

## Ketoisocaproic acid, a metabolite of leucine, suppresses insulin-stimulated glucose transport in skeletal muscle cells in a BCAT2-dependent manner

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**Moghei M, Tavajohi-Fini P, Beatty B, Adegoke OA.** Ketoisocaproic acid, a metabolite of leucine, suppresses insulin-stimulated glucose transport in skeletal muscle cells in a BCAT2-dependent manner. *Am J Physiol Cell Physiol* 311: C518–C527, 2016. First published August 3, 2016; doi:10.1152/ajpcell.00062.2016.—Although leucine has many positive effects on metabolism in multiple tissues, elevated levels of this amino acid and the other branched-chain amino acids (BCAAs) and their metabolites are implicated in obesity and insulin resistance. While some controversies exist about the direct effect of leucine on insulin action in skeletal muscle, little is known about the direct effect of BCAA metabolites. Here, we first showed that the inhibitory effect of leucine on insulin-stimulated glucose transport in L6 myotubes was dampened when other amino acids were present, due in part to a 140% stimulation of basal glucose transport ( $P < 0.05$ ). Importantly, we also showed that  $\alpha$ -ketoisocaproic acid (KIC), an obligatory metabolite of leucine, stimulated mTORC1 signaling but suppressed insulin-stimulated glucose transport ( $-34\%$ ,  $P < 0.05$ ) in an mTORC1-dependent manner. The effect of KIC on insulin-stimulated glucose transport was abrogated in cells depleted of branched-chain aminotransferase 2 (BCAT2), the enzyme that catalyzes the reversible transamination of KIC to leucine. We conclude that although KIC can modulate muscle glucose metabolism, this effect is likely a result of its transamination back to leucine. Therefore, limiting the availability of leucine, rather than those of its metabolites, to skeletal muscle may be more critical in the management of insulin resistance and its sequelae.

insulin resistance; leucine;  $\alpha$ -ketoisocaproic acid; glucose transport; mTORC1

TYPE 2 diabetes mellitus (T2DM) is a global health concern and a major risk factor for morbidity and mortality. It is preceded by insulin resistance, a condition that is characterized by a reduction in the body's sensitivity to insulin (7, 41). Many factors can contribute to the development of insulin resistance and T2DM, with nutrition being one of the major ones. In addition to high-fat diets, high-protein diets have been linked to the development of insulin resistance and T2DM (18, 33).

Dietary protein and amino acids, in particular the branched-chain amino acids (BCAAs: leucine, isoleucine, and valine), have many positive metabolic effects, including regulation of body weight [especially in obese individuals (6, 23, 49)], skeletal muscle protein synthesis (22, 29), and glucose homeostasis (11, 30, 48). BCAAs, particularly leucine, stimulate glucose transport and improve insulin sensitivity in skeletal muscle (16, 19, 28). The effects of leucine and other BCAAs in regulating protein synthesis and skeletal muscle mass are mediated at least in part via the activation of the mammalian/mechanistic target of rapamycin complex 1 (mTORC1) (22).

Despite their beneficial effects, high plasma concentrations of BCAAs are associated with insulin resistance and a higher risk of T2DM (1, 20, 24, 27). This link is at least in part explained by the fact that activated mTORC1 can impair insulin signaling via the inhibitory serine phosphorylation of insulin receptor substrate 1 (IRS1) (12, 23, 44, 45). Given the fact that other studies have shown that leucine stimulates glucose transport and improves insulin sensitivity in skeletal muscle (16, 19, 28), controversy still exists as to if and how leucine is involved in the development of insulin resistance and T2DM. The controversy may be attributed to the concentrations of leucine used in those studies. It is also not clear if the presence of other amino acids would modulate the effect of leucine.

In addition to the amino acids themselves, metabolomics studies have linked elevated blood levels of BCAA metabolites to insulin resistance and T2DM (1, 24, 27). High concentration of the leucine metabolite,  $\alpha$ -ketoisocaproic acid (KIC), is associated with insulin resistance and T2DM in humans (31) and animals (9). Although KIC can activate mTORC1, stimulate the protein synthesis pathway (8, 21, 50), and induce insulin secretion (25), much less is known about its direct effect on insulin action in skeletal muscle, except for a study that showed that at supraphysiological concentrations (up to 2 mM) KIC stimulates glucose transport in skeletal muscle (28).

Here, we showed that the effects of leucine on insulin-stimulated glucose transport and activation of mTORC1 in skeletal muscle cells were modulated by the presence of other amino acids in the incubation medium. Importantly, we demonstrated that KIC inhibited insulin-stimulated glucose transport and that this required mTORC1 activity. Finally, we showed that in cells depleted of BCAT2, the effect of KIC was abrogated.

### MATERIALS AND METHODS

**Reagents.** Alpha Modification of Eagle's Medium (AMEM) and antibiotic-antimycotic preparations were purchased from Wisent (St Bruno, Quebec, Canada); fetal bovine serum (FBS), horse serum, Lipofectamine RNAiMAX and Opti-MEM 1X Reduced Serum Medium from Thermo Fisher Canada (Burlington, Ontario Canada); leucine- (no. R8999-12) and amino acid-free (no. R8999-04A) RPMI 1640 media from US Biologicals (Salem MA); L-leucine, sodium 4-methyl-2-oxovalerate (KIC), 2-deoxyglucose, rapamycin, protease inhibitor cocktail, phosphatase inhibitor cocktail, anti-BCAT2 and anti-gamma tubulin antibodies, and siRNA oligonucleotides from Sigma Aldrich (Oakville, Ontario, Canada). Phospho (ph) S6K1 (T389), ph-IRS1 (S612 or S616 for the human protein), ph-S6 (S235/236), ph-Akt (S473), horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse secondary antibodies were obtained from Cell Signaling Technology (Danvers, MA). Phospho-tyrosine IRS1 (Y612) was from Abcam (Cambridge, MA) while [ $^3$ H]-2-deoxyglucose was obtained from Perkin Elmer (Markham, Ontario, Canada). HRP substrate was obtained from Millipore (Etobicoke, Ontario, Canada).

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**Cell culture.** L6 rat skeletal muscle myoblasts (American Type Culture Collection) were cultured in 6-well plates in growth medium (AMEM supplemented with 10% FBS and 1% Antibiotic-Antimycotic preparations) until they reached ~90–100% confluency. Cells were then switched into the differentiation medium (AMEM, 2% horse serum, 1% antibiotic-antimycotic preparations) and replenished with fresh medium every 48 h. Myotubes were used on day 5 of differentiation.

