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ORIGINAL ARTICLE

Leucine facilitates the insulin-stimulated glucose uptake and insulin signaling in skeletal muscle cells: involving mTORC1 and mTORC2

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Abstract Leucine, a branched-chain amino acid, has been shown to promote glucose uptake and increase insulin sensitivity in skeletal muscle, but the exact mechanism remains unestablished. We addressed this issue in cultured skeletal muscle cells in this study. Our results showed that leucine alone did not have an effect on glucose uptake or phosphorylation of protein kinase B (AKT), but facilitated the insulin-induced glucose uptake and AKT phosphorylation. The insulin-stimulated glucose uptake and AKT phosphorylation were inhibited by the phosphatidylinositol 3-kinase inhibitor, wortmannin, but the inhibition was partially reversed by leucine. The inhibitor of mammalian target of rapamycin complex 1 (mTORC1), rapamycin, had no effect on the insulin-stimulated glucose uptake, but eliminated the facilitating effect of leucine in the insulin-stimulated glucose uptake and AKT phosphorylation. In addition, leucine facilitation of the insulin-induced AKT phosphorylation was neutralized by knocking down the core component of the mammalian target of rapamycin complex 2 (mTORC2) with specific siRNA. Together, these findings show that leucine can facilitate the insulin-induced insulin signaling and glucose uptake in skeletal muscle cells through both mTORC1 and mTORC2, implicating the potential importance of this amino acid in glucose homeostasis and providing new mechanistic insights.

Keywords Leucine · Glucose uptake · Insulin signaling · Mammalian target of rapamycin

Abbreviations

BCAA	Branched-chain amino acids
2-DG	2-Deoxy-D-glucose
2DG6P	2-Deoxyglucose-6-phosphate
4EBP1	eIF4E-binding proteins
G6PDH	Glucose-6-phosphate dehydrogenase
IRS-1	Insulin receptor substrate-1
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
mTORC2	Mammalian target of rapamycin complex 2
PI3-K	Phosphatidylinositol 3-kinase
PKB/AKT	Protein kinase B
Rictor	Rapamycin-insensitive companion of mTOR
S6K	Ribosomal protein S6 kinase

Introduction

Insulin resistance is a precursor and key component of type 2 diabetes, and is characterized by the decreased response

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to insulin in peripheral target tissues such as liver, adipose tissue and skeletal muscle (Petersen and Shulman 2002; Bernard et al. 2013). Normally, circulation insulin binds to its specific receptors, triggers a series of intracellular signaling cascades, leads to translocation of the glucose transporter 4 from cytosol to plasma membrane, and then drives the uptake of glucose into adipocytes and skeletal muscle cells (Kleinert et al. 2011). Skeletal muscle is responsible for 70–80 % of the postprandial blood glucose disposal. So, impaired insulin signaling pathway will lead to decreased glucose uptake in skeletal muscle and disrupt blood glucose homeostasis (Kleinert et al. 2011; Li et al. 2010). Recently, the regulation of insulin signaling and glucose metabolism by certain amino acids has become a major focus of interest (Bernard et al. 2011; Doi et al. 2003, 2007; Nishitani et al. 2002). Branched-chain amino acids (BCAA) such as leucine have been shown to function as an important nutrient-signaling molecule to improve insulin sensitivity by multiple mechanisms (Floyd et al. 1966; Kalogeropoulou et al. 2008; Zhang et al. 2007; Guo et al. 2010; Macotela et al. 2011; Cota et al. 2006). It has been revealed that leucine stimulates glucose uptake in skeletal muscle via a pathway that is independent from the classic insulin cascades. However, the exact mechanism of leucine involvement in glucose metabolism and insulin signaling has not been defined.

