An amino acid mixture is essential to optimize insulin-stimulated glucose uptake and GLUT4 translocation in perfused rodent hindlimb muscle

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An amino acid mixture is essential to optimize insulin-stimulated glucose uptake and GLUT4 translocation in perfused rodent hindlimb muscle. J Appl Physiol 113: 97–104, 2012. First published May 17, 2012; doi:10.1152/japplphysiol.01484.2011.—The purpose of this study was to investigate whether an amino acid mixture increases glucose uptake across perfused rodent hindlimb muscle in the presence and absence of a submaximal insulin concentration, and if the increase in glucose uptake is related to an increase in GLUT4 plasma membrane density. Sprague-Dawley rats were separated into one of four treatment groups: basal, amino acid mixture, submaximal insulin, or amino acid mixture with submaximal insulin. Glucose uptake was greater for both insulin-stimulated treatments compared with the non-insulin-stimulated treatment groups but amino acids only increased glucose uptake in the presence of insulin. Phosphatidylinositol 3-kinase (PI 3-kinase) activity was greater for both insulin-stimulated treatments with amino acids having no additional impact. Akt substrate of 160 kDa (AS160) phosphorylation, however, was increased by the amino acids in the presence of insulin, but not in the absence of insulin. AMPK was unaffected by insulin or amino acids. Plasma membrane GLUT4 protein concentration was greater in the rats treated with insulin compared with no insulin in the perfusate. In the presence of insulin, amino acids increased GLUT4 density in the plasma membrane but had no effect in the absence of insulin. AS160 phosphorylation and plasma membrane GLUT4 density accounted for 76% of the variability in muscle glucose uptake. Collectively, these findings suggest that the beneficial effects of an amino acid mixture on skeletal muscle glucose uptake, in the presence of a submaximal insulin concentration, are due to an increase in AS160 phosphorylation and plasma membrane-associated GLUT4, but independent of PI 3-kinase and AMPK activation.

isoleucine; leucine; phosphatidylinositol 3-kinase activity; blood glucose clearance

INeSULIN IS THE PRIMARY REGULATOR OF glucose homeostasis. The concentration of blood glucose at any given time is determined by the entry of glucose into the blood from the digestive system, its removal by glucose-sensitive tissues, and its release from the liver. In the postprandial state, a rise in blood glucose stimulates the release of insulin from the pancreas. Insulin controls blood glucose within a narrow range by simultaneously increasing peripheral glucose uptake, primarily by the skeletal muscle, and decreasing liver glucose output. Recently, our laboratory (3, 13) as well as others (5, 7, 24) have reported that the glucose uptake process can be improved by amino acid supplementation.

Amino acids have been reported to increase skeletal muscle glucose uptake in vivo (3, 5, 7, 12) and in vitro (5, 13, 23, 24). Of the amino acids, the branched-chain amino acids isoleucine and leucine have received the most attention. Although these amino acids can significantly lower blood glucose, the exact mechanism remains elusive. In a recent report our laboratory showed that rats gavaged with an amino acid mixture had an improved glucose response to an oral glucose challenge compared with rats gavaged with carbohydrate only (3). In addition we demonstrated that the improved glucose tolerance was associated with enhanced Akt substrate of 160 kDa (AS160) phosphorylation and increased skeletal muscle glucose uptake (3). Using the isolated muscle preparation, our in vivo findings were confirmed in vitro by our laboratory (13). Given the strong relationship between the number of glucose transporters at the plasma membrane and glucose uptake (9, 14, 21), we became intrigued by the potential affects our mixture may have on GLUT4 translocation, a critical step for blood glucose clearance. We therefore hypothesized that the increase in amino acid-stimulated skeletal muscle glucose uptake we observed both in vitro and in vivo resulted from increased plasma membrane-associated GLUT4. To test this hypothesis, in the present investigation, we used the hindlimb perfusion technique. This technique enabled us to directly observe the effects of our amino acid mixture on GLUT4 translocation and to evaluate both the singular and combined effects of insulin and amino acids on plasma membrane GLUT4 content. The hindlimb perfusion technique also allowed us to precisely control the amounts and time in which the skeletal muscle was exposed to the carbohydrate, amino acids, and insulin, variables that we could not control during our previous in vivo experiments.