**Treatment with leucine or KIC.** Myotubes were starved for 4 h in serum- and amino acid-free RPMI medium. They were then incubated either in an RPMI medium that contained all amino acids except leucine, or in the same medium supplemented with different leucine concentrations (150, 350, 600, 800, and 1,800  $\mu\text{M}$ ) for 30 min. Cells were then cultured in fresh medium supplemented with 100 nM insulin for 20 min. Myotubes were then harvested or used for glucose transport assay (see below). Additional experiments were carried out as above but with leucine supplementation being done in amino acid-free RPMI medium. For experiments with KIC, following the 4 h starvation in amino acid-free medium, myotubes were cultured for 30 min in amino acid-free medium supplemented with KIC (0, 200, and 400  $\mu\text{M}$  final concentrations). In other experiments, incubation with KIC was carried out in the presence of 50 nM rapamycin, an inhibitor of mTORC1.

**Glucose transport.** Cells were cultured in 12-well plates. Following treatments, myotubes were rinsed twice with HEPES [4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid]-buffered saline. They were then incubated in 300  $\mu\text{l}$  of transport solution (HEPES buffer, pH 8, 10  $\mu\text{M}$  2-deoxyglucose, 0.5  $\mu\text{Ci/ml}$  [ $^3\text{H}$ ]-2-deoxyglucose) for 5 min at 37°C, as described previously (14).

**siRNA gene silencing.** L6 myoblasts were plated in 6- or 12-well plates. On day 3 of differentiation, they were transfected with 50 nM of BCAT2 siRNA oligonucleotides (no. 1: sense 5'-GAGUGCAUC-CGCCAGCUCA, anti-sense 5'-UGAGCUGGCGGAUGCACUC; or no. 2: sense 5'-CUAUGUGCGGCCGGUGCUU, anti-sense 5'-AAGCACCGGCCGCACAUAU), or 50 nM of scrambled siRNA oligonucleotides using Lipofectamine RNAiMAX reagent according to the manufacturer's instructions. On day 5 of differentiation (48 h following transfection), myotubes were subjected to KIC treatment as described above. Cells were then used for glucose transport or Western blot analysis.

**Western blot analysis.** Following treatments, cells were processed as described previously (14, 51). Briefly, cells were harvested in lysis buffer [1 mM EDTA, 2% sodium dodecyl sulfate (SDS), 25 mM Tris-HCl, pH 7.5, 1 mM DTT, and 10  $\mu\text{l/ml}$  of each of protease inhibitor and phosphatase inhibitor cocktails]. Equal amounts of protein were separated on 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer onto polyvinylidene difluoride (PVDF) membranes. Incubation in primary and secondary antibodies, image acquisition, and quantification were as described (14, 51).

**Data presentation and statistical analyses.** Glucose transport data, calculated as picomoles per microgram protein, were normalized to the control group, the group that was not treated with amino acids/KIC or insulin (CTL-ins). In Fig. 2F, glucose transport data were expressed as picomoles of deoxyglucose per micrograms protein. Western blots for ph-S6K1, ph-IRS1, and ph-Akt were normalized to the insulin control group [not treated with amino acid/KIC, but treated with insulin (CTL + ins)]. This was because in some experiments (for example Fig. 2B), CTL-ins signal was barely detectable.

Data are presented as means  $\pm$  SE. Where the data were normalized (Fig. 1, A–E, Fig. 2, A–E, Fig. 3, A–D, Fig. 4, A–D, and Fig. 5, B–E), column statistics with a one-sample *t*-test was used to test treatment effects. One-way analysis of variance (ANOVA) was used to test treatment effects in Fig. 2, F and G, and Fig. 5A; when the effects were significant, Tukey's post hoc tests were done to identify significant differences among means. Where we sought to examine insulin effect, Student's two-tailed *t*-tests were performed. Signifi-

cance was determined as  $P < 0.05$ . Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, San Diego CA).

## RESULTS

**Leucine suppresses insulin-stimulated glucose transport, but only in the absence of other amino acids.** Because dietary proteins contain amino acids other than leucine, we sought to determine how the presence of other amino acids (medium composition) affects insulin-stimulated glucose uptake in L6 myotubes, since glucose uptake and metabolism in skeletal muscle is a key factor in whole body glucose homeostasis and muscle insulin resistance is a hallmark of T2DM (32, 41). In the presence of other amino acids, leucine (0–1,600  $\mu\text{M}$ ) did not significantly alter insulin-stimulated glucose transport, although there was a trend toward increased basal glucose transport (Fig. 1A). Under this condition, leucine increased basal (2.5- to 4-fold) and insulin-stimulated (40–65%) phosphorylation of S6K1<sup>T389</sup> at all concentrations tested ( $P < 0.05$  for 150, 350, 600, and 800  $\mu\text{M}$  of leucine and  $P = 0.09$  for 1,600  $\mu\text{M}$ , Fig. 1B). Interestingly, within the control (leucine-free) group, insulin caused a 9X increase in ph-S6K1<sup>T389</sup> levels while insulin effect in the leucine groups ranged from 1.5 to 3X, indicating a suppression of insulin effect by leucine. Basal and insulin-stimulated phosphorylation of IRS1<sup>Ser616</sup> (S612 in mouse sequence) showed similar trends (Fig. 1C). A lack of effect of insulin on IRS1 serine phosphorylation in the leucine groups, robustly (3X) seen in the control group, is likely due to increased basal IRS1 serine phosphorylation. Insulin caused a 3X increase in ph-IRS1<sup>Ser616</sup> in the control group; however, within the leucine groups, the effect of insulin ranged from 80 to 140%. Whereas insulin stimulated tyrosine phosphorylation of IRS1 under leucine free-conditions, insulin effect was not significant in groups treated with leucine, except at 350 and 1,600  $\mu\text{M}$  (Fig. 1D). The effect of insulin on AKT<sup>S473</sup> was preserved irrespective of [Leu] (Fig. 1E). Therefore, although it did not affect glucose transport, leucine increased basal and insulin-stimulated phosphorylation of mTORC1 pathway substrates and impaired insulin-stimulated tyrosine phosphorylation of IRS1 but without an effect on AKT phosphorylation.