The mammalian target of rapamycin (mTOR) is a crossroad of amino acid-mediated signaling and insulin signaling pathways (Kim et al. 2013; Kim and Guan 2011; Goberdhan et al. 2009), and its role in glucose metabolism has recently garnered a lot of attention. Specifically, mTOR can be activated by amino acids like leucine and by many other factors such as insulin, growth factors and cytokines (Dodd and Tee 2012). The insulin-induced activation of mTOR involves phosphatidylinositol 3-kinase (PI3-K) and protein kinase B (PKB, also known as AKT) (Bhaskar and Hay 2007; Rosner et al. 2010). mTOR is activated through its phosphorylation at serine 2448, a consensus site for both AKT- and amino acid-mediated phosphorylation (Hinault et al. 2004; Bhaskar and Hay 2007). There are two distinct complexes, mTORC1 and mTORC2, and they act downstream or upstream of AKT, respectively. Activation of mTORC1 by insulin or amino acids promotes cellular growth and protein synthesis via phosphorylation of the ribosomal protein S6 kinase (S6K) and eIF4E-binding proteins (4EBP1) (Hinault et al. 2004). Activated mTORC2 can promote AKT phosphorylation at Ser473 (Sarbassov et al. 2005). Due to these complex and mutual regulations between AKT and mTOR, the mechanism by which leucine regulates glucose metabolism and insulin signaling via mTOR has been extensively studied but remains unestablished. Initially, amino acids including leucine have been shown to attenuate glucose uptake by the mTORC1/S6K-mediated serine/threonine phosphorylation of insulin receptor substrate-1/2 (IRS-1/2) and the subsequent negative feedback (Balage et al. 2011). However, increasing evidence has shown the opposite effects of amino acids on insulin signaling. For example, some amino acids such as leucine can actually improve insulin signaling and glycemic control (Nishitani et al. 2002; Bernard et al. 2011, 2012; Kleinert et al. 2011). A mixture of amino acids has been shown to improve glucose uptake in muscle in both healthy, non- insulin resistant tissue and insulin resistant tissues (Kleinert et al. 2011; Bernard et al. 2011). This effect was associated with AS160 phosphorylation but independent of AKT and mTOR although the amino acid mixture increased phosphorylation of both AKT and mTOR. In adipocytes, leucine has been previously shown to promote the insulin-induced glucose transport through activation of AKT and mTOR when PI3K is inhibited, and this effect involved mTORC2 (Hinault et al. 2004, 2006; Hresko and Mueckler 2005). We have recently shown that leucine supplementation increases AKT and mTOR phosphorylation in skeletal muscle of rats on high-fat diet (Li et al. 2013) but leucine facilitates insulin signaling independent of mTORC1 and mTORC2 in cultured hepatocytes (Yang et al. 2013). Nevertheless, the leucine-mediated activation of mTORC1 and mTORC2 in regulation of insulin signaling and glucose uptake in skeletal muscle has not been defined. In this study, we have addressed these issues.

Materials and methods

Materials

Antibodies against β-actin, AKT, phosphoserine 473 AKT, mTOR and phosphoserine 2448 mTOR were obtained from Cell Signaling Technology (Beverly, MA). Cell culture reagents were from Gibco BRL Life Technologies (paisley, Scotland). Hexokinase, 2-deoxy-D-glucose (2-DG), glucose-6-phosphate dehydrogenase (G6PDH), Resazurin-sodium salt and triethanolamine hydrochloride, Adenosine 5′-triphosphate disodium salt (ATP), β-nicotinamide adenine dinucleotide phosphate (β-$NADP^+$), diaphorase, wortmannin, and rapamycin were from Sigma Chemical (MO, USA). LY294002 were purchased from Calbiochem (CA, USA). Leucine was obtained from Wako Pure Chemical (Osaka, Japan). The siRNA against rapamycin-insensitive companion of mTOR (rictor) and related scrambled siRNA were from Sigma. Lipofectamine™ RNAi-MAX transfection reagents were from Invitrogen (NY, USA). All other chemicals were of highest analytical grade.

Skeletal muscle cell culture

The culture of primary rat skeletal muscle cells was performed with a micro-explant culture technique as described (Smith and Merrick 2010). Briefly, skeletal muscles of the fore and hind limbs from 4 to 5 days old SD rats were collected aseptically and washed 3 times in the ice-cold PBS. Fat, bone and connective tissue were removed. The muscle pieces were then cut into 400 μm^3 cubes and placed in collagen-coated dishes or plates containing DMEM/F12 with 10 % fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin in an atmosphere of 5 % CO_2 at 37 °C until cells reached 80 % confluence. To obtain fully differentiated myotubes, the medium was changed to DMEM/F12 with 2 % FBS and replaced after 2 and 4 days of culture. After 4–6 days, myotube differentiation was complete, and experimental procedures were initiated. Phase-contrast microscopy and electron microscope were used to observe the morphology of the skeletal muscle cells and myotubes. C2C12 myoblasts were purchased from ATCC and maintained in DMEM with 10 % fetal bovine serum. Myogenic differentiation was induced by replacement of the medium with the serum-reduced differentiation medium (DMEM containing 2 % horse serum).

Treatment of skeletal muscle cells

Differentiated myotubes were starved for 30 min with the serum-free DMEM medium, pre-treated with an inhibitor (100 nM Wortmanin, 10 μM LY294002 or 50 nM rapamycin in serum-free DMEM medium) for 30 min, and then treated with leucine or/and insulin (in serum-free DMEM medium) as noted.

