An amino acid mixture enhances insulin-stimulated glucose uptake in isolated rat epitrochlearis muscle

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Kleinert M, Liao Y-H, Nelson JL, Bernard JR, Wang W, Ivy JL.. An amino acid mixture enhances insulin-stimulated glucose uptake in isolated rat epitrochlearis muscle. J Appl Physiol 111: 163–169, 2011. First published April 28, 2011; doi:10.1152/japplphysiol.01368.2010—Protein and certain amino acids (AA) have been found to lower blood glucose. Although these glucose-lowering AA are important modulators of skeletal muscle metabolism, their impact on muscle glucose uptake remains unclear. We therefore examined how an AA mixture consisting of 2 mM isoleucine, 0.012 mM cysteine, 0.006 mM methionine, 0.0016 mM valine, and 0.014 mM leucine impacts skeletal muscle glucose uptake in the absence or presence of a submaximal (sINS) or maximal insulin (mINS) concentration. The AA mixture, sINS, and mINS significantly increased 2-[3H]deoxyglucose (2-DG) uptake by 63, 79, and 298% above basal, respectively. When the AA mixture was combined with sINS and mINS, 2-DG uptake was further increased significantly by 26% (P = 0.028) and 14% (P = 0.032), respectively. Western blotting analysis revealed that the AA mixture increased basal and sINS Akt substrate of 160 kDa (AS160) phosphorylation, while AA mixture did not change phosphorylation of Akt or mammalian target of rapamycin (mTOR) under these conditions. Interestingly, addition of the AA mixture to mINS increased phosphorylation of mTOR, Akt as well as AS160, compared with mINS alone. These data suggest that certain AA increase glucose uptake in the absence of insulin and augment insulin-stimulated glucose uptake in an additive manner. Furthermore, these effects appear to be mediated via a pathway that is independent from the canonical insulin cascade and therefore may prove effective as an alternative therapeutic treatment for insulin resistance.

Akt substrate of 160 kDa; isoleucine; skeletal muscle

DIABETES MELLITUS, ESPECIALLY type 2 diabetes, is a pandemic. The common hallmark and fundamental defect of type 2 diabetes is the body’s resistance to the effects of insulin, which is the hormone responsible for moving sugar from the blood into the cells. This inability to properly handle a glucose load stems from diminished peripheral insulin-stimulated glucose transport (9). Problems are most prominent in the fed state when glucose molecules absorbed from the gut elevate blood glucose levels, which trigger insulin release from the pancreas. Normally, circulating insulin binds to specific receptors on skeletal muscle, triggering an intracellular signaling cascade that induces translocation of specialized insulin-responsive glucose transport (GLUT-4) from an intracellular compartment to the cell’s surface (23). This increases the muscle’s permeability to glucose, which enters through the GLUT-4 transporter via facilitated diffusion. With insulin resistance, however, fewer GLUT-4 transporters translocate to the plasma membrane for a given physiological insulin stimulus, making the muscle less permeable to glucose (9). Perturbations in the signal transduction distal to the insulin receptor (7) are most likely responsible for the decreased number of GLUT-4 transporters that reach the cell surface with insulin resistance. Because skeletal muscle tissue is chiefly responsible for postprandial blood glucose disposal (10), diminished capacity to absorb glucose presents a major challenge to blood glucose homeostasis. Euglycemia requires a balance between the rate of glucose appearance and glucose removal. With insulin resistance, the inability to properly remove glucose from the blood leads to prolonged hyperglycemia, which can result in blindness, renal failure, neuropathy, and cardiovascular disease.

Recent reports (37) suggest that the branched-chain amino acids (BCAA) leucine (Leu) and isoleucine (Ile) can ameliorate hyperglycemia by stimulating skeletal muscle to take up glucose. Initial efforts to find the molecular effectors responsible have identified phosphatidylinositol 3-kinase (PI3-kinase) and atypical protein kinase C (aPKC) as mediators (11, 36), which are part of the insulin signaling cascade. Analogous to the effect of insulin, BCAA treatment also increases GLUT-4 concentration at the plasma membrane in skeletal muscle, which ultimately accounts for the increased glucose uptake (37).

