation. To confirm that leucine activates the mTOR/p70S6K pathway, we performed Western blot analysis using a p70S6K antibody that recognizes phosphorylated Thr^{389}, the key residue for the kinase activity of p70S6K in skeletal muscle (17). Basal and contraction-stimulated p70S6K

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**Fig. 4.** ATP, phosphocreatine (PCr), and glycogen content, and force production during contraction in rat epitrochlearis muscle. Isolated muscle was preincubated for 40 min and incubated for 40 min. Leucine was added as required during the incubation period. Muscles were electrically stimulated during the last 10 min of the incubation period, after which levels of ATP (A), PCr (B), and glycogen (C) were measured. Values are expressed as means ± SE (n = 7–10 per group). Means with different letters are statistically different from each other (P < 0.01). Force production (initial peak tension) during the contraction was also evaluated (D). Values are expressed as means ± SE [n = 12 per group; not significant (NS)].

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Thr\textsuperscript{389} phosphorylation was significantly enhanced by leucine (Fig. 5). Contraction increased p70S6K phosphorylation by 62% in the absence of leucine and by 161\% in the presence of leucine ($P < 0.05$), and the stimulatory effect of leucine on contraction-stimulated p70S6K phosphorylation was abolished by rapamycin (Fig. 5).

**Leucine decreases insulin-stimulated glucose transport in skeletal muscle.** We next examined whether leucine affects insulin-stimulated glucose transport. In our previous study, we obtained maximal stimulation of 3-MG transport using 1 \( \mu \text{M} \) insulin (12, 13). In contrast to muscle contraction, insulin-stimulated activity of 3-MG transport (above basal) was significantly decreased by 33\% by leucine (0.76 \( \pm \) 0.02 and 0.51 \( \pm \) 0.06 \( \mu \text{mol·g muscle}^{-1}·\text{h}^{-1} \) in the absence and presence of leucine, respectively; $P < 0.01$) (Fig. 6). The inhibitory effect of leucine on insulin-stimulated glucose transport was blocked in the presence of rapamycin (Fig. 6).

**Leucine increases insulin-stimulated p70S6K Thr\textsuperscript{389} phosphorylation.** We determined whether leucine affects the activation of p70S6K in isolated skeletal muscle. Insulin increased phosphorylation 4-fold compared with the basal rate in the absence of leucine and 11-fold in the presence of leucine (Fig. 7). Consistent with the inhibitory effect of leucine on insulin-stimulated glucose transport, the stimulatory effect of leucine on p70S6K phosphorylation was blocked in the presence of rapamycin (Fig. 7).

**Leucine increases insulin-stimulated IRS1 Ser\textsuperscript{636/639} phosphorylation.** To examine whether leucine induces the inhibitory phosphorylation of IRS1, we performed Western blot analysis using an IRS1 antibody that recognizes phosphorylated Ser\textsuperscript{636/639}, a key residue for functional modulation of IRS1 in skeletal muscle (42). Insulin increased Ser\textsuperscript{636/639} phosphorylation by 113\% compared with the basal rate in the absence of leucine, and by 218\% in the presence of leucine (Fig. 8). Consistent with the effect of leucine on insulin-stimulated glucose transport...
port and p70S6K phosphorylation, the stimulatory effect of leucine on Ser636/639 phosphorylation was abolished in the presence of rapamycin (Fig. 8).

Leucine decreases insulin-stimulated Akt Ser473 phosphorylation.

To determine whether leucine impairs reactions downstream of IRS1, we measured the phosphorylation status of Ser473 of Akt in skeletal muscle. Insulin markedly stimulated Akt phosphorylation. Leucine mildly but significantly decreased insulin-stimulated Akt phosphorylation by 14% (Fig. 9), but this effect was not evident in the presence of rapamycin (Fig. 9). Muscle contraction also increased Akt Ser473 phosphorylation significantly, but this increase was not affected by the presence of leucine (Fig. 10).