Glucose uptake assay

Glucose uptake in skeletal muscle cells was determined by a non-radioisotope, enzymatic fluorescence assay as described previously (Yamamoto et al. 2006). This method enabled the detection of 2-deoxyglucose-6-phosphate (2DG6P) accumulation inside cells by measuring the fluorescence of NADPH produced from $NADP^+$, which is coupled to the oxidation of 2-DG–2DG6P by G6PDH. In brief, after the pre-incubation with an inhibitor and the incubation with leucine or/and insulin for the noted time, cells were washed twice with KRPH buffer (20 mM Hepes, 5 mM KH2PO4, 1 mM MgSO4, 1 mM CaCl2, 136 mM NaCl, 4.7 mM KCl, pH 7.4) containing 0.1 % BSA. The washed myotubes were incubated with KRPH buffer containing 1 mmol/L 2-DG and 0.1 % BSA at 37 °C in 5 % CO2 for 30 min, and then rinsed twice with KRPH containing 0.1 % BSA. Cells in each well were treated with 25 μL of 0.1 N NaOH, subjected to one freeze and thaw cycle, and finally incubated at 85 °C for 40 min in a temperature-controlled bath. The components in the wells were then treated with 25 μL of 0.1 N HCl and incubated in an freshly prepared assay cocktail (50 mM TEA, 50 mM KCl, 0.5 mM MgCl2, 0.02 % BSA, 670 μM ATP, 0.12 μM $NADP^+$, 25 μM resazurin-sodium salt, 5.5 units/mL hexokinase, 16 units/mL G6PDH, and 1 unit/mL diaphorase) for 90 min at 37 °C. At the end of the incubation, fluorescence at 590 nm with excitation at 530 nm was quantified using an automatic microplate reader (Spectra Max M2).

Gene silencing with siRNA

The siRNA against Rictor or scrambled siRNA was introduced into C2C12 myoblasts by reverse transfection with reagents from Lipofectamine™ RNAiMAX according to the manual from the provider. In brief, the transfection mixture was applied to 6-well plate right before plating cells in complete DMEM without antibiotics. The medium was replaced with fresh medium after 3–4 h and cells were incubated overnight. The medium was switched to the differentiation medium (DMEM + 2 % horse serum) and cells were allowed to differentiate for 2 days before any treatment.

Immunoblotting

Cells were lysed in ice-cold lysis buffer (150 mmol/L Tris–HCl, 1 % NP-40, 0.1 %Tween-20) supplemented with 0.1 % PMSF. Protein concentrations were determined by BCA protein assay kit (Pierce). Lysates (40 μg/lane) were resolved in 7.5 % SDS-PAGE gels and transferred to nitrocellulose membranes (Bio-Rad). The presence of proteins was detected by immunoblotting with primary antibodies as indicated and alkaline phosphatase-conjugated secondary antisera. Specifically, one membrane was cut according to the protein size of molecular weight marker for probing different antibodies. For phosphorylated and total protein with the same size, the membrane was incubated with phosphorylated antibody first, then treated with stripping buffer and reprobed with total antibody. Bands were visualized with Molecular Imager VersaDoc MP 4000 System (Bio-Rad). The intensity was quantified by densitometry analysis using QuantityOne (Bio-Rad) and normalized to β-actin.

Statistical analysis

Results are presented as mean ± SD. Data were analyzed with one-way ANOVA followed by Student–Newman–Keuls test. The SPSS 15.0 software package was used for all statistical analyses. The level of significance was set at $p < 0.05$.