It is not known, however, if BCAA-induced glucose uptake occurs in the presence of insulin, which has implications for insulin-resistant individuals. Further, it is unclear whether Akt substrate of 160 kDa (AS160) is activated with BCAA treatment. AS160 Thr325 is a key residue for insulin-stimulated GLUT-4 translocation (28, 42). Similarly, Akt appears to be indispensable for insulin-stimulated glucose uptake (6). Insulin treatment also increases mammalian target of rapamycin (mTOR) activity in skeletal muscle (40). This effect is in part mediated by Akt (34), which phosphorylates mTOR on the Ser2448 residue. Leu is also known to activate mTOR, and this increase in mTOR activity has been linked with activation of glycogen synthase (39).

Therefore, the purpose of this study was to investigate the effect of a novel AA mixture consisting predominantly of Ile on muscle glucose uptake, and its interaction with a physiological and maximally stimulating concentration of insulin. Insulin signaling proteins Akt, mTOR, and AS160 were also investigated. We expected that insulin treatments and the AA mixture would increase glucose uptake compared with basal glucose uptake. We further hypothesized that the AA mixture combined with insulin would result in an additional increase in glucose uptake compared with each insulin treatment alone.
MATERIALS AND METHODS

Animals. All research and handling procedures were approved by the Institutional Animal Care and Use Committee of The University of Texas at Austin and animals were cared for accordingly. Male Sprague-Dawley rats 23 days of age were purchased from Charles River Laboratories (Wilmington, MA). All rats were housed in cages (3 rats/cage) in an air-conditioned room (22°C) with a 12:12-h dark-light cycle and were fed standard laboratory chow and water ad libitum. After ~2 wk of acclimation when rats weighed between 100 and 120 g, they were used in the experiments.

Experimental protocol. Using the isolated epitrochlearis muscle preparation, we first investigated the dose response of our AA mixture on muscle glucose uptake. For the dose-response experiment, the uptake solution contained either no additional treatment or one of the following concentrations of the AA mixture: 0.5 × AA [0.003 mM cysteine (Cys), 0.002 mM methionine (Met), 0.004 mM Val, 0.5 mM Ile, and 0.003 mM Leu], 1 × AA [0.006 mM Cys, 0.003 mM Met, 0.008 mM Val, 1.0 mM Ile, and 0.007 mM Leu], 2 × AA [0.012 mM Cys, 0.006 mM Met, 0.016 mM Val, 2.0 mM Ile, and 0.014 mM Leu], or 4 × AA [0.024 mM Cys, 0.012 mM Met, 0.032 mM Val, 4.0 mM Ile, and 0.028 mM Leu]. Ile was the predominante AA in our mixture because it was previously reported to be the most effective of the BCAA in lowering blood glucose.

Next, we studied the effect of our AA mixture on insulin-stimulated glucose uptake and proteins associated with insulin signaling. Our dose-response experiment did not reveal a difference between the AA mixtures tested. Therefore, the AA mixture we chose to use was based on the concentration of Ile previously reported to have the most potent effect on glucose uptake in vitro under insulin-free conditions (11). To investigate the effect of the AA mixture on insulin-stimulated glucose uptake, epitrochlearis muscles were incubated in uptake solution containing either no treatment (control), the 2 × AA mixture (AA), 75 μU/ml insulin (iIns), 2 μU/ml insulin (mIns), iIns plus AA, or mIns plus AA. In addition, to determine glucose uptake, the uptake solution contained radioactive tracers as described below. Muscles that were incubated without radioactive tracers were used for measurement of high-energy phosphates and Western blot analysis of insulin signaling proteins.

