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ORIGINAL ARTICLE

An amino acid mixture improves glucose tolerance and lowers insulin resistance in the obese Zucker rat

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Abstract The purpose of this investigation was to test an amino acid mixture on glucose tolerance in obese Zucker rats [experiment (Exp)-1] and determine whether differences in blood glucose were associated with alterations in muscle glucose uptake [experiment (Exp)-2]. Exp-1 rats were gavaged with either carbohydrate (OB-CHO), carbohydrate plus amino acid mixture (OB-AA-1), carbohydrate plus amino acid mixture with increased leucine concentration (OB-AA-2) or water (OB-PLA). The glucose response in OB-AA-1 and OB-AA-2 were similar, and both were lower compared to OB-CHO. This effect of the amino acid mixtures did not appear to be solely attributable to an increase in plasma insulin. Rats in Exp-2 were gavaged with carbohydrate (OB-CHO), carbohydrate plus amino acid mixture (OB-AA-1) or water (OB-PLA). Lean Zuckers were gavaged with carbohydrate (LN-CHO). Fifteen minutes after gavage, a radiolabeled glucose analog was infused through a catheter previously implanted in the right jugular vein. Blood glucose was significantly lower in OB-AA-1 compared to OB-CHO while the insulin responses were similar. Glucose uptake was greater in OB-AA-1 compared with OB-CHO, and similar to that in LN-CHO in red gastrocnemius muscle (5.15 ± 0.29, 3.8 ± 0.27, 5.18 ± 0.34 μmol/100 g/min, respectively). Western blot analysis showed that Akt substrate of 160 kDa (AS160) phosphorylation was enhanced for OB-AA-1 and LN-CHO compared to OB-CHO. These findings suggest that an amino acid mixture improves glucose tolerance in an insulin resistant model and that these improvements are associated with an increase in skeletal muscle glucose uptake possibly due to improved intracellular signaling.

Keywords Oral glucose tolerance test · Isoleucine · Leucine · Akt substrate of 160 kDa (AS160) · Skeletal muscle

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Introduction

Type 2 diabetes is a disease that is characterized by severe insulin resistance of insulin sensitive tissues such as skeletal muscle, liver and adipose tissue. When the body is presented with a glucose challenge, insulin is released into the general circulation and binds to its plasma membrane receptor. The binding of insulin to its receptor triggers a series of protein phosphorylation events within the cell, which leads to the translocation of glucose transporter 4 (GLUT4) to the plasma and transverse-tubule membranes and the removal of glucose from the extracellular medium. Because skeletal muscle is the primary site for postprandial glucose disposal (DeFronzo et al. 1981; Katz et al. 1983), it is therefore, important in regulating glucose homeostasis. In the case of type 2 diabetes, the ability of insulin to initiate translocation of GLUT4 in skeletal muscle is

defective primarily due to disruption in the insulin signaling cascade (Christ et al. 2002).

Dietary supplementation has become popular in many populations for improving insulin sensitivity and impaired glucose tolerance. These supplements are often advertised as a natural alternative to a pharmacologic-based approach. Recent investigations have focused on amino acid supplementation as a means of controlling the postprandial glucose response. Of the amino acids studied, the branched-chain amino acids (BCAAs) have generated the most research interest as they have been shown to markedly lower blood glucose (Bernard et al. 2011; Doi et al. 2003, 2007; Nishitani et al. 2002). Results from these studies suggest both leucine and isoleucine to be the most potent of the amino acids in enhancing skeletal muscle glucose uptake. Thus, the ability of amino acid supplementation to lower blood glucose holds great clinical significance for individuals with impaired glucose tolerance or type 2 diabetes.

Our laboratory has recently shown that an amino acid mixture composed of isoleucine and several additional amino acids improves glucose tolerance and muscle glucose uptake both in vivo and in vitro, in healthy, non-insulin resistant tissue (Bernard et al. 2011, 2012; Kleinert et al. 2011). We also determined that this amino acid mixture increases muscle glucose uptake in the absence and presence of a maximally stimulating concentration of insulin (Kleinert et al. 2011). This indicates that this mixture stimulates glucose uptake via an insulin-independent mechanism.