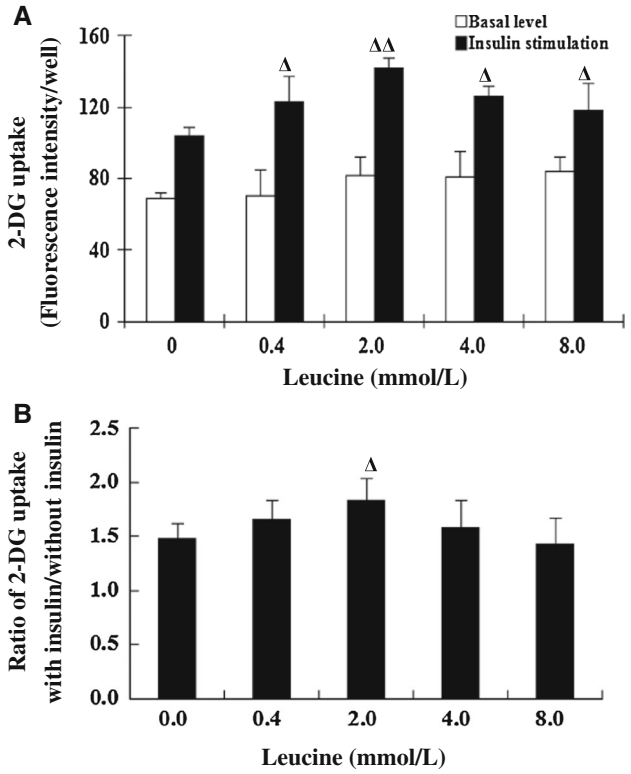

Fig. 1 Leucine facilitates the insulin-stimulated glucose uptake in a dose-dependent manner. The differentiated myotubes were incubated with 0, 0.4, 2.0, 4.0 or 8.0 mmol/L of leucine (Leu) in the presence or absence of 100 nmol/L insulin (Ins) for 45 min. **a** Glucose uptake was determined by a non-radioisotope, enzymatic fluorescence assay as described in "Materials and methods". Results represent the mean ± SD of six independent experiments. **b** Ratios of the insulin-induced glucose uptake over the basal level of glucose uptake were calculated and analyzed. $^{\Delta}p < 0.05$ vs. control group treated without leucine; $^{\Delta\Delta}p < 0.01$ vs. control group treated without leucine

Results

Leucine facilitates the insulin-stimulated glucose uptake in a dose-dependent manner in skeletal muscle cells

Primary skeletal muscle cells were isolated from rat and cultured as detailed in "Materials and methods". As shown in Suppl. Fig. 1, myoblasts proliferated after a 3-day culture (Suppl. Fig. 1A), and started to fuse into small multinucleated myotubes on day 7 (Suppl. Fig. 1B). Both number and size of the myotubes were increased through further fusion and differentiated when they became mature myotubes (Suppl. Fig. 1C) and striated (Suppl. Fig. 1D). Effects of leucine and insulin on glucose uptake in mature myotubes were then evaluated. As shown in Fig. 1a, leucine alone did not alter the basal level of glucose uptake but did increase the insulin-induced glucose uptake at 0.4, 2.0, 4.0 and 8.0 mmol/L ($p < 0.05$) with the peak effect at 2.0 mmol/L ($p < 0.01$). The ratios of the insulin-induced glucose uptake over the basal levels were calculated. As shown in Fig. 1b, the facilitating effect of leucine on the insulin-mediated glucose uptake reached a statistical significance at 2 mmol/L ($p < 0.05$). Therefore, 2.0 mmol/L of leucine was used as the optimal level in the following experiments.

Leucine promotes the insulin-stimulated glucose uptake in the presence of PI3-kinase inhibitor in skeletal muscle cells

To determine whether the facilitating effect of leucine in the insulin-stimulated glucose uptake is mediated by upstream components of the insulin signaling pathway, a PI3-kinase inhibitor (wortmannin) or an mTORC1 inhibitor (rapamycin) was used. As expected, insulin stimulated glucose uptake by twofold and wortmannin totally inhibited the insulin-stimulated glucose uptake in myotubes ($p < 0.05$, Fig. 2). Leucine further enhanced the insulin-induced glucose uptake by 15 % ($p < 0.05$, Fig. 2), and reversed inhibitory effect of wortmannin on the insulin-induced glucose uptake by 60 % ($p < 0.05$, Fig. 2). The same profile of glucose uptake was observed with LY294002, another PI 3-kinase inhibitor (Suppl. Fig. 2). Rapamycin alone had no effect on the insulin-stimulated glucose uptake, but eliminated the facilitating effect of leucine in the insulin-stimulated glucose uptake ($p < 0.05$, Fig. 2). Combination of wortmannin and rapamycin significantly decreased the insulin-mediated glucose uptake in the presence or absence of leucine.