It is well documented that insulin-stimulated skeletal muscle glucose uptake requires that activation of phosphatidylinositol 3-kinase (PI 3-kinase). However, less is known about the relationship between PI 3-kinase, amino acids, and glucose uptake. Using the isolated muscle technique Nishitani et al. (24) demonstrated that leucine-induced glucose uptake was significantly decreased when the incubation medium included specific inhibitors for either PI 3-kinase and its downstream target atypical protein kinase C (aPKC) in the absence of insulin. Doi et al. (5) also reported that isoleucine-induced glucose uptake in C2C12 myotubes was inhibited in the presence of a specific inhibitor of PI 3-kinase. Although it appears that PI 3-kinase is an important signal in amino acid-stimulated glucose uptake, it has also been reported that PI 3-kinase activ-

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ity may be impaired by amino acids (2, 30). Therefore, in the presence of amino acids, the activity of PI 3-kinase, and how this kinase affects downstream proteins, requires further investigation.

Thus the primary purpose of this study was to investigate whether an amino acid mixture increased glucose uptake across perfused rodent hindlimb muscle in the presence and absence of a submaximal insulin concentration, and if the increase in glucose uptake was related to an increase in GLUT4 plasma membrane density. A secondary purpose was to determine if differences in glucose uptake were associated with changes in the activity of PI 3-kinase activity and/or the phosphorylation status of 5'-AMP activated protein kinase (AMPK), aPKC, and AS160.

MATERIALS AND METHODS

Animal care and housing. Twenty-nine male Sprague-Dawley rats ~7 wk old were obtained from Harlan (Indianapolis, IN). Upon arrival rats were randomly assigned to one of four groups: basal (BAS, n = 6), amino acid mixture (AA, n = 7), submaximal insulin (sINS, n = 7), or amino acid mixture with submaximal insulin (AA-sINS, n = 9). Rats were housed two per cage and provided standard laboratory chow (Prolab RMH 1800 SLL2, LabDiet, Brentwood, MO) and water ad libitum. The temperature of the animal room was maintained at 21°C, with an artificial 12:12-h light-dark cycle. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas at Austin and conformed to the guidelines for the use of laboratory animals published by the United States Department of Health and Human Resources.

Hindlimb perfusion. All animals were tested at ~9 wk old. After an overnight fast rats were subjected to the hindlimb perfusion technique. Details for the surgical preparation and hindlimb perfusion technique have been described previously (11, 27). Perfusion flow was directed only to the right hindlimb by cannulating the right iliac artery and vein to the tip of the femoral artery of the rat. After the cannulas were in place the rat was euthanized by a cardiac injection (65 mg/kg body wt) of pentobarbital sodium. The cannulas were then placed in line with the perfusion system and the right hindlimb was washed out with Krebs-Henseleit buffer (pH 7.4) containing 2 mM pyruvate at a flow rate of 6 ml/min. Following the 10-min washout period the arterial line was switched to the cell-free perfuse containing 2 mM pyruvate, 6 mM glucose, 2 mM mannitol, 0.2 μCi/ml 2-[14]H]deoxyglucose (2-DG), and 0.15 μCi/ml [U-14]C)sucrose ([14]C)sucrose) in Krebs-Henseleit buffer (pH 7.4) with the flow rate set at 4 ml/min. Although the perfusate was cell-free, we have found in previous studies that with this perfusion medium basal and insulin-stimulated glucose uptakes are normal in quiescent muscle (8, 22). Also, muscle ATP (preperfusion 6.7 ± 0.3 μmol/g, postperfusion 6.6 ± 0.2 μmol/g) and creatine phosphate (preperfusion 18.6 ± 0.5 μmol/g, postperfusion 17.8 ± 0.5 μmol/g) concentrations are similar before and after perfusion and are comparable to those found by Kushmerick and colleagues (15) for rat muscle.

For the insulin-stimulated treatment groups (AA-sINS and sINS), 200 μU/ml insulin was added to both the washout buffer and perfusate medium. For the amino acid treatment groups (AA-sINS and AA) 2 mM of an amino acid mixture was also added to both the washout buffer and perfusate medium. The amino acid mixture was described previously (3, 13) and contained 5.28 mg cysteine, 3.36 mg methionine, 6.68 mg valine, 944.8 mg isoleucine, and 6.68 mg leucine per 50 ml solution. Although the majority of the amino acid mixture is composed of isoleucine, we previously demonstrated that the effects of the mixture on muscle glucose uptake were superior to isoleucine alone (3). The perfusions were performed at 37°C and continued for a total of 25 min, at which time the right gastrocnemius was excised, freeze clamped in liquid nitrogen, and stored at −80°C for later analysis.