When studied alone, leucine at all concentrations tested suppressed insulin-stimulated glucose transport ( $P < 0.05$  for 150, 350, and 600  $\mu\text{M}$  of leucine and  $P = 0.06$  for 800 and 1,600  $\mu\text{M}$ , Fig. 2A), although the effect appeared strongest at 150  $\mu\text{M}$ , where there was a 41% reduction in glucose transport ( $P < 0.01$ ). There was no significant effect of leucine on basal glucose transport except at 150  $\mu\text{M}$ . The effect of leucine appeared linked to the activation of mTORC1 as the amino acid increased S6K1<sup>T389</sup> phosphorylation, especially at 150, 800, and 1,600  $\mu\text{M}$  ( $P < 0.05$ , Fig. 2B). Interestingly, unlike in Fig. 1B, basal phosphorylation of S6K1 was barely detectable, irrespective of the leucine concentration. Leucine in the presence of insulin also stimulated IRS1<sup>Ser612</sup> phosphorylation, but the effects were more variable and therefore not significant (Fig. 2C). As in Fig. 1D, insulin robustly stimulated IRS1<sup>Y612</sup> phosphorylation in the absence of leucine but its effect was insignificant or highly variable in leucine-treated groups, except at 150 and 600  $\mu\text{M}$  (Fig. 2D). Leucine did not have an effect on ph-Akt<sup>S473</sup> levels in the presence or absence of insulin (Fig. 2E).

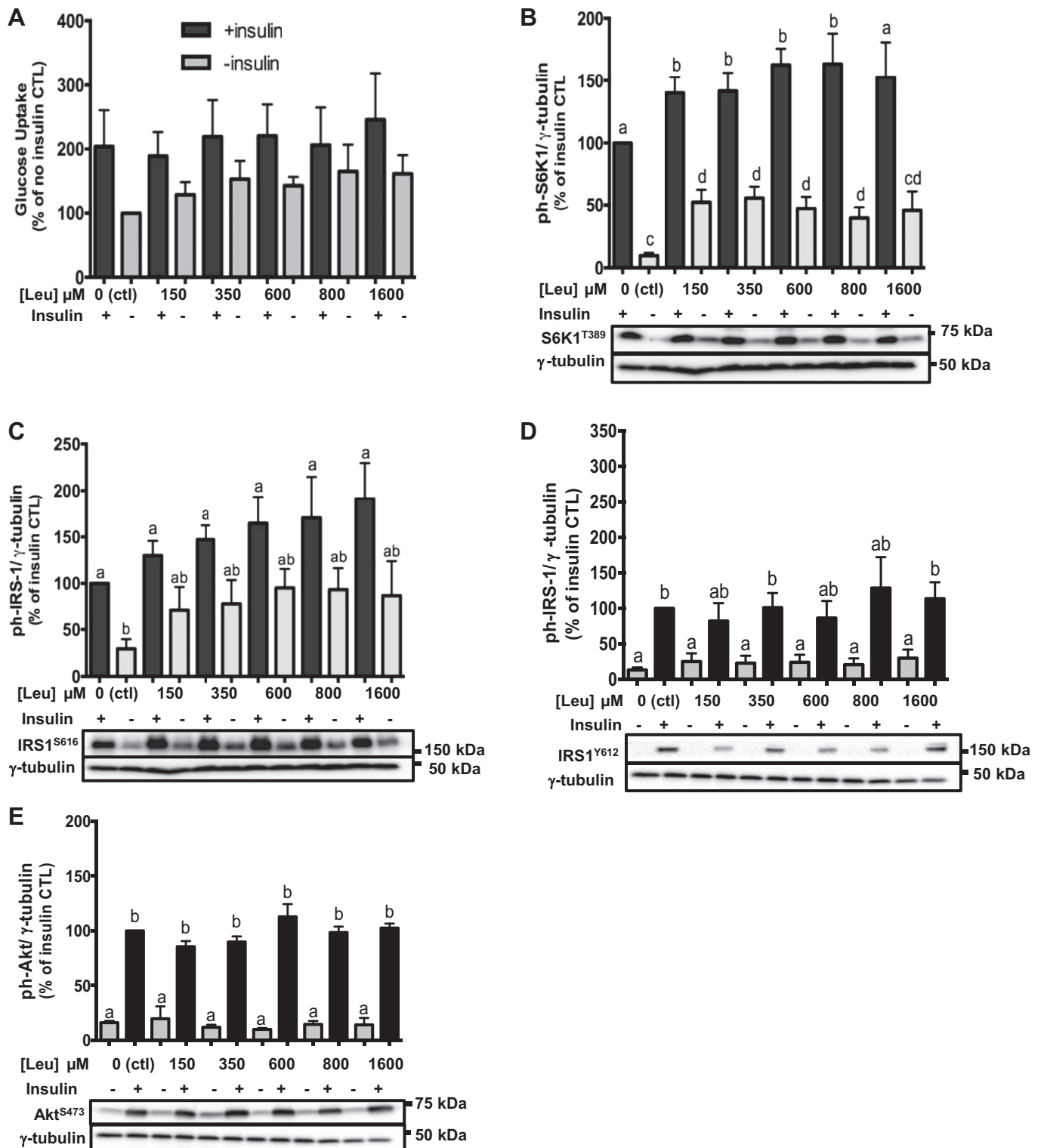


Fig. 1. Glucose uptake and mTORC1 signaling in response to different leucine concentrations in the presence of other amino acids. L6 myotubes were starved for 4 h in serum- and amino acid-free RPMI medium. They were then incubated in a medium that contained all amino acids except leucine and then supplemented with various [Leu] for 30 min. Finally, the myotubes were incubated for another 20 min in the presence or absence of 100 nM of insulin, followed by glucose uptake assay or Western blot analysis. A: glucose transport, expressed as %CTL (no amino acids or insulin), is not different among groups. Graphical representation and Western blot images of ph-S6K1<sup>T389</sup> (B), ph-IRS1<sup>Ser616</sup> (C), ph-IRS1<sup>Y612</sup> (D), and ph-Akt<sup>S473</sup> (E). Data are presented as % CTL (no amino acids + ins). In A–E, data are means  $\pm$  SE;  $n = 3$  independent experiments with 3–6 replicates per treatment within each experiment. Bars with different letters are significantly different ( $P < 0.05$ ). In B, the effect of insulin is significant in all groups. In the presence and absence of insulin, CTL is significantly different from all groups except 1,600  $\mu$ M Leu. In C, insulin effect is significant only in CTL. In D, the effect of insulin is significant at 0, 350, and 1,600  $\mu$ M Leu. In E, while insulin effect is significant in all groups, other treatments are not.