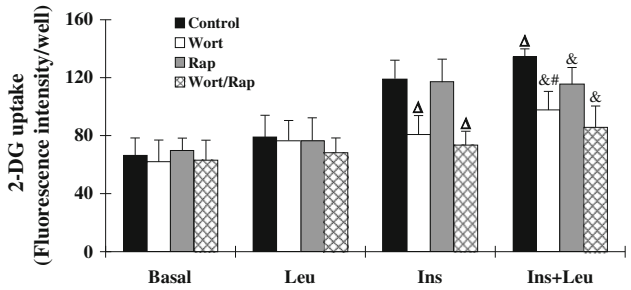

Fig. 2 Leucine facilitates the insulin-stimulated glucose uptake in the presence of PI3-K inhibitor wortmannin. The differentiated myotubes were pretreated with 100 nmol/L wortmannin (Wort) or/and 50 nmol/L rapamycin (Rap) as noted, and then treated with 2 mmol/L leucine (Leu) or/and 100 nmol/L insulin (Ins) as noted in the continuous presence of inhibitors for 45 min. Glucose uptake was determined by a non-radioisotope, enzymatic fluorescence assay as described in "Materials and methods". Results represent the mean ± SD of 6 independent experiments. $^{\Delta}p < 0.05$ vs. group treated with insulin alone; $^{\#}p < 0.05$ vs. group treated with insulin and wortmannin; $^{\&}p < 0.05$ vs. group treated with insulin and leucine

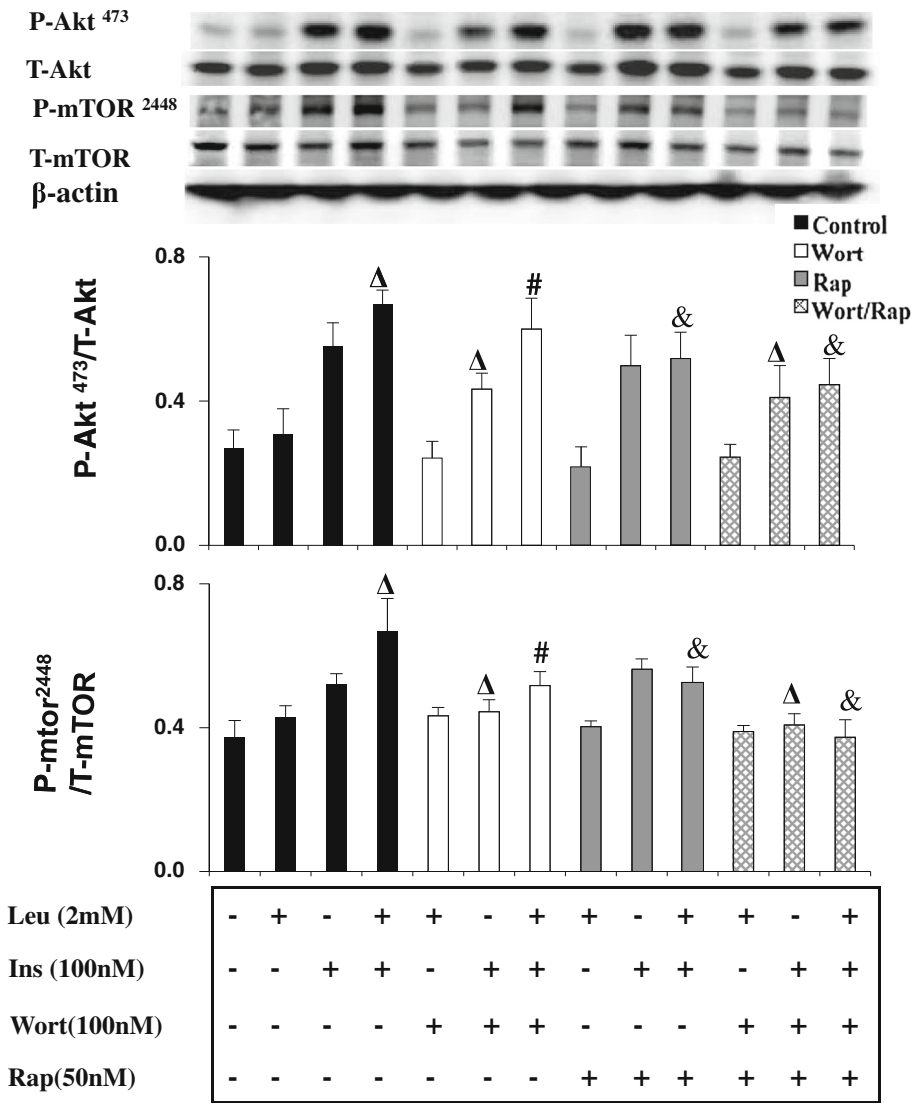

Fig. 3 The facilitating effect of leucine on insulin signaling is blunted by the mTORC1 inhibitor rapamycin but not by the PI3-K inhibitor wortmannin. The differentiated myotubes were pretreated with 100 nmol/L wortmannin (Wort) or/and 50 nmol/L rapamycin (Rap) as indicated, and then incubated with 2 mmol/L leucine (Leu) or/and 100 nmol/L insulin (Ins) as noted for 45 min. Levels of total and phosphorylated AKT (T-AKT and P-AKT), total and phosphorylated mTOR (T-mTOR and P-mTOR) were evaluated by immunoblottings with specific antibodies and quantified. Results represent the mean ± SD of three independent experiments. $^{\Delta}p < 0.05$ vs. group treated with insulin alone; $^{\#}p < 0.05$ vs. group treated with insulin and wortmannin; $^{\&}p < 0.05$ vs. group treated with insulin and leucine