The obese Zucker rat is a widely accepted animal model for the study of insulin resistance. These rats are characterized by a genetic disorder that causes them to be obese and develop insulin resistance. Obese Zucker rats display severe hyperinsulinemia, glucose intolerance and elevated plasma insulin response to a glucose challenge, similar to that observed in insulin resistant humans. The insulin resistance of the obese Zucker rat has been traced to defects early in the insulin signaling pathway (Christ et al. 2002). Because amino acids appear to improve muscle glucose uptake independently of insulin, we hypothesized that amino acid supplementation prior to an oral glucose challenge would improve the glucose tolerance of the obese Zucker rat. We also hypothesized that this improvement would be due to a greater rate of glucose clearance by the skeletal muscle. Therefore, the purpose of this investigation was to determine if an amino acid mixture, previously demonstrated to improve glucose tolerance under non-insulin resistance conditions, can improve glucose tolerance in the obese Zucker rat [experiment (Exp)-1], and if so, are alterations in glucose tolerance due to improved skeletal muscle glucose uptake and cell signaling [experiment (Exp)-2].

Materials and methods

Animals

Exp-1: Thirty-five female obese Zucker rats approximately 7 weeks old were obtained from Charles River (Wilmington, MA). The obese Zucker rats were randomly assigned to one of four groups: obese-carbohydrate (OB-CHO, $n = 9$), obese-carbohydrate plus a 5-amino acid mixture (OB-AA-1, $n = 9$), obese-carbohydrate plus a 5-amino acid mixture with increased leucine concentration (OB-AA-2, $n = 9$) or obese-placebo (OB-PLA, $n = 8$). Rats were housed individually and provided standard laboratory chow (Prolab RMH 1800 5LL2, LabDiet, Brentwood, MO) and water ad libitum. The temperature of the animal room was maintained at 21 °C and an artificial 12:12 h light–dark cycle set. 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.

Exp-2: Eighteen female obese Zucker rats and six lean Zucker rats approximately 7 weeks old were obtained from Charles River. The obese Zucker rats were then randomly assigned to either obese-carbohydrate (OB-CHO, $n = 6$), obese-carbohydrate plus a five amino acid mixture (OB-AA-1, $n = 7$) or obese-placebo (OB-PLA, $n = 5$). Lean Zucker rats were assigned to the carbohydrate group (LN-CHO, $n = 6$). Rats were housed individually and provided standard laboratory chow and water ad libitum as described in Exp-1. Animal care and university approval were similar to that described for Exp-1.

Experimental protocol

Exp-1: Following 2 weeks of acclimation, rats were orally gavaged with distilled water and wrapped in a towel each day for 6 days prior the testing to become accustomed to the experimental procedures. After a 12-h fast, each rat was wrapped in a towel and the tip of their tail cut and bled prior the oral glucose tolerance test (OGTT). Rats were orally gavaged (8 ml/kg body weight) with 1 of 4 solutions: OB-CHO (22.5 % glucose), OB-AA-1 (amino acid mixture in 22.5 % glucose), OB-AA-2 (amino acid mixture with increased leucine concentration in 22.5 % glucose) or OB-PLA (distilled water). The amino acid mixture 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. The leucine concentration was increased to 50 mg/50 ml for the OB-AA-2 treatment. The specific amino acids were chosen because each has been shown to have a positive impact on glucose metabolism and previous

experiments in our laboratory found it to be more efficacious than isoleucine or other amino acids tested independently (Bernard et al. 2011). Blood was taken from the tail immediately before the gavage and 15, 30, 60 and 120 min after. Immediately after 120 min, rats were released from the towel wrap and returned to their cage until the next blood collection. Blood was collected in one test tube containing ethylenediaminetetraacetic acid (EDTA) (24 mg/ml, pH 7.4) and another with 10 % perchloric acid (PCA) for later analysis. Immediately after the 120 min blood sample, rats were sacrificed with a cardiac injection of sodium pentobarbital (65 mg/kg body weight).