Muscle incubation procedure. Following a 12-h fast, rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (65 mg/kg body wt), and the left and right epitrochlearis muscles were rapidly excised. After muscle removal, rats were euthanized via an intracardiac injection of sodium pentobarbital (50 mg/kg body wt). Excised epitrochlearis muscles were incubated according to previous work (19). In brief, the muscles were incubated at 29°C for 50 min in 3 ml of continuously gassed (95%O2, 5%CO2) preincubation medium, consisting of Krebs-Henseleit bicarbonate buffer (KHB), 8 mM glucose, 32 mM mannitol, and 0.1% BSA. Following the preincubation, the muscle was transferred to another vial and incubated at 29°C for 10 min in 3 ml of continuously gassed washout medium, consisting of KHB, 2 mM pyruvate, 38 mM mannitol, 0.1% BSA for 10 min. Finally, the muscle was incubated at 29°C for 20 min in 3 ml of uptake medium, which consisted of KHB, 2 mM pyruvate, 6 mM glucose, 32 mM mannitol, and 0.1% BSA, with or without 280 μCi/ml 2-[3H]deoxyglucose (2-DG) and 10 μCi/ml [14C]mannitol and the designated treatment. Immediately after the incubations, muscles were briefly blotted on gauze (McKesson, Richmond, VA) wetted with 0.9% saline solution and freeze clamped with Wallenberg tongs cooled in liquid nitrogen. For glucose uptake measurement, muscles were immediately analyzed. Samples for Western blotting or high-energy phosphate measurements were stored at −80°C until assayed.

Muscle glucose uptake measurements. Glucose uptake was calculated from the incorporation rate of 2-DG into the muscle fibers during the 20 min of incubation in the uptake medium. Frozen muscle samples were digested in 1 ml 1 M KOH at 60°C for 20 min. Muscle homogenates were neutralized with 1 ml 1 M HCl, and 300 μl were added into 6 ml BioSafe II scintillation cocktail (Research Products International, Mt. Prospect, IL). Duplicate samples were counted for 3H and 14C in an LS-6000 liquid scintillation spectrophotometer (Beckman, Fullerton, CA). Muscle 2-DG uptake was calculated as the difference between total muscle 2-DG and 2-DG in the extracellular space. 2-DG concentration in the extracellular space was determined by the amount of [14C]mannitol in the tissue (19).

ATP and creatine phosphate determination. Frozen epitrochlearis muscles were pulverized in 333 μl of 8% perchloric acid (PCA) in 40% ethanol with a ceramic pestle and mortar while cooled with liquid nitrogen. Pulverized samples were homogenized at 0°C in 500 μl of 8% PCA in 40% ethanol with a glass tissue grinder pestle and glass mortar (Corning Life Sciences, Lowell, MA). Glass pestle and mortar were rinsed with 500 μl of 6% PCA, and the homogenate was centrifuged at 13,000 g for 15 min at 4°C. Supernatants were neutralized with 267 μl 2 M K2CO3 and 0.4 M triethanolamine and centrifuged at 13,000 g for 10 min. One milliliter of the supernatant was assayed for ATP and creatine phosphate levels according to Lamprecht et al. (30, 31).

Tissue processing for signaling protein immunoblotting. Tissues were homogenized in ice-cold homogenization buffer (20 mM HEPES, 2 mM EGTA, 50 mM NaF, 100 mM KCl, 0.2 mM EDTA, 50 mM glycerophosphate, 1 mM DTT, 0.1 mM PMSF, 1 mM benzamidine, and 0.5 mM Na vanadate) with a glass tissue grinder pestle (Corning Life Sciences). Homogenates were centrifuged at 14,000 g for 10 min at 4°C. Supernatants were used for immunoblotting analysis, and their protein concentrations were determined as previously described (32). Aliquots of supernatants were stored at −80°C.

Antibodies for signaling protein immunoblotting. All antibodies were purchased from Cell Signaling Technology (Beverly, MA). To examine how the AA mixture would affect insulin-responsive phosphorylation sites, membranes were probed with anti-phospho-AS160-Thr410, anti-phospho-mTOR-Ser2448, and anti-phospho-Akt-Thr308. In addition, all membranes were probed with an α-tubulin antibody to ensure that equal amounts of protein were loaded. All primary antibodies were detected by horseradish peroxidase-conjugated secondary anti-rabbit IgG.