The facilitating effect of leucine on insulin signaling is blunted by rapamycin, but not by wortmannin in skeletal muscle cells

To investigate the direct effect of leucine on insulin signaling, activations of AKT and mTOR were evaluated. As shown in Fig. 3, leucine alone did not stimulate AKT phosphorylation at residue 473 while insulin did. However, leucine alone caused a small but significant increase in mTOR phosphorylation at residue 2448 similarly as insulin alone. Addition of leucine enhanced the insulin-mediated phosphorylations of AKT^{473} and $mTOR^{2448}$ significantly ($p < 0.05$). The insulin-induced phosphorylations of AKT^{473} and $mTOR^{2448}$ were inhibited by wortmannin, and the inhibition was blunted by the presence of leucine. The insulin-induced phosphorylations of AKT^{473} and $mTOR^{2448}$ were not altered by rapamycin, but the facilitating effect of leucine on the insulin-stimulated phosphorylations of AKT^{473} and $mTOR^{2448}$ was blunted by rapamycin ($p < 0.05$). The insulin-induced phosphorylations of AKT^{473} and $mTOR^{2448}$ were suppressed by the combination of wortmannin and rapamycin significantly in the presence or absence of leucine. Together, these results demonstrate that insulin signaling is inhibited by wortmannin but the facilitating effect of leucine in insulin signaling is blunted by rapamycin, a specific inhibitor of mTORC1.

The facilitating effect of leucine on insulin signaling also involves mTORC2

It has been shown that AKT phosphorylation at residue 473 requires mTORC2. To determine whether mTORC2 was involved in the leucine facilitation of the insulin-induced AKT phosphorylation at residue 473, the core