Muscle glucose uptake. Rates of 2-DG uptake were determined in mixed gastrocnemius muscle samples. Muscle was weighed and dissolved in 1 N potassium hydroxide (KOH) by incubating for 15 min at 65°C. Samples were then vortexed and incubated for an additional 5 min. An equal volume of 1 N hydrochloric acid (HCl) was then added to neutralize the samples. Next, an aliquot of neutralized muscle sample was added to a vial containing 6 ml Bio-Safe II counting cocktail (Research Products International, Mount Prospect, IL). Arterial perfusate samples were taken during the hindlimb perfusion and treated the same as the muscle homogenate. Duplicate samples were counted in a liquid scintillation counter (Beckman LS 6500, Beckman Coulter, Fullerton, CA) preset for simultaneous counting of 13H and 14C DPM. Quenching was determined by counting prepared standards. The accumulation of intramuscular 2-DG was indicative of muscle glucose uptake. Intramuscular 2-DG was calculated by subtracting the concentration of 2-DG in the extracellular space from the total muscle 2-DG concentration. The extracellular space for each muscle sample was determined by measuring the [14]C)sucrose concentration in the muscle homogenate.

PI 3-kinase activity. For insulin receptor substrate-1 (IRS-1)-associated PI 3-kinase activity, ~150 mg of muscle was homogenized (1:9) in an ice-cold homogenization buffer (pH 7.4) containing 20 mM HEPES, 2 mM EGTA, 50 mM sodium fluoride (NaF), 100 mM potassium chloride (KCl), 0.2 mM EDTA, 50 mM glycerolphosphate, 1 mM DTT, 0.1 mM PMSF, 1 mM benzamidine, and 0.5 mM sodium orthovanadate (Na3VO4) with a glass tissue grinder pestle (Corning Life Sciences, Acton, MA). The homogenate was then centrifuged at 14,000 g for 10 min at 4°C. Aliquots of the supernatant were stored at −80°C for later analysis. The protein concentration of the homogenate was determined using the Lowry method (20).

One milligram of sample protein was immunoprecipitated with 8 μg of anti-IRS-1 (Millipore, Billerica, MA) and homogenization buffer overnight at 4°C. Protein-A Sepharose (PRO-A) beads were then prepared by washing once with phosphate triton azide (PTA) and twice with homogenization buffer and centrifuged at 14,000 g for 5 min at 4°C between each wash. After the final wash, the packed PRO-A beads were resuspended in homogenization buffer at a 1:1 [14]C)sucrose concentration in the muscle homogenate. For the determination of total PI 3-kinase activity, ~150 mg of mixed gastrocnemius muscle was homogenized (1:9) in ice-cold 0.5 M TCA. The homogenate was then centrifuged at 1,500 rpm for 5 min at 4°C. The supernatant was discarded and the pellet was resuspended in 3 ml of 5% TCA/1 mM EDTA. The protein concentration was then determined using the Lowry method (20). One milligram of protein was then subjected to the extraction protocol in accordance to the manufacturer’s instructions. The extracted substrate was incubated for 1 h and the product PI(3,4,5)P3 was detected using an ELISA kit (catalog no. K1000s, Echelon Biosciences, Salt Lake City, UT). The amount of phosphatidylinositol (3,4,5)-triphosphate (PIP3) produced by extracted PI 3-kinase from the sample was proportional to the total PI 3-kinase activity.

For the determination of total PI 3-kinase activity, ~150 mg of mixed gastrocnemius muscle was homogenized (1:9) in ice-cold 0.5 M TCA. The homogenate was then centrifuged at 1,500 rpm for 5 min at 4°C. The supernatant was discarded and the pellet was resuspended in 3 ml of 5% TCA/1 mM EDTA. The protein concentration was then determined using the Lowry method (20). One milligram of protein was then subjected to the extraction protocol in accordance to the manufacturer’s instructions. The extracted substrate was incubated for 1 h and the product PI(3,4,5)P3 was detected using an ELISA kit (catalog no. K1000s, Echelon Biosciences). The amount of PIP3 produced by extracted PI 3-kinase from the sample was proportional to the total PI 3-kinase activity.
ATP and creatine phosphate assays. Muscle samples were pulverized in the frozen state under liquid N2. Pulverized samples were added to 300 μl of 8% perchloric acid in 40% ethanol and homogenized at 0°C. The supernatants were then neutralized in 2M K2CO3 and 0.4 M triethanolamine, centrifuged for 10 min, and assayed for ATP and creatine phosphate according to Lamprecht and colleagues (17, 18).