Signaling protein immunoblotting. Muscle protein (50 μg for phosphor-Akt, α-tubulin, phospho-mTOR, and phospho-AS160) was subjected to SDS-PAGE, and the proteins were separated on a 10% resolving gel. The resolved proteins were transferred to a PVDF membrane using a semidry transfer unit. With the use of a visible antibody incubation period, membranes were washed in TTBS and antibody binding was visualized by ECL in a chemiluminescence detection system. Membrane 2-DG uptake was calculated as the difference between total muscle 2-DG and 2-DG in the extracellular space. 2-DG concentration in the extracellular space was determined by the amount of [14C]mannitol in the tissue (19).

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stripping solution (100 mM β-mercaptoethanol, 2% SDS, and 62.5 mM Tris base and pH 6.7) and heated at 60°C for 1 h to remove the primary antibodies. Membranes were washed in TTBS to remove the stripping solution and then blocked in NFDM/TTBS for 1 h at room temperature. The upper membrane section was probed for mTOR, and the lower membrane section was probed for α-tubulin. The procedures for primary and secondary incubation, visualization, and quantification were the same as those described above.

Statistical analysis. Data from the dose-response study were subjected to one-way ANOVA, and upon verification of overall significance means were compared using the LSD post hoc test. All other data were tested for variance with two-way ANOVA with AA and INS being the two independent variables. When the two-way ANOVA identified a significant AA × INS interaction, mean differences were compared according to a priori planned comparison tests. A level of \( P < 0.05 \) was required to be deemed statistically significant. All values are expressed as means ± SE.

RESULTS

Incubated epitrochlearis muscle has normal ATP and PCr levels. To test the validity of our incubation procedure, we measured the ATP and PCr concentrations in 12 epitrochlearis muscles that had been subjected to the incubation protocol as described in the methods and compared them to freshly excised muscles. The ATP and PCr concentrations for the incubated muscles were 7.07 ± 0.34 and 20.34 ± 0.79 μmol/g, respectively, which is comparable with previous reports that incubated muscles that had been subjected to the incubation protocol as described in the methods and compared them to freshly excised muscles. The ATP and PCr concentrations in 12 epitrochlearis muscles were 7.07 ± 0.34 and 20.34 ± 0.79 μmol/g, respectively, which is comparable with previous reports that incubated muscles that had been subjected to the incubation protocol as described in the methods and compared them to freshly excised muscles.

Muscle glucose uptake. As shown in Fig. 1, the 0.5× AA mixture was sufficient to significantly increase basal glucose uptake by 79%. The 1×, 2×, and 4× AA mixtures had similar effects and increased basal glucose uptake by 73, 81, and 95%, respectively.

As demonstrated in Fig. 2, the sINS treatment increased glucose uptake compared with basal (\( P = 0.006 \)) and mINS increased glucose uptake compared with sINS (\( P < 0.001 \)). Furthermore, the 2× AA mixture significantly increased basal, sINS-, and mINS-stimulated glucose uptake by 0.34, 0.33, and 0.31 \( \mu \text{mol·g}^{-1}·\text{20 min}^{-1} \), respectively. This represented increases in glucose uptake of 65 (\( P = 0.022 \)), 26 (\( P = 0.028 \)), and 14% (\( P = 0.032 \)), respectively.

Figure 2. Effect of an AA mixture on insulin-stimulated glucose uptake in isolated rat epitrochlearis muscle. Isolated muscle strips were preincubated for 50 min, washed out for 10 min, and incubated in glucose uptake medium with either no additional treatment (basal; \( n = 6 \)), AA mixture (AA; \( n = 7 \)), 75 μU/ml insulin [submaximal (s)INS; \( n = 7 \)], 2 μU/ml insulin [maximal (m)INS; \( n = 7 \)], sINS/AA (\( n = 7 \)), or mINS/AA (\( n = 9 \)). Values are means ± SE in \( \mu \text{mol·g}^{-1}·\text{20 min}^{-1} \).