We used data from Figs. 1A and 2A to compare basal and insulin-stimulated glucose transport in their respective control medium [a medium that contained all amino acids except leucine (Fig. 1A), or amino acid-free medium (Fig. 2A)]. When expressed in absolute units, amino acids (without leucine) significantly increased basal glucose transport compared with incubation in the amino acid-free medium (140% increase, Fig. 2F). As a result, insulin effect on glucose transport was dampened in the presence of amino acids (Fig. 2F). Similar effects were seen in S6K1 phosphorylation, whereby basal phosphorylation of the protein is seen only in the presence of amino acids. Unlike the glucose transport data, however, the insulin effect remained robust (Fig. 2G).

*Effect of KIC on glucose transport and mTORC1 signaling.* We next examined the effect of the obligatory leucine metabolite, KIC, on glucose transport and mTORC1 signaling. Supplementation with 200  $\mu$ M of KIC suppressed insulin-stimulated glucose transport by 34% ( $P < 0.05$ , Fig. 3A). It also suppressed basal glucose transport. The effect of KIC at a higher concentration (400  $\mu$ M) was not significant.

Like leucine, the effect of KIC on glucose transport occurred in parallel with increased insulin-stimulated phosphorylation of S6K1<sup>T389</sup> (~4X, Fig. 3B) and of IRS1<sup>Ser612</sup> (55%, Fig. 3C). Basal IRS1<sup>Ser612</sup> too was higher. KIC did not modify the effect of insulin on Akt<sup>Ser473</sup> phosphorylation (Fig. 3D).

*KIC-mediated suppression of insulin-stimulated glucose transport requires mTORC1 functions.* If the suppression of insulin-stimulated glucose transport by KIC is mediated by mTORC1, inhibiting mTORC1 signaling should ameliorate the effect. As shown before, 200  $\mu$ M of KIC suppressed insulin-stimulated glucose transport ( $P < 0.05$ , Fig. 4A). However, coincubation with 50 nM of rapamycin, an mTORC1 inhibitor, attenuated the inhibitory effect of KIC. S6K1<sup>T389</sup> phosphorylation was completely abolished while phosphorylation of S6K1 substrates ribosomal protein S6 (S6<sup>Ser235/236</sup>) and IRS1<sup>Ser612</sup> was suppressed (Fig. 4, B–D).

*KIC-mediated suppression of insulin-stimulated glucose transport in myotubes requires BCAT2.* Since transamination of leucine to its metabolite KIC is reversible, one could speculate that KIC inhibition of insulin-stimulated glucose transport is merely a proxy effect of leucine. This possibility is underlined by the fact that, under basal conditions, myotubes incubated with KIC and leucine have similar BCAA concentrations ( $113 \pm 9$  and  $112 \pm 3$  mM, respectively); both were higher than the concentration in control group ( $43 \pm 4$   $\mu$ M,  $P < 0.0001$ ). In the presence of insulin, BCAA concentrations were higher in myotubes incubated with KIC ( $160 \pm 6$   $\mu$ M) compared with those incubated with leucine ( $128 \pm 7$   $\mu$ M,  $P < 0.01$ ); both were higher than in control (no leucine or KIC) group ( $69 \pm 4$   $\mu$ M,  $P < 0.0001$ ). Therefore, to examine the contribution of leucine to the effect of KIC, we used RNA interference (RNAi) to knock-down BCAT2 (Fig. 5A). Depletion of BCAT2 attenuated the effect of KIC on insulin-induced glucose transport (Fig. 5B) and suppressed the insulin-stimulated increases in S6K1<sup>T389</sup> (Fig. 5C) and IRS1<sup>Ser612</sup> (Fig. 5D) phosphorylation observed in KIC-treated cells. Unexpectedly, Akt<sup>Ser473</sup> phosphorylation was also suppressed (Fig. 5E).

## DISCUSSION

Whereas recent metabolomics studies have linked elevated levels of BCAA and their metabolites to insulin resistance and development of T2DM, cause-and-effect studies are lacking. Specifically, it is unknown if the implicated metabolites in themselves cause insulin resistance, or if they merely reflect the condition. Using skeletal muscle-derived L6 myotubes, we demonstrated that 1) the suppressive effect of leucine on insulin-stimulated glucose transport is modulated by the presence of other amino acids; 2) KIC suppresses insulin-stimulated glucose transport and that this effect requires mTORC1 activity, and 3) the effect of KIC on glucose transport requires BCAT2, suggesting that the effects of KIC are merely proxies of those of leucine.

Earlier studies on the effect of amino acids on insulin sensitivity typically studied individual amino acids. Because these nutrients are consumed in the context of a meal or at least in proteins and therefore in the presence of other amino acids, our studies showing that the presence of other amino acids modulate the effect of leucine reflect what likely occurs in vivo. Nevertheless, studies on the effects of individual amino acid on insulin actions are relevant because of the increasing prevalence of the use of individual amino acids either because of their anabolic effects (5, 34) or to take advantage of specific health benefits (4, 26, 43). Such studies are also useful in examining the likely upper toxicity effects of individual amino acids, especially for leucine, an amino acid that appears unique in terms of its effect on metabolism.