AMPK, αPKC, and AS160 phosphorylation. Approximately 150 mg of muscle was homogenized and the protein concentration of each sample determined as described above. Sample protein (60 μg) was combined with an equal amount (1:1) of Laemmli sample buffer (125 mM Tris, 20% glycerol, 2% SDS, 0.008% bromophenol blue, pH 6.8) (16) and boiled for 5 min. Next, samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the proteins separated on an 8% resolving gel for 1.5 h. The resolved proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane using a semidy transfer unit and blocked in 7% nonfat dry milk in Tris-Tween-buffered saline (NFDM/TTBS) for 1 h at room temperature. The membranes were then incubated with either affinity purified anti-phospho-AMPKα (Thr-172) (Cell Signaling Technology, Danvers, MA), anti-phospho-αPKC/z (Thr-410/403) (Cell Signaling Technology), or anti-phospho-AS160 (Thr-642) (Millipore, Billerica, MA) at 4°C. Following the overnight incubation the membranes were washed for three 5-min washes in TTBS, then incubated for 2 h at room temperature with the species-specific secondary antibody. The membranes were then washed with five 8-min washes with TTBS, and antibody binding was visualized by enhanced chemiluminescence in accordance with the manufacturer’s instructions (Perkin Elmer, Boston, MA). Images were captured using a charge-coupled device camera in a ChemiDoc system (Bio-Rad, Hercules, CA) and saved to a computer. Density of the bands was quantified with Quantity One analysis software (Bio-Rad) and expressed as a percentage of a standard run on each gel.

After the phosphorylation status was determined, the primary phosphorylated antibody was stripped from the membrane to determine alpha-tubulin protein concentration to demonstrate equal protein loading across samples. Membranes were placed in a stripping solution containing 100 mM β-mercaptoethanol, 2% SDS, and 62.5 mM Tris base (pH 6.7) and heated at 60°C for 1 h. Membranes were washed for three 15-min washes with TTBS to remove the stripping solution. The primary and secondary antibody incubation times, washing, exposure, and quantification were the same as that described above.

Plasma membrane fractionation. The plasma membrane was isolated as described previously (19, 28). Briefly, a portion of the gastrocnemius was homogenized in an ice-cold buffer (8 × wt/vol) containing 20 mM HEPES (pH 7.2), 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM DTT, 1 mM Na3VO4, 10% glycerol, 3 mM benzamidane, 10 μM leupeptin, 5 μM pepstatin A, and 1 mM PMSF. The homogenate was centrifuged at 33,000 rpm for 30 min at 4°C, and the supernatant was collected as the crude homogenate fraction. The pellet was resuspended in an ice-cold buffer (4 × wt/vol) in which 1% Triton X was added. The resuspended pellet was then centrifuged at 11,000 rpm for 10 min at 4°C. The supernatant, representing the plasma membrane fraction, was collected. To determine plasma membrane purity the enzymatic activity of the membrane marker 5′-nucleotidase in the plasma membrane fraction was compared with its activity in the crude homogenate (Table 1) as described previously (29). The method of Fiske and SubbaRow (10) was used for determination of 5′-nucleotidase activity. The specific activities and purity indexes for the plasma membrane marker 5′-nucleotidase are displayed in Table 1. The protein concentration for the plasma membrane and crude homogenate was determined using the Lowry method (20).

Plasma membrane GLUT4 protein concentration. The plasma membrane GLUT4 protein concentration was determined from plasma membrane fractions obtained from mixed gastrocnemius muscle samples. Sample protein was subjected to SDS-PAGE and the protein was separated on a 12% resolving gel. Next, the resolved proteins were transferred to a PVDF membrane using a semidy transfer unit and blocked in NFDM-NTBS saline for 1 h at room temperature. Using a visible molecular weight marker (Bio-Rad) as a guide the PVDF membranes were cut into an upper and lower membrane section. The upper membrane section was probed with affinity-purified anti-sodium-potassium-ATPase (Na+—K+—ATPase) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. The Na+—K+—ATPase pump is a ubiquitous plasma membrane marker used to demonstrate equal protein loading across samples. The lower membrane section was probed with affinity-purified anti-GLUT4 antibody (donated by Dr. Samuel Cushman, National Institute of Diabetes and Digestive Kidney Disease, Bethesda, MD) overnight at 4°C. The secondary antibody, incubation times, washing, exposure, and quantification were similar to that described above.