DISCUSSION

A novel finding of the present study is that contraction-stimulated glucose transport is enhanced by leucine (Fig. 1). This increase is accompanied by enhanced AMPKαThr172 phosphorylation, an essential step for full kinase activation (39) (Fig. 2A). AMPK is a heterotrimeric kinase, consisting of a catalytic α-subunit and two regulatory subunits, β and γ. Two distinct α isoforms of AMPK (α1 and α2) exist in skeletal muscle (38). In our previous studies, we used the same stimulation protocol as used in the present study to demonstrate that muscle contraction activates both α1 and α2 activity via AMPKαThr172 phosphorylation (12, 24, 41).

That leucine increased AMPKαThr172 phosphorylation suggests that leucine induces covalent modification of AMPKαThr172 via an AMPK kinase. The LKB1 complex (34, 36) and CaMKK (15, 45) have been identified as AMPK kinases in skeletal muscle. The LKB1 complex is constitutively active and is not activated directly by AMP, but the binding of AMP to AMPK facilitates Thr172 phosphorylation by the LKB1 complex (34, 36). Thus LKB1 is believed to be a crucial upstream kinase for energy-dependent activation of AMPK. In the present study, the enhancement of AMPK activation by leucine was not accompanied by a profound decrease in ATP, PCr, or glycogen content (Fig. 4), indicating that CaMKK, not LKB1, is likely involved in the leucine-induced increase in AMPKαThr172 phosphorylation. In support of this concept, the stimulatory effect of leucine on contraction-stimulated 3-MG transport and AMPK phosphorylation was abolished in the presence of CaMKK blockade by STO-609 (Figs. 1 and 2B). We note that the phosphorylation status of CaMKII Thr287 (indicative of cytosolic Ca2+) was unaffected by leucine (Fig. 3), suggesting that leucine enhances contraction-stimulated CaMKK activity without a major increase in cytosolic Ca2+.

p70S6K is thought to play a critical role in regulating muscle protein synthesis after exercise (5, 31). The BCAAs, and leucine in particular, and resistance exercise are strong stimulators of p70S6K and have synergistic effects on p70S6K in humans (17). We also found that contraction-induced phosphorylation of p70S6K was markedly and consistently enhanced in the presence of leucine (Fig. 5). The stimulatory effect of leucine on contraction-induced 3-MG transport (Fig. 1) is not surprising because the large energy expenditure is devoted to protein synthesis in skeletal muscle cells. In support of this idea, the stimulatory effect of leucine on 3-MG transport was absent in the presence of rapamycin (Fig. 1), which

Fig. 8. Basal and insulin-stimulated insulin-receptor substrate 1 (IRS1) Ser636/639 phosphorylation (P-IRS1) in rat epitrochlearis muscle. Isolated muscle was preincubated for 40 min and incubated for 60 min in the absence or presence of rapamycin. Leucine was added as required during the incubation period; insulin was added during the last 30 min of the incubation period. The tissue lysate was subjected to Western blot analysis with a phosphospecific IRS1 antibody. Values are expressed as means ± SE (n = 4–8 per group). Means with different letters are statistically different from each other (P < 0.05).

Fig. 9. Basal and insulin-stimulated Akt Ser473 phosphorylation (P-Akt) in rat epitrochlearis muscle. Isolated muscle was preincubated for 40 min and incubated for 60 min in the absence or presence of rapamycin. Leucine was added as required during the incubation period; insulin was added during the last 30 min of the incubation period. The tissue lysate was subjected to Western blot analysis with a phosphospecific Akt antibody. Values are expressed as the mean ± SE (n = 4–8 per group). Means with different letters are statistically different from each other (P < 0.01).
We demonstrated previously that a single tetanic contraction was enough to begin to increase 3-MG transport in incubated rat epitrochlearis muscle; maximal activity was observed with 10 contractions and there was no further increase with 15 contractions (24). There was also a striking similarity between the increase in AMPK activity and the increase in 3-MG transport: maximal AMPK-α1 and -α2 activity was observed with 10 contractions, and there was no further increase with 15 contractions (24). Therefore, our present results could be interpreted as follows: leucine increases the maximal rate of contraction-induced glucose transport and the maximal contraction-stimulated activity of AMPK in skeletal muscle. It should be emphasized that tetanic contraction is not the strongest stimulator of insulin-independent glucose transport and AMPK activity in skeletal muscle. For example, 10 tetanic contractions increased 3-MG transport 5-fold compared with basal transport, and pharmacological inhibition of oxidative phosphorylation using the chemical uncoupler dinitrophenol (0.5 mM for 20 min) increased 3-MG transport 11-fold, which was accompanied by more pronounced increases in AMPK-α1 and -α2 activity (12). Thus it is reasonable to speculate that even when muscle is maximally stimulated by contraction, it still has the ability to evoke additional activation of glucose transport and AMPK activity in response to leucine.