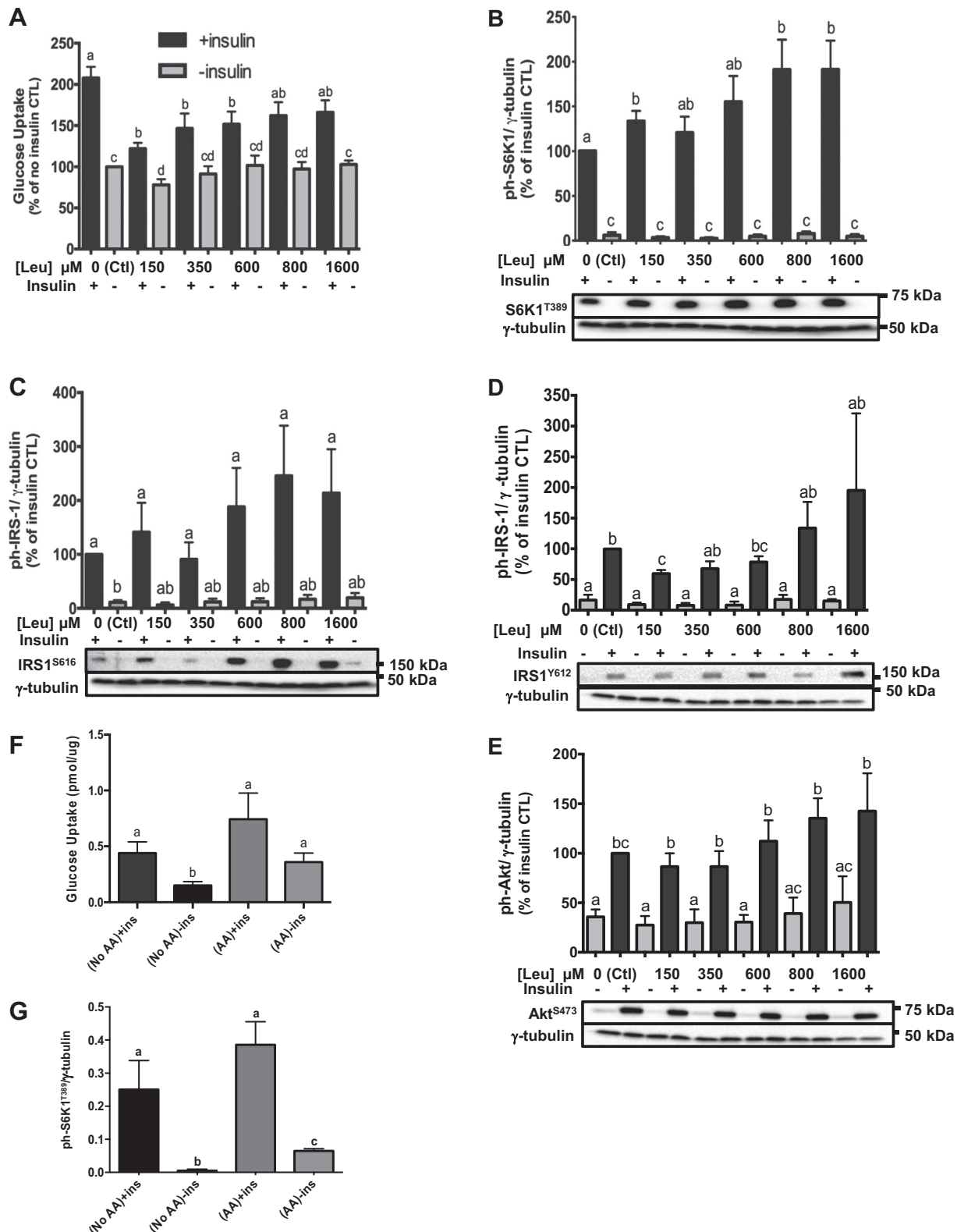
In our study, a lack of effect of leucine in the presence of other amino acids was due to an augmentation of basal (insulin-free) glucose transport (see Fig. 2F). Insulin-independent glucose disposal, especially as can be modulated by exercise, is a critical component of whole body glucose disposal (3, 35). Little is known about the effects of amino acids on this, although one study showed that leucine stimulated contraction-induced increase in glucose transport in isolated muscles (13). Consistent with the significance of the contribution of basal glucose transport to overall muscle glucose uptake, a recent report showed that pharmacological inhibition of S6K1 increases basal, but not insulin-stimulated, glucose transport in L6 myotubes (40).

Interestingly, the suppressive effect of leucine on insulin-stimulated glucose uptake was maximal at 150  $\mu$ M. Other studies have reported a stimulatory effect of leucine and other amino acids on insulin-stimulated glucose transport but at supraphysiological concentrations (19, 28, 36). In mice lacking BCAT2, in which concentrations of the BCAAs were elevated from a total of ~300  $\mu$ M to ~7 mM, insulin sensitivity increased rather than worsened (38). This is contrary to an obesity-associated increase in the levels of these amino acids where such elevations are associated with poor insulin sensitivity (1, 20, 24). Therefore, at supraphysiological concentrations of leucine and perhaps other amino acids, additional mechanisms, perhaps including simultaneous activation of both protein synthesis and proteolysis (38), are activated to promote glucose utilization and therefore transport.

Previous studies have implicated mTORC1 signaling in the negative effect of leucine on insulin signaling. This is because leucine, especially in the presence of insulin, activates mTORC1/S6K1 signaling, which leads to inhibitory serine

(S302, S307, S612, S1101) phosphorylation of IRS1 (10). Although our data are in general agreement with previous data, we note some differences. In cells incubated with only leucine, stimulation of S6K1<sup>T389</sup> phosphorylation occurred in parallel with suppression of insulin-stimulated glucose

transport. In the presence of other amino acids, however, leucine stimulated basal and insulin-induced S6K1<sup>T389</sup> phosphorylation, even though there was no effect on glucose transport. Under these conditions, there were only modest effects on IRS1(S/Y), and leucine-induced impairment of