Muscle proteins assessed by Western blot analysis were not evaluated for their total concentrations, and therefore we do not know if they differed among the treatment groups. However, since all rats were from the same colony, and of the same strain and age, it is unlikely that muscle proteins would differ significantly.

Statistical analysis. A one-way ANOVA was performed on all data. When a significant F-ratio was obtained, a Fisher’s least significant difference post hoc test was performed to identify statistically significant differences (P < 0.05) between means. A sequential regression analysis was performed to determine the relationship between AS160, GLUT4, and muscle glucose uptake. Statistical analyses were completed using SPSS software (SPSS, Chicago, IL), and all values are expressed as means ± standard error (SE).

RESULTS

Animal characters. There were no significant differences in body mass among treatment groups (AA-sINS 293.7 ± 6.5 g; sINS 291.4 ± 3.1 g; AA 288.1 ± 5.7 g; BAS 284.8 ± 7.4 g) on the day of testing.

Muscle glucose uptake. Rates of 2-DG uptake in mixed gastrocnemius muscle samples were elevated for the AA-sINS and sINS treatments compared with the AA and BAS treatment groups (Fig. 1). Furthermore, rates of glucose uptake were greater in the AA-sINS treatment group compared with sINS. However, under non-insulin-stimulated conditions, the addition of amino acids to the perfusate had no effect on glucose uptake compared with BAS.

PI 3-kinase activity. For both IRS-1-associated and total PI 3-kinase, there was greater activity for the insulin-stimulated treatments compared with the non-insulin-stimulated treatment groups (Fig. 2, A and B). However, among the insulin-stimulated treatment groups, there was no difference observed between AA-sINS and sINS treatment groups. Similarly, among

| Table 1. 5′-Nucleotidase activity in the plasma membrane and crude homogenate
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<td>PM</td>
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Values are means ± SE. 5′-Nucleotidase activity is expressed as μmol·mg−1·min−1. AA-sINS, perfusate contained 6 mM carbohydrate, 200 μM insulin, and a 2 mM amino acid mixture; sINS, perfusate contained 6 mM carbohydrate and 200 μM insulin; AA, perfusate contained 6 mM carbohydrate and 2 mM amino acid mixture; BAS, perfusate contained 6 mM carbohydrate.
the non-insulin-stimulated treatment groups, there was no difference between AA and BAS treatment groups.

Western blotting. There was no difference between treatment groups for the phosphorylation of AMPK (Fig. 3). In the presence of a submaximal insulin concentration, the amino acid mixture significantly increased αPKC phosphorylation compared with the treatment group with no amino acids (Fig. 4). Similarly, amino acids increased αPKC phosphorylation in the absence of insulin compared with basal. Insulin stimulation resulted in a significantly increased AS160 phosphorylation compared with the non-insulin-stimulated treatment groups (Fig. 5). Moreover, AS160 phosphorylation was increased in the AA-sINS treatment compared with the sINS treatment group. There was no difference between AA compared with BAS treatment groups. There was no difference in the alpha-tubulin protein concentration, indicating the same amount of protein was loaded for each sample.

Insulin stimulation resulted in a significant elevation in plasma membrane GLUT4 protein concentration compared with the non-insulin-stimulated treatment groups (Fig. 6). The concentration of GLUT4 at the plasma membrane was also significantly greater in the AA-sINS treatment compared with the sINS treatment group. There was, however, no difference in GLUT4 plasma membrane concentration in AA compared with BAS treatment groups. There was no difference in the plasma membrane concentration of the Na⁺-K⁺-ATPase pump, indicating the same amount of protein was loaded for each sample.

Using sequential multiple regression analysis, we regressed AS160 and GLUT4 on skeletal muscle glucose uptake and found that the change in the coefficient of determination (ΔR²) due to AS160 was 0.716 and 0.044 for GLUT4 (Fig. 7). The total variance of glucose uptake explained by the model was 76%.