AA mixture increases AS160 Thr642 phosphorylation. As shown in Fig. 3, the sINS treatment increased AS160 Thr642 phosphorylation compared with basal (\( P = 0.003 \)) and mINS increased AS160 Thr642 phosphorylation compared with sINS (\( P < 0.001 \)). In addition, the 2× AA mixture significantly increased basal (\( P = 0.012 \)) and sINS (\( P = 0.046 \))- and mINS-stimulated (\( P = 0.011 \)) AS160 Thr642 phosphorylation.

AA mixture increases maximal insulin-stimulated Akt Thr308 phosphorylation. As seen in Fig. 4, the sINS treatment increased Akt Thr308 phosphorylation compared with basal (\( P = 0.008 \)) and mINS increased Akt Thr308 phosphorylation compared with basal (\( P = 0.008 \)) and mINS increased Akt Thr308 phosphorylation compared with basal (\( P = 0.008 \)) and mINS increased Akt Thr308 phosphorylation compared with basal (\( P = 0.008 \)).
pared with sINS (P < 0.001). In addition, the 2× AA mixture significantly increased mINS-stimulated Akt Thr
308 phosphorylation (P = 0.04) but had no additional effect on basal or sINS-stimulated Akt Thr
308 phosphorylation.

AA mixture increases maximal insulin-stimulated mTOR Ser2448 phosphorylation. As depicted in Fig. 5, the sINS treatment failed to increase mTOR Ser2448 phosphorylation compared with basal, but mINS increased mTOR Ser2448 phosphorylation compared with sINS (P < 0.001). In addition, the 2× AA significantly increased mINS-stimulated mTOR Ser2448 phosphorylation (P = 0.043) but had no additional effect on basal or sINS-stimulated mTOR Ser2448 phosphorylation.

Abundance of α-tubulin was the same for all treatments. We probed our membranes with an antibody that detects endogenous levels of total α-tubulin protein. This served as a loading control, as all proteins were detected on the same membrane. Analysis of endogenous levels of total α-tubulin protein indicated no statistical differences among the treatment groups, suggesting that equal amounts of protein were loaded for all treatments (Fig. 6).

**DISCUSSION**

In the present study, it was demonstrated that an AA mixture increased basal and insulin-stimulated glucose uptake in isolated rat epitrochlearis muscle. Most notably the AA mixture increased glucose uptake in the presence of maximally stimulating concentrations of insulin. In addition, phosphorylation of AS160 increased with AA mixture treatment. These findings suggest that certain AA can, independent of the effects of insulin, directly increase skeletal muscle glucose uptake. Furthermore, it appears that insulin and the AA mixture increase glucose uptake via at least partially separate pathways. Lastly, the data suggest that AS160 might be a point of convergence between these pathways.

The present finding that an AA mixture increased basal and insulin-stimulated glucose uptake in isolated rat epitrochlearis muscle implies that certain AA can directly stimulate skeletal muscle glucose uptake. This is consistent with previous in vitro research that demonstrated that Leu (36), Ile (11), and Ile-Leu dipeptides (33) increased glucose uptake under insulin-free conditions in C2C12 myotubes, isolated soleus, and isolated epitrochlearis muscle respectively. More evidence for AA-induced glucose uptake via mechanisms unrelated to insulin...
comes from in vivo studies. In rats, administration of a bolus of Ile did not raise plasma insulin levels compared with saline-treated animals. However, Ile treatment significantly lowered plasma glucose levels and increased skeletal muscle glucose compared with controls (12, 13). Although these previous findings make a strong argument that certain AA enhance insulin-stimulated glucose uptake, we sought to confirm this hypothesis using an isolated muscle technique. This experimental paradigm eliminates many confounding in vivo factors, ranging from muscle capillarization to hormones (5), while retaining the metabolic and functional properties of the muscle tissue (35).