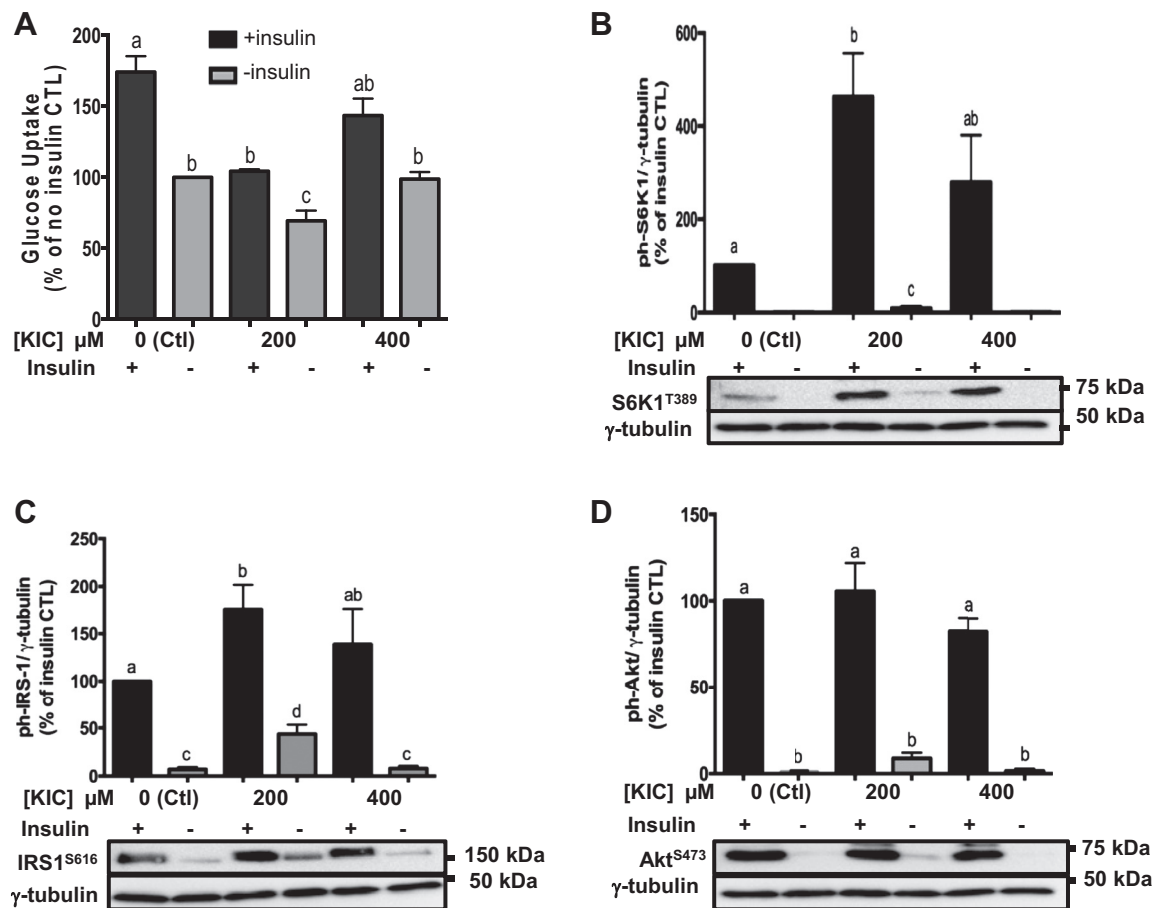


Fig. 3. KIC suppresses insulin-stimulated glucose transport in parallel with increased mTORC1 activity in myotubes. Myotubes were starved for 4 h as described in Fig. 1. They were then treated with 200 or 400  $\mu$ M of KIC for 30 min followed by incubation in 100 nM insulin for 20 min. Glucose transport was then measured, or myotubes were harvested and used for Western blot analysis. A: glucose transport is expressed as %CTL (no KIC or insulin). Means  $\pm$  SE;  $n = 4$  independent experiments with 3–6 replicates per treatment within each experiment. Insulin effect is significant at 0 and 200  $\mu$ M KIC. In the presence and absence of insulin, CTL is significantly different from 200  $\mu$ M KIC. Graphical representation and Western blot images of ph-S6K1<sup>T389</sup> (B), ph-IRS1<sup>Ser612</sup> (C), ph-Akt<sup>Ser473</sup> (D). Data are expressed as % CTL (no KIC + insulin). In B–D, data are means  $\pm$  SE;  $n = 3$  independent experiments with 3–6 replicates per treatment within each experiment. Bars with different letters are significantly different ( $P < 0.05$ ). In B and C, the effect of insulin is significant in all groups. In the presence and absence of insulin, CTL is significantly different from 200  $\mu$ M KIC. In D, the effect of insulin is significant in all groups.

insulin-stimulated IRS1 tyrosine phosphorylation was not always associated with impairment of glucose transport (Fig. 1, A and D). In all conditions tested, there was no effect on AKT (S473) phosphorylation. These data suggest a role for additional factors, other than S6K1-IRS1 axis, in mediating the effects of leucine on insulin-stimulated glucose transport in skeletal muscle.

Elevated levels of metabolites of BCAA are seen in blood samples of obese and T2DM patients (1, 24, 27). It is unknown

whether these metabolites have a direct effect on insulin sensitivity. We showed here that KIC suppressed insulin-stimulated glucose transport in muscle cells, one of a few peripheral cell types in which this compound is formed. In Figs. 3A, 4A, and 5B, we noticed a consistent trend toward reduced basal glucose transport in KIC-treated cells, even though those experiments were performed under diverse conditions. When we pulled relevant groups from these studies and analyzed them together, basal glucose transport was sup-

Fig. 2. Leucine, in the absence of other amino acids, impairs insulin-stimulated glucose in parallel with increased activation of mTORC1 in myotubes. L6 myotubes were treated as in Fig. 1, except that incubation in various [Leu] was done in an RPMI medium that did not contain any other amino acid. A: glucose transport is expressed as %CTL (no amino acids or insulin). Means  $\pm$  SE;  $n = 4$  independent experiments with 3–6 replicates per treatment within each experiment. The effect of insulin is significant in all groups. In the presence of insulin, CTL is significantly different from 150  $\mu$ M ( $P < 0.01$ ), 350  $\mu$ M, and 600  $\mu$ M Leu ( $P < 0.05$ ). In the absence of insulin, CTL is significantly different from 150  $\mu$ M Leu. Graphical representation and Western blot images of ph-S6K1<sup>T389</sup> (B), ph-IRS1<sup>Ser616</sup> (C), ph-IRS1<sup>Y612</sup> (D), and ph-Akt<sup>S473</sup> (E). An amino acid mixture that lacked leucine enhances basal glucose uptake (F) and ph-S6K1<sup>T389</sup> levels (G). In B–G, data are means  $\pm$  SE;  $n = 3$  independent experiments with 3–6 replicates per treatment within each experiment. Bars with different letters are significantly different ( $P < 0.05$ ). In B, the effect of insulin is significant in all groups. In the presence of insulin, CTL is significantly different from 150, 800, and 1,600  $\mu$ M Leu, whereas in the basal state (absence of insulin) there is no significant difference between groups. In C, the effect of insulin is significant only in CTL. In D, the effect of insulin is significant at 0, 150, and 600  $\mu$ M Leu. In the presence of insulin but in not in basal, CTL is significantly different from 150  $\mu$ M Leu. In E, while insulin effect is significant, other treatments are not.