DISCUSSION

The primary objective of the present study was to investigate the effects of our amino acid mixture to increase skeletal muscle glucose uptake in relation to cellular signaling, activation of PI 3-kinase, and GLUT4 translocation in the presence and absence of a submaximal insulin concentration. To achieve this objective, we compared the effects of four different experimental treatments, AA-sINS, sINS, AA, and BAS, on glucose uptake using the rat hindlimb perfusion technique. Our study results demonstrated that skeletal muscle glucose uptake during hindlimb perfusion was significantly greater for the AA-sINS treatment compared with sINS. This finding confirms our previous studies reporting that our amino acid mixture enhances glucose uptake both in vitro (13) and in vivo (3) in the presence of insulin. We were able to extend our findings in the present investigation by demonstrating that the increase in amino acid-stimulated glucose uptake was directly related to an increase in AS160 phosphorylation and plasma membrane-associated GLUT4 protein concentration, but was not associated with increased activation of PI 3-kinase in addition to that induced by insulin alone. In addition, we could not confirm our previous results reporting that our amino acid mixture increases skeletal muscle glucose uptake in the absence of insulin (13).

Activation of the insulin receptor results in cellular mechanisms that directly mediate the number of glucose transporters translocated to the plasma membrane. Once insulin is bound to its receptor, the insulin receptor phosphorylates tyrosine residues on IRS-1. In turn, phosphorylated IRS-1 binds and activates PI 3-kinase, which then catalyzes the conversion of phosphatidylinositol (4,5)-bisphosphate (PIP2) to PIP3. This is a critical step in classical insulin signaling as blocking PI 3-kinase is known to inhibit insulin-stimulated glucose uptake. The subsequent activation of the downstream proteins protein kinase B (Akt/PI3K), αPKC, and AS160 leads to GLUT4 translocation to the plasma membrane. Thus glucose uptake is a highly coordinated process and is directly correlated with the number of GLUT4 transporters located at the plasma membrane (9, 14, 21).

In the present investigation we found that insulin had a strong stimulatory effect on skeletal muscle glucose uptake. Submaximal insulin stimulation resulted in a threefold increase in glucose uptake compared with the non-insulin-stimulated conditions. Furthermore, the combination of amino acids and insulin resulted in a 36% increase in glucose uptake compared with insulin alone.
Both IRS-1-associated and total PI 3-kinase activity were significantly increased in the presence of insulin compared with a perfusate with no insulin. In fact, there was approximately a threefold difference between insulin- and non-insulin-stimulated PI 3-kinase activity, which correlated well with the ~3-fold difference found with glucose uptake. However, adding the amino acid mixture to the perfusate had no further impact on IRS-1-associated PI 3-kinase or total PI 3-kinase activity in the presence or absence of insulin, suggesting that amino acid-stimulated glucose uptake does not require activation of PI 3-kinase beyond that produced by insulin.

The absence of amino acid activation of PI 3-kinase activity does not agree with the research of Doi et al. (5) and Nishitani et al. (24). Doi et al. (5) reported that glucose uptake was enhanced when isoleucine was added to C2C12 myotubes, but when LY294002, a potent PI 3-kinase inhibitor, was added to the same medium, a significant decrease in glucose uptake was observed. Also, Nishitani et al. (24), using the isolated muscle preparation, reported that leucine stimulated glucose uptake, but leucine-induced glucose uptake was inhibited in the presence of LY294002. These in vitro studies imply that amino acids induce glucose uptake via PI 3-kinase activation leading to GLUT4 translocation. It should also be noted, however, that the inhibitor used in the studies by Doi et al. (5) and Nishitani et al. (24), LY294002, blocks all classes of PI 3-kinase (31). A major difference between our current study and the in vitro studies of Doi et al. (5) and Nishitani et al. (24) is that we actually measured PI 3-kinase activity, rather than determining the necessity of PI 3-kinase in amino acid-stimulated glucose uptake. Our results are similar to Baum et al. (2) who reported that rats gavaged with either carbohydrate or a carbohydrate plus leucine supplement had similar IRS-1-associated PI 3-kinase activity 15 min postsupplementation. Moreover, it has been demonstrated that amino acids attenuate the length of time PI 3-kinase is activated (2, 26, 30), suggesting continuous exposure to high levels of amino acids could over time reduced glucose uptake, possibly via a mammalian target of rapamycin (mTOR)-mediated negative-feedback mechanism on IRS-1 (30). One possibility that could explain the discrepancy in findings is that PI 3-kinase may play a permissive role in amino acid-stimulated glucose uptake, and by eliminating its activity, glucose uptake is compromised.

In our effort to determine a mechanism for amino acid-induced skeletal muscle glucose uptake, we next focused on the impact of our amino acid mixture on AS160 phosphorylation. 