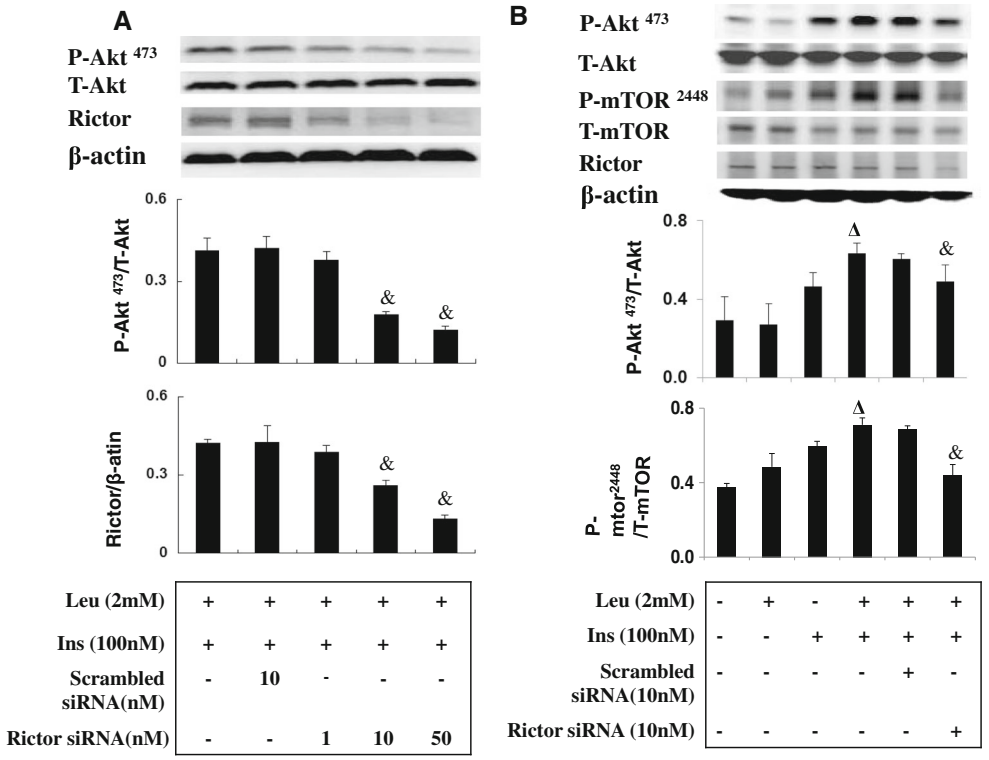

Fig. 4 The facilitating effect of leucine on insulin signaling also involves mTORC2. The C2C12 myoblasts were transfected with siRNAs against Rictor or a scrambled siRNA as described in "Materials and methods", and then treated with insulin (Ins) alone, leucine (Leu) alone, or insulin plus leucine for 45 min. Levels of rictor, total and phosphorylated AKT (T-AKT and P-AKT), and total and phosphorylated mTOR (T-mTOR and P-mTOR) were evaluated by immunoblottings with specific antibodies and quantified. **a** C2C12 myoblasts were transfected with different amounts of Rictor-siRNA (1, 10 and 50 nmol/L), and were treated with insulin or leucine as noted, followed by evaluations of AKT phosphorylation at serine-473. **b** C2C12 myoblasts were transfected with Rictor-siRNA or the scrambled siRNA, and then treated with insulin or leucine as noted, followed by evaluations of AKT phosphorylation at serine-473 and mTOR phosphorylation by immunoblotting with specific antibodies. Results represent the mean ± SD of at least 3 independent experiments. $^{\Delta}p < 0.05$ vs. group treated with insulin alone; $^{\&}p < 0.05$ vs. group treated with insulin and leucine

component of mTORC2, Rictor, was knocked down with specific siRNAs in C2C12 myoblasts. As shown in Fig. 4a and b, Rictor was knocked down efficiently by the specific siRNA but not by the scrambled siRNA. Knockdown of Rictor inhibited the phosphorylation of AKT^{473} induced by both insulin and leucine in a dose-dependent manner (Fig. 4a). Insulin alone and insulin plus leucine induced phosphorylation of AKT^{473} but leucine alone failed to do so (Fig. 4b). However, leucine alone, insulin alone, and leucine plus insulin all stimulated phosphorylation of $mTOR^{2448}$. It is noteworthy that leucine enhanced the insulin-stimulated phosphorylations of AKT^{473} and $mTOR^{2448}$. The facilitating effect of leucine on the insulin-induced phosphorylations of AKT^{473} and $mTOR^{2448}$ was significantly blocked by the specific siRNA against Rictor, but not by the scrambled siRNA ($p < 0.05$). These results demonstrate that leucine may facilitate the insulin-mediated AKT phosphorylation at residue 473 in skeletal muscle cells through a pathway involving mTORC2.

Discussion

Leucine, as a signaling nutrient, has been shown to facilitate the insulin-promoted anabolic processes, such as protein synthesis in skeletal muscle and the insulin-mediated glucose metabolism such as hepatic gluconeogenesis and glucose uptake in skeletal muscle and adipose tissues (Yoshizawa 2012; Hinault et al. 2004; Kleinert et al. 2011; Peyrollier et al. 2000). In this study, we provide evidence that leucine alone has no effect on glucose uptake but can facilitate the insulin-induced glucose uptake in primary cultured skeletal muscle cells. This observation appears to be conflicting with previous studies. Nishitani et al. reported that 2 mM leucine in the insulin-free medium stimulated glucose uptake on soleus muscles to the same extent as a physiological level of insulin (Nishitani et al. 2002). However, Nishitani's study was performed with freshly isolated soleus muscles from adult rats while our study was done with cultured primary skeletal muscle cells. Glucose uptake in soleus muscles in Nishitani's study

could not completely eliminate the influence of residual insulin. Our results are consistent with those from Bernard et al. In their study with isolated rat muscles, an mixture of amino acids increased glucose uptake significantly in the absence of insulin and enhanced the insulin-mediated glucose uptake (Kleinert et al. 2011); however, after the isolated rat muscles were washed thoroughly with perfusion, the same mixture of amino acids did not influence the basal level of glucose uptake while enhancing the insulin-induced glucose uptake (Bernard et al. 2011, 2013). Together, these results support the notion that leucine or amino acids alone may not be able to stimulate glucose uptake in skeletal muscle but can facilitate the insulin-stimulated glucose uptake.