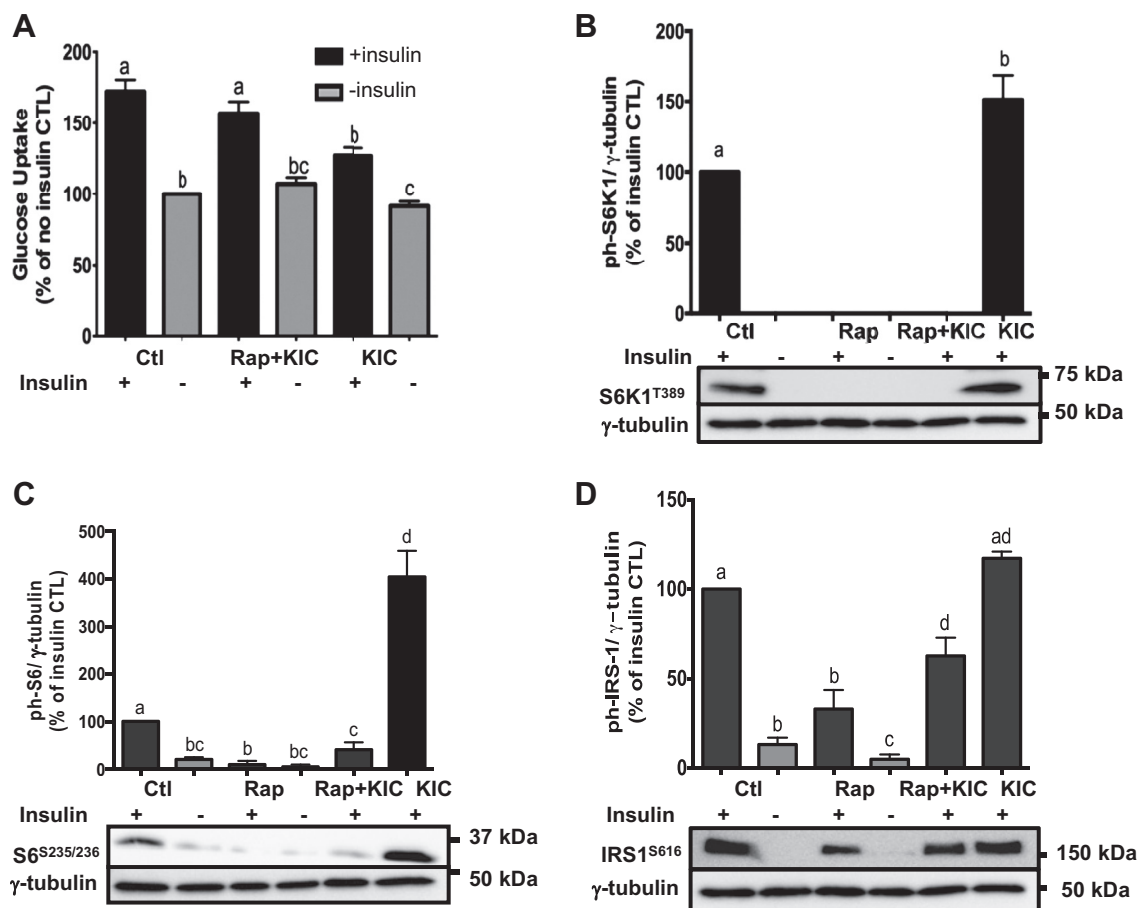


Fig. 4. A: KIC-mediated suppression of insulin-stimulated glucose transport requires mTORC1 activity. Following starvation as described in Fig. 3, myotubes were incubated in 200  $\mu$ M KIC with or without 50 nM rapamycin (Rap) for 30 min. They were then incubated in 100 nM insulin for another 20 min, followed by glucose transport assay or Western blot analysis. A: glucose transport is expressed as %CTL (no KIC or insulin). Insulin effect is significant in all groups. In insulin-stimulated cells, CTL is significantly different from KIC. Graphical representation and Western blot images of ph-S6K1<sup>T389</sup> (B), ph-S6<sup>Ser235/236</sup> (C), and ph-IRS1<sup>Ser612</sup> (D). In B–D, data are expressed as %CTL (no KIC + insulin). Means  $\pm$  SE;  $n = 3$  independent experiments with 3–6 replicates per treatment within each experiment. Bars with different letters are significantly different ( $P < 0.05$ ). In B–D, in the presence of insulin, CTL is significantly different from KIC (B and C), Rap, and Rap + KIC (C and D).

pressed by 28% in KIC-treated groups ( $P < 0.001$ ,  $n = 10$ ). This likely explains some of the effect of KIC on insulin-stimulated glucose transport.

KIC, like leucine, can activate mTORC1 signaling (8) and the observed effects of KIC on insulin-stimulated glucose transport occurred in parallel with the activation of mTORC1/S6K1 signaling. Furthermore, as for leucine (14, 27, 46), our inhibition studies implicated mTORC1 in the KIC-induced suppression of insulin-stimulated glucose transport.

Because KIC can be transaminated back to leucine, it was necessary to ascertain whether the effects observed were due to KIC or if the metabolite was acting as proxy for leucine. Our finding of a requirement for BCAT2 for KIC's effect on glucose transport and mTORC1 activity is consistent with another study attributing the stimulatory effect of KIC on insulin secretion to its transamination back to leucine (52). Given the negative effect of KIC-mediated activation of mTORC1 on insulin action, an increase in Akt<sup>S473</sup> phosphorylation would have been expected in cells treated with KIC in BCAT2-knockdown background. We did not observe this (Fig. 5E), a finding consistent with a recent study that showed that the suppressive effect of dietary protein on leg glucose disposal

is independent of any changes in Akt phosphorylation (42). This may suggest additional mechanisms for the effect of KIC on insulin action.

A limitation of this work is that it is an *in vitro* study. Although it would be necessary to see whether KIC inhibits insulin sensitivity *in vivo* and whether such effects are modulated by BCAT2, deduction of KIC effects in muscle in such studies would unavoidably be complicated by contributions of other tissues and growth factors. In addition, it would be useful to examine the effect of KIC in the background of inflammation, since the link between elevated metabolites of BCAA and insulin resistance is usually seen in obesity and T2DM, conditions associated with elevated circulating levels of inflammatory factors (15, 39, 47).