Leucine has been implicated in the modulation of insulin-stimulated glucose transport in skeletal muscle cells. In cultured L6 cells, exposure to amino acids, including leucine, for 1 h reduced insulin-stimulated glucose transport by as much as 55% (43). Krebs et al. (19) and Tremblay et al. (42) showed that an acute elevation in plasma levels of amino acids, including leucine, during an euglycemic hyperinsulinemic clamp decreases insulin-stimulated glucose transport into skeletal muscle and reduces insulin-stimulated whole body glucose disposal in healthy humans. In our study, the inhibitory effect of leucine on insulin-stimulated glucose transport was clearly abolished by rapamycin (Fig. 6). Furthermore, the stimulatory effect of leucine on Thr389 phosphorylation of p70S6K (Fig. 7) and IRS1 Ser636/639 phosphorylation (Fig. 8) was abolished in the presence of rapamycin. In support of our observations, Tremblay et al. (43) showed that the inhibitory effect of amino acids on insulin-stimulated glucose transport in cultured L6 cells is fully prevented by rapamycin. More recently, Tremblay et al. (42) used skeletal muscle biopsies from healthy subjects to show that elevation of plasma amino acid levels, including that of leucine, during an euglycemic hyperinsulinemic clamp increased the ability of insulin to activate p70S6K, which was accompanied by a corresponding increase in inhibitory IRS1 phosphorylation of Ser312 and Ser636/639. They also showed that rapamycin prevents amino acid-dependent increases in serine phosphorylation of IRS1 and insulin-stimulated glucose transport in cultured L6 cells. These observations suggest that leucine overactivates the mTOR/p70S6K pathway, which results in inhibitory serine phosphorylation of IRS1 and impairs insulin action in skeletal muscle.

An intriguing finding is that leucine only mildly decreased insulin-stimulated phosphorylation of Akt on Ser473 (Fig. 9) even though this residue plays a key role in insulin-stimulated signaling involving glucose transport (44). However, similar observations have been reported by Tremblay et al. (43), who showed that, in cultured L6 cells, insulin-stimulated Akt phosphorylation and activity were unaltered by amino acids, includ-
ing leucine, although such conditions resulted in a marked inhibition of IRS1-associated PI 3-kinase activity and 2-deoxy-glucose transport activity. Akt may only require partial stimulation of upstream signaling by insulin to achieve full or almost full activation in muscle cells.

As with insulin stimulation, muscle contraction increases Akt Ser473 phosphorylation in skeletal muscle (35). However, the role of Akt activation in contraction-stimulated glucose transport is unclear. Although pharmacological blockade of PI 3-kinase signaling by wortmannin and LY294002 inhibits contraction-stimulated Akt phosphorylation and activity (35), it does not decrease contraction-stimulated glucose transport in skeletal muscle (13, 20, 22, 47). In addition, the skeletal muscle of the Akt2 knockout mouse does not exhibit impaired basal or exercise-stimulated glucose transport (33). This dissociation between Akt activation and glucose transport indicates that, in contrast to insulin-stimulated glucose transport, Akt does not play a central role in contraction-stimulated glucose transport in skeletal muscle. Consistent with these findings, Akt Ser473 phosphorylation was increased by muscle contraction irrespective of the presence or absence of leucine (Fig. 10).

In summary, we found that leucine has a stimulatory effect on contraction-stimulated glucose transport and an inhibitory effect on insulin-stimulated glucose transport in rat skeletal muscle, and these effects are both absent in the presence of rapamycin. Concomitant increases in contraction-stimulated AMPK Thr172 phosphorylation and insulin-stimulated IRS1 Ser409/410 phosphorylation might have been responsible for these opposing effects of leucine. We propose that leucine modulates contraction- and insulin-stimulated glucose transport in skeletal muscle by activating mTOR/p70S6K signaling.

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DISCLOSURES

No conflicts of interest are declared by the authors.

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