**Fig. 3.** AMPK phosphorylation in mixed gastrocnemius samples obtained from rats subjected to the hindlimb perfusion technique. Values are means ± SE.

**Fig. 4.** Atypical protein kinase C (aPKC) phosphorylation in mixed gastrocnemius samples obtained from rats subjected to the hindlimb perfusion technique. Values are means ± SE. *P < 0.05 vs. sINS, ¶P < 0.05 vs. AA, †P < 0.05 vs. BAS.
tion. AS160, a distal signal in the insulin-signaling cascade, prevents GLUT4 translocation. Once phosphorylated, however, its inhibitory effect on GLUT4 translocation is removed and GLUT4 is able to move from an intracellular storage pool to the plasma membrane. We previously reported that our amino acid mixture increased AS160 phosphorylation in vivo (3) and in vitro (13) and the increase in AS160 phosphorylation was associated with an amino acid-induced increase in muscle glucose uptake. In agreement, the current investigation found that perfusing our amino acid mixture also increased AS160 phosphorylation, although this effect was only observed in the presence of a submaximal insulin concentration and not in the absence of insulin. It is unlikely that Akt/PKB was responsible for the increased phosphorylation of AS160. We have previously reported that our AA mixture does not increase Akt/PKB phosphorylation in the presence or absence of insulin (13), as also observed by Peyrollier et al. (26). This would also agree with our current results that our amino acid mixture does not activate PI 3-kinase, which is directly upstream of Akt/PKB.

Therefore, these results suggest that amino acids increase the phosphorylation of AS160 via a mechanism independent of the canonical insulin-signaling pathway.

In the present investigation, plasma membrane GLUT4 protein concentration was significantly increased when insulin was added to the perfusate. The number of glucose transporters located at the plasma membrane was even greater when the perfusate contained both insulin and our amino acid mixture, demonstrating amino acids have a direct role in regulating GLUT4 translocation. Nishitani et al. (25) found that administration of both leucine and isoleucine to carbon tetrachloride-induced liver cirrhotic rats lowered their blood glucose response to an oral glucose challenge, which was associated with an increase in their skeletal muscle glucose uptake and sarcolemma-associated GLUT4 protein concentration (25). However, amino acid administration could have resulted in in vivo changes in insulin secretion or resulted in systemic changes that could account for the increased GLUT4 translocation observed. Our results therefore extend the findings of Nishitani et al. (25) by demonstrating that amino acids have a
direct effect on GLUT4 translocation, at least in the presence of insulin.

Together, AS160 phosphorylation and GLUT4 translocation were found to account for \( \sim 76\% \) of the variability in muscle glucose uptake. However, when using sequential regression analysis we found that the increase in AS160 phosphorylation accounted for \( \sim 72\% \) (\( R^2 = 0.716 \)) of the increase in glucose uptake while GLUT4 translocation could account for only 4\% (\( R^2 = 0.044 \)). These results suggest that the increase in muscle glucose uptake observed were due to GLUT4 translocation subsequent to the phosphorylation of AS160.

Although we found a strong correlation between AS160, GLUT4 translocation, and muscle glucose uptake, given what is currently known about insulin signaling, it is interesting that the increase in plasma membrane GLUT4 occurred without an increase in IRS-1-associated PI 3-kinase activity. There are several possibilities that may explain this observation. First, activation of mTOR, which is activated by amino acids and plays a role in amino acid-stimulated protein synthesis (1), may be involved. However, this appears unlikely as it has been demonstrated in several studies that blocking mTOR with its specific inhibitor, rapamycin, has no impact on amino acid-stimulated glucose uptake (5, 24). Furthermore, the primary amino acid in our mixture, isoleucine, is not known to activate mTOR.

Second, the cellular energy sensor AMPK also recruits GLUT4 to the plasma membrane. This is an intriguing possibility because AMPK mediates the insulin-independent translocation of GLUT4. However, we were unable to find a beneficial effect of our amino acid mixture on AMPK phosphorylation regardless of whether insulin was included in the perfusate. Our findings are in agreement with Doi et al. (6) demonstrating that isoleucine-stimulated glucose uptake is not mediated through an AMPK-dependent mechanism. Thus it appears unlikely that AMPK plays a role in amino acid-stimulated glucose uptake.