It has been indicated that the mutual influences between leucine and insulin signaling pathways may be associated with modulations of the intracellular signaling components (Balage et al. 2001; Greiwe et al. 2001). The signaling pathway for protein synthesis regulated by leucine and insulin has been well studied. Specifically, insulin binds to its receptor and activates downstream components, such as AKT. AKT then activates S6K and protein synthesis via phosphorylation of mTOR. Leucine activates S6K through direct activation of mTOR without involving AKT. Thus, leucine and insulin promote protein synthesis in skeletal muscle through different mechanisms, and may synergistically promote S6K activation and protein synthesis when their levels are both increased (Greiwe et al. 2001). The synergistic effect of leucine and insulin in regulation of glucose metabolism may be different mechanistically. In this study, our results showed that leucine alone did not promote glucose uptake in cultured myotubes and is probably due to the failure of leucine alone to activate AKT. Interestingly, although leucine alone did not activate AKT and glucose uptake, it did enhance the insulin-stimulated AKT phosphorylation and glucose uptake. The enhancement was only partially blocked by wortmannin, indicating that leucine plays its role via a pathway that is independent of the classic insulin signaling cascade. The mTORC1 inhibitor, rapamycin, had no effect on the insulin-stimulated glucose uptake and AKT phosphorylation, but inhibited the facilitating effect of leucine in the insulin-stimulated glucose uptake and AKT phosphorylation. Therefore, mTORC1 may be involved in the facilitating effect of leucine. The mTOR-promoted activation of AKT at serine 473 has been shown to be mediated by mTORC2 (Sarbassov et al. 2005). Recent studies have suggested that mTORC2 phosphorylation may be regulated by mTORC1 and thus a new regulatory link between the two mTOR complexes has come to life (Julien et al. 2010). In this study, we found that the leucine-facilitated insulin activation of AKT^{473} was decreased by knocking down the core component of mTORC2, Rictor, suggesting that mTORC2 might be also involved in facilitation of insulin signaling by leucine. The role of mTORC2 in metabolic process has not been well documented. Tato et al. showed that amino acids activated both mTORC1 and mTORC2 and induced a rapid phosphorylation of AKT at Thr308 and Ser473. Whereas both phosphorylations were dependent on the presence of mTOR, only AKT phosphorylation at Ser473 was dependent on the presence of Rictor (Tato et al. 2011). These observations support our results that mTORC2 is involved in the intracellular signaling of amino acids such as leucine. Nevertheless, due to the possibility of off-target effects caused using the single siRNA targeting rictor, our findings need to be validated in more studies.

The facilitating effect of leucine in glucose uptake observed in this study disagrees with some previous reports that leucine stimulates glucose uptake in skeletal muscle independent of mTOR. Some studies have even shown that amino acid activation of the mTORC1/S6K1 pathway can decrease glucose uptake in skeletal muscle by causing insulin resistance via serine/threonine phosphorylation of IRS1 (Melnik 2012; Tremblay et al. 2005). However, application of rapamycin, an inhibitor of mTORC1 in the clinic as an effective immunosuppressant, was found to promote glucose intolerance and a diabetes-like syndrome (Sipula et al. 2006; Houde et al. 2010), implicating that a certain degree of mTORC1 activity is required for insulin signaling. Recent results from us and others have also shown that chronic leucine supplementation can improve activation of mTOR and AKT and insulin sensitivity in rodents on high-fat diet (Li et al. 2013; Zhang et al. 2007). Leucine supplementation does not necessarily cause IRS-1 serine phosphorylation, but actually leads to phosphorylation of IRS-1 at tyrosine 632 (Li et al. 2013), which may explain the increased insulin sensitivity (Gual et al. 2005). Therefore, mTOR, as a sensor of nutrients and cellular energy, is a two-side sword. Appropriate mTOR activity is necessary for the full activation of insulin signaling, but too much activation of mTOR will cause insulin resistance. Thus, the outcome of the amino acid-mediated activation of mTOR may vary and depends on many factors such as composition or concentration of amino acids (Hinault et al. 2004; Nishitani et al. 2002; Kleinert et al. 2011), the level of ambient insulin (Kleinert et al. 2011), and the composition of culture medium or buffer (Tato et al. 2011). Further investigations are required to optimize the conditions under which amino acids play a positive role in different models.

In summary, results from this study demonstrate that leucine alone does not promote insulin signaling or glucose uptake but can facilitate the insulin-induced insulin signaling and glucose uptake in skeletal muscle cells. The facilitating effect of leucine may involve both mTORC1 and mTORC2. These results shed new light on the

mechanism of interactions between leucine and insulin, and provide new evidence of potential role of leucine in improving insulin sensitivity and glucose homeostasis.

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Conflict of interest The authors declare that they have no competing financial interests.

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