In conclusion, we demonstrated that the effect of leucine on insulin sensitivity of glucose transport was modulated by the presence of other amino acids because of the effect of other amino acids on basal glucose transport. We also showed that KIC, in an mTORC1-dependent manner, inhibited insulin-stimulated glucose transport and that this inhibition was relieved in cells depleted of BCAT2. Because liver (2, 37) and muscle (17) activity of branched-chain keto acid dehydroge-



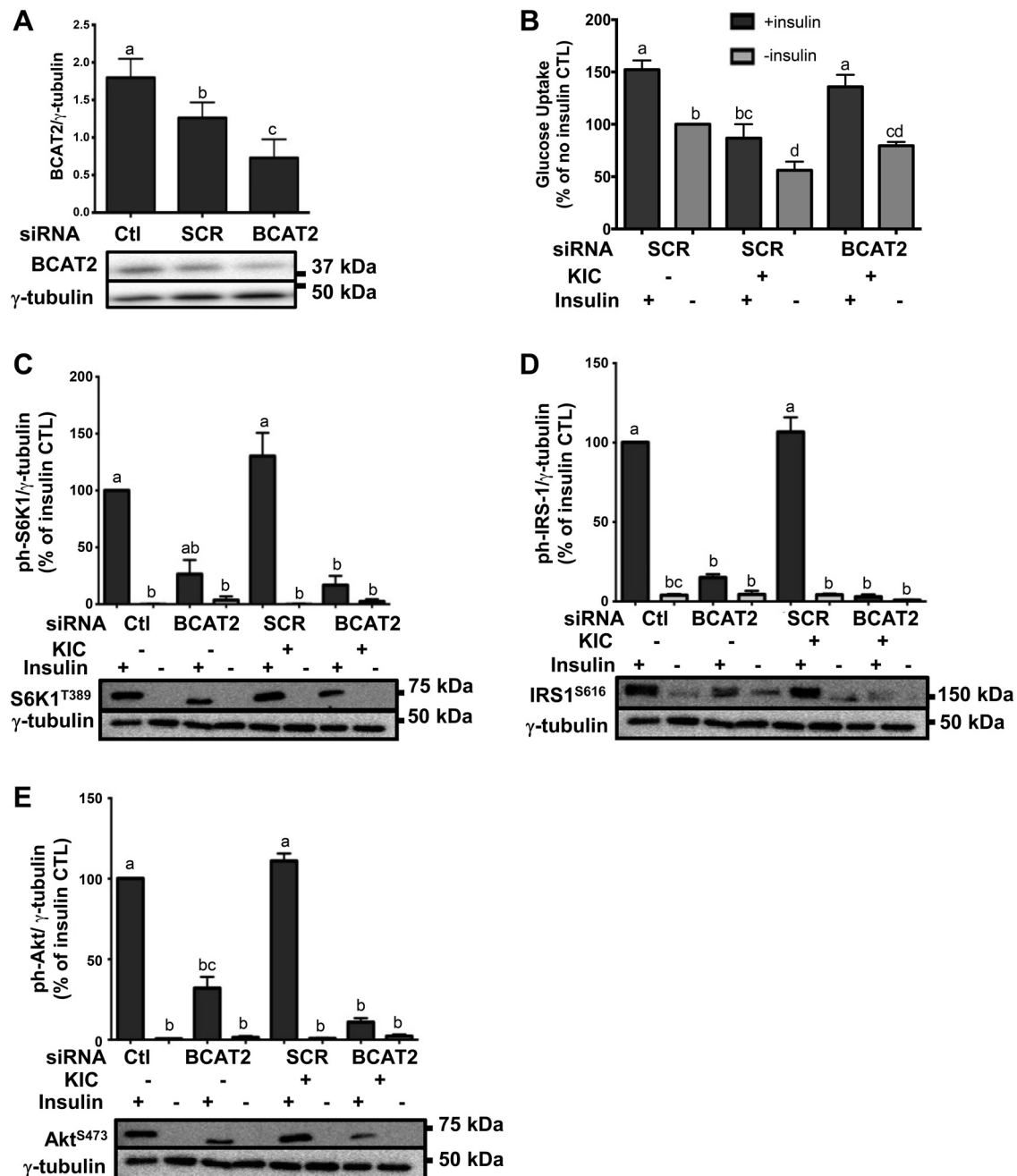


Fig. 5. BCAT2 knockdown attenuates the inhibitory effect of KIC on insulin-stimulated glucose transport in myotubes. On *day 3* of differentiation, L6 myotubes were transfected with 50 nM scrambled or BCAT2-targeting siRNA oligonucleotide. On *day 5* of differentiation, following 4 h of starvation as described in Fig. 1, myotubes were supplemented with or without 200  $\mu$ M KIC for 30 min. Incubation then continued in the presence of 100 nM insulin for another 20 min. Afterward, glucose transport was conducted or myotubes were harvested and used for Western blot analysis. **A**: immunoblot analysis showing BCAT2 knockdown. **B**: glucose transport is expressed as %CTL (SCR siRNA, no KIC, no insulin). The effect of insulin is significant in all groups. In the presence of insulin, SCR is significantly different from SCR + KIC. In the absence of insulin, SCR is significantly different from the other groups. Graphical representation and Western blot images of ph-S6K1<sup>T389</sup> (**C**), ph-IRS1<sup>S616</sup> (**D**), ph-Akt<sup>S473</sup> (**E**). Data are expressed as %SCR (no KIC + ins) (**C**–**E**). Data are means  $\pm$  SE;  $n = 3$  independent experiments with 3–6 replicates per treatment within each experiment. Bars with different letters are significantly different ( $P < 0.05$ ). In **C**, the effect of insulin is significant in CTL and SCR + KIC groups. In the presence of insulin, CTL is significantly different from BCAT2 + KIC. In **D**, insulin effect is significant in CTL and SCR + KIC. In the presence of insulin, CTL is significantly different from BCAT2 + KIC. In **E**, the effect of insulin is significant in all groups except BCAT2 + KIC. In the presence of insulin, CTL is significantly different from BCAT2 + KIC.

nase (BCKDH), the enzyme that irreversibly catabolizes KIC, is diminished in obesity and T2DM, mechanisms that prevent KIC accumulation may have therapeutic potential for the management of insulin resistance and its sequelae.

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## DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

## AUTHOR CONTRIBUTIONS

M.M., P.T.-F., and O.A.J.A. conception and design of research; M.M., P.T.-F., and B.B. performed experiments; M.M. and P.T.-F. analyzed data; M.M., P.T.-F., and O.A.J.A. interpreted results of experiments; M.M. prepared figures; M.M. drafted manuscript; M.M., P.T.-F., B.B., and O.A.J.A. edited and revised manuscript; M.M., P.T.-F., B.B., and O.A.J.A. approved final version of manuscript.

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