A third possibility involves a downstream signal from PI 3-kinase, aPKC. In vitro studies report that blocking aPKC with its specific inhibitor, GF109203X, results in significantly reduced amino acid-stimulated glucose uptake (5, 24). Thus these studies suggest that aPKC may be involved in amino acid-stimulated glucose uptake, presumably by increasing GLUT4 translocation to the plasma membrane. In agreement with these in vitro studies, we found that our amino acid mixture increased aPKC phosphorylation both in the presence and absence of insulin in vivo. The present investigation suggests that activation of aPKC might be involved in amino acid-stimulated glucose uptake. However, these results need to be viewed with caution as activation of aPKC in the absence of insulin had no effect on GLUT4 translocation or muscle glucose uptake. Nevertheless, because PI 3-kinase activity was not enhanced by amino acids, but aPKC was, this finding suggests that amino acids may prompt the phosphorylation of aPKC independent of PI 3-kinase.

Despite the fact perfusing rat hindlimb muscle with our amino acid mixture and insulin significantly increased glucose uptake, we did not observe increased amino acid-stimulated glucose uptake in the absence of insulin. In contrast, using the isolated muscle preparation, our laboratory previously demonstrated that our amino acid mixture increased glucose uptake across the epitrochlearis muscle in the absence of insulin (13). This is in agreement with earlier in vitro studies reporting that leucine (24), isoleucine (5) and isoleucine-leucine dipeptides (23) increased glucose uptake independent of insulin. Furthermore, in vivo studies, insulin appears to only play a permissive role in amino acid-stimulated glucose uptake. Oral administration of isoleucine significantly lowered blood glucose and increased skeletal muscle glucose uptake but did not raise plasma insulin levels compared with rats treated with saline (6, 7).

Consistent with these reports, our laboratory found that there was no difference in insulin secretion between rats orally gavaged with an amino acid mixture and those gavaged with a carbohydrate supplement, but the amino acid mixture resulted in a reduced blood glucose response (3). Collectively, these findings suggest that the effects of amino acids on glucose uptake are independent of insulin. In addition, when we evaluated the effect of our amino acid mixture on glucose uptake in isolated epitrochlearis muscle in the presence of either a submaximal or maximal insulin concentration, we found that the insulin and amino acid effects were additive (13). This would suggest that not only are the effects of amino acids on glucose uptake independent of insulin, but that the amino acid signaling pathway controlling glucose uptake is unique.

The discrepancy between studies that report a stimulatory effect of amino acids on glucose uptake in the absence of insulin, and the present study in which we observed no effect, is likely due to differences in the experimental models employed (4). Studies reporting that amino acids enhanced glucose uptake in the absence of insulin were performed using in vitro procedures such as cell culture and the isolated muscle preparation. In these studies blood flow is not a factor. For the isolated muscle preparation the relatively small soleus strip (24) and epitrochlearis (13) muscle allow excellent muscle fiber access to amino acids in solution. This is obviously the situation with cell culture experiments as well. In contrast, the hindlimb perfusion technique uses an intact circulatory system. Insulin exerts important actions on vascular cells and therefore may influence perfusate flow through the capillary beds or amino acid movement from the capillary to the interstitial space during perfusion. Thus we may not have observed increased amino acid-stimulated glucose uptake in the absence of insulin because capillary flow through the hindlimb muscleature or amino acid transit from capillary to interstitial space was compromised. In contrast, when a submaximal insulin concentration was added to the perfusate, flow through the capillary beds of the muscle was improved, contributing to the significantly greater amino acid-stimulated glucose uptake observed in the presence of insulin.
In summary, our amino acid mixture was found to increase skeletal muscle glucose uptake compared with a perfusate with no amino acids in the presence of insulin. However, we were unable to demonstrate that our amino acid mixture could increase muscle glucose uptake in the absence of insulin. While the mechanism by which amino acids work is not fully understood, our findings demonstrated that amino acid-stimulated skeletal muscle glucose uptake is not due to increased PI 3-kinase activity or AMPK phosphorylation. Rather, the amino acid induced improvements in glucose uptake appear due to increased aPKC and AS160 phosphorylation and enhanced GLUT4 translocation to the plasma membrane by a process that may be unique to amino acid stimulation.

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DISCLOSURES

J. L. Nelson is employed by Abbott Laboratories, the company that funded the study. His involvement in the study was study design, reviewing statistical analyses, editing the manuscript, and providing final approval of the manuscript.

AUTHOR CONTRIBUTIONS


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