At variance with our findings, Doi et al. (11) observed no additional glucose uptake in C2C12 myotubes when 2 mM Ile was added to an insulin treatment. This discrepancy may arise from the different in vitro models used. Doi et al. (11) used C2C12 myotubes, while we employed mature epitrochlearis muscles. C2C12 cells have insufficient levels of GLUT-4 and lack developed t-tubules, which is reflected by their modest insulin responsiveness, even after differentiation (26, 43). In concurrence with these limitations, glucose uptake increased only ~10% with insulin treatment in the C2C12 myotubes. In comparison, we observed increases of 79 and 298% with physiological and maximally stimulating levels of insulin, respectively. In addition, the different results may be due to the fact that in the present study an AA mixture was used, while Doi et al. (11) investigated the effects of Ile alone. It may be for the same reasons that Doi et al. (11) reported a different dose-response relationship. While in the present study we demonstrated a robust increase in glucose uptake with an AA mixture containing only 0.5 mM Ile that was not further enhanced with the AA mixtures containing 1, 2, or 4 mM of Ile, Doi et al. (11) reported that glucose uptake peaked in response to 2 mM Ile.

AA have been reported to reduction insulin-stimulated glucose uptake (23). Iwanaka et al. (22) found that Leu blunts insulin-stimulated glucose uptake in vitro. Moreover, a number of studies (29) in humans have demonstrated that infusion of AA causes hyperinsulinemia, hyperglycemia, and insulin resistance. These dissimilarities to our results can be explained by the different experimental approaches and research questions. In the present study, the AA mixture contained only 0.014 mM Leu, while Iwanaka et al. (22) exposed the epitrochlearis muscle to 2 mM Leu. Moreover, we incubated muscle strips with the AA mixture for 20 min, while Iwanaka et al. (22) exposed the muscle to Leu for 60 min. Whether the different results are caused by the choice and concentration of the AA or the incubation time awaits future investigation. Studies that infuse subjects with AA for a prolonged period of time attempt to mimic a diet overly rich in protein to investigate how prolonged elevated plasma AA levels affect the body. For similar reasons hyperlipidemic, hyperglycemic, or hyperinsulinemic clamps are used, which can all induce insulin resistance (16, 17). The goal of the present investigations was to examine how a bolus of certain AA would affect skeletal muscle glucose uptake. Therefore, the time skeletal muscle is exposed to AA and the types of AA used are vastly different between the present and the infusion studies.

The AA mixture’s ability to enhance mINS-stimulated glucose uptake in an additive manner suggests that insulin and the AA mixture induce glucose uptake via at least partially separate pathways, analogous to contraction-stimulated glucose uptake. To date this possibility has not been thoroughly investigated. Earlier attempts to characterize the pathway for AA-induced glucose uptake revealed that phosphatidylinositol 3-kinase (PI3-kinase) and aPKC, but not mTOR, are necessary for the effect. Using specific inhibitors for PI3-kinase, aPKC, and mTOR, Nishitani et al. (36) reported that Leu-stimulated glucose uptake by isolated soleus muscle requires PI3-kinase and aPKC but not mTOR. Similarly, Morifuji et al. (33) demonstrated that increased glucose uptake in isolated epitrochlearis muscle in response to Ile-Leu dipeptides was blocked with specific PI3-kinase and aPKC inhibitors. Furthermore, Doi et al. (11) demonstrated that PI3-kinase and aPKC are necessary for Ile-induced glucose uptake in C2C12, while mTOR is not. None of these studies, however, investigated changes in phosphorylation states of these proteins.

In light of the possible involvement of PI3-kinase, we decided to investigate the phosphorylation states of Akt and AS160 because both are downstream of PI3-kinase and to date their role in AA-induced glucose uptake has not been comprehensively examined. Furthermore, AS160 is an important regulator of GLUT-4 translocation (reviewed in Ref. 41). Since it has been suggested that AA-induced glucose uptake is ultimately mediated by increased GLUT-4 translocation (37), increased AS160 phosphorylation with AA mixture treatment could provide a mechanism for enhanced GLUT-4 translocation to the plasma membrane.

Although AA-induced glucose uptake is thought to occur independently of mTOR, the present study assessed whether the AA mixture had any effect on mTOR phosphorylation. It has been demonstrated that Leu increases mTOR phosphorylation (3, 24), while Ile does not (3). Leu also appears to be the only BCAA that has a stimulatory effect on protein synthesis in the rat (1), a process largely mediated by mTOR (45). Measuring the effect of the AA mixture on mTOR phosphorylation has implications for future studies investigating its effect on glycogen synthesis. It has been reported that mTOR activation is associated with inactivation of glycogen synthase kinase 3, which in turn increased glycogen synthase activity (2, 21), a key enzymatic regulator of glycogen synthesis (23).

Our data suggest that the increased basal, sINS, and mINS glucose uptake in response to the AA mixture was mediated by increased AS160 phosphorylation but that changes in Akt or mTOR phosphorylation, at least under basal and sINS conditions, were not required. Akt is important for insulin-stimulated glucose uptake, because it mediates the insulin-induced phosphorylation of AS160 (27). In agreement, sINS and mINS treatments both increased Akt phosphorylation together with AS160 phosphorylation. Yet, the AA mixture appeared to mediate an increase in AS160 phosphorylation that was independent of increased Akt phosphorylation. Although the Thr542 residue on AS160 is thought to be a key site for Akt-mediated GLUT-4 translocation in response to insulin stimulation (42), it has been reported that other stimuli, such as in situ contraction or exercise, induce AS160 Thr542 phosphorylation in skeletal muscle (27). This emphasizes that the phosphorylation of this particular AS160 residue is not exclusively regulated by Akt. Therefore, it may be that certain AA can modulate AS160 phosphorylation in the absence of insulin. These observations lend credence to the argument that certain AA induce glucose uptake via a pathway separate from the insulin signaling pathway.
cascade and that AS160 is a point of convergence between these two signaling conduits. Furthermore, the inability of the AA mixture to increase Akt phosphorylation is in agreement with cell culture studies (2,38,39) and a report investigating the effects of Leu on contraction- and insulin-stimulated glucose uptake (22).

Although it is unlikely that AA-induced muscle glucose uptake requires activation of Akt or mTOR, we did observe an enhanced phosphorylation of these proteins under mINS in the presence of the AA mixture. As to how the AA mixture increased mINS-stimulated Akt and mTOR phosphorylation is subject to speculation. It may be that with maximally stimulating concentrations of insulin the uptake of AA was disproportionally increased. This may have magnified the effects of the AA mixture on the insulin and mTOR signaling pathways. It has been reported that insulin stimulates peripheral BCAA uptake in fasted men (14). Interestingly, however, the AA transport system L, which largely mediates BCAA transport (8), appears to be insulin insensitive (18). Alternatively, the insulin-sensitive AA transport system A (25) has been reported to have some affinity for cysteine, methionine, and Leu (44). Insulin-induced activation of system A appears to be PI3-kinase and Akt dependent (15,20). In light of the extreme hyper-phosphorylation of Akt following treatment with mINS, it could be that an equally hyperactivated AA transport system A allowed for greater AA influx into the muscle.

In conclusion, we have demonstrated that an AA mixture consisting predominately of L-leu enhanced glucose uptake in an additive manner in rat epircholeal muscle treated with physiological and maximally stimulating concentrations of insulin. These effects on glucose uptake appear to be mediated by a molecular pathway that is independent from the insulin signaling cascade. Future research should strive to better characterize the molecular events through which certain AA can induce glucose uptake, as this may prove beneficial to individuals suffering from impaired glucose tolerance or type 2 diabetes.

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DISCLOSURES
J. Nelson is employed by Abbott Nutrition, Abbott Laboratories, who funded the research. He helped develop the experimental design of the study, provided feedback on interpretation of results, and provided constructive criticism for the manuscript. He did not participate in data collection.

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