Exp-2: Following 2 weeks of acclimation, rats were orally gavaged with distilled water and wrapped in a towel each day for 6 days prior testing to become accustomed to the experimental procedures. Four days before the OGTT, rats were prepared for surgical implantation of a jugular vein catheter. Rats were placed under isoflurane gas anesthesia to perform the jugular vein catheterization. Briefly, the right jugular vein was accessed above the clavicle by making a small incision over the ventral thorax. After removing the surrounding tissue to expose the jugular vein, ligatures were placed around the vein and a catheter containing heparinized saline inserted into the vein and guided down to the right atrium. The catheter was then guided from the thorax and through a small incision on the head. To insure that the catheter was in the proper position, a small amount of blood was drawn, flushed and heparinized saline stored in the catheter to prevent clotting. The incision sites over the thorax and head were then sutured and the catheter was sealed with a silicone patch to prevent backflow of the stored heparinized saline. A small strip of Velcro with a hole was sutured over the incision behind the head. The catheter was then threaded through the Velcro and the remaining catheter was coiled and placed between 2 Velcro strips for protection (Axelson and Bruot 1982).

Each morning leading up to the experimental day, the rats had their catheter flushed to maintain catheter patency and gavaged with saline to familiarize them with the OGTT procedure. Four days post-surgery, and following a 12-h fast, rats were wrapped in a towel and the tip of their tail cut and bled as discussed in the above section. The rats were then orally gavaged (8 ml/kg body weight) with either 22.5 % glucose (OB-CHO and LN-CHO), an amino acid mixture in 22.5 % glucose (OB-AA-1) or distilled water (OB-PLA). The amino acid mixture was as described previously in Exp-1. Fifteen minutes after the gavage, a bolus containing 40 μCi/kg body weight [^{3}H] 2-deoxyglucose (2-DG) and 20 μCi/kg body weight [U-^{14}C] mannitol was injected by syringe via the jugular catheter. The tail was bled and blood collected as described above at 2, 10, 20 and 45 min after the radioactive tracers were infused for later analysis. Immediately after the 45 min blood sample, corresponding to 60 min after gavage, the rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (75 mg/kg body weight) at which time the red and white gastrocnemius were excised, freeze clamped in liquid nitrogen and stored at −80 °C for later analysis. Rats were then euthanized by cardiac injection of sodium pentobarbital.

Blood analysis

A drop of blood was used to measure blood glucose with a portable glucose analyzer (One Touch Ultra 2; LifeScan Inc., Milpitas, CA). EDTA samples were then centrifuged 14,000×g for 10 min at 4 °C and the plasma insulin determined by a radioimmunoassay kit according to the manufactures' instructions (Linco, St. Charles, MO).

Muscle glucose uptake

Rates of 2-DG uptake were determined in both red and white gastrocnemius muscle samples. Approximately 80–100 mg of muscle was dissolved in 1 N potassium hydroxide (KOH) by incubating for 15 min at 65 °C, vortexed then incubated for an additional 5 min at 65 °C. Next, an equal volume of 1 N hydrochloric acid (HCl) was added to the digested samples and vortexed to neutralize the samples. To determine the specific activity of the blood, an aliquot of the PCA extract was added to 1 N KOH, then neutralized with an equal volume of 1 N HCl. A 300-μl aliquot of neutralized muscle and blood samples were then added to a vial containing 6 ml Bio-Safe II counting cocktail (Research Products International, Mount Prospect, IL). Duplicate samples were counted in a liquid scintillation counter (Beckman LS 6500, Beckman Coulter, Fullerton, CA) preset for simultaneous counting of [^{3}H] and [^{14}C] DPM. Quenching was determined by counting prepared standards. The accumulation of intracellular 2-DG was indicative of muscle glucose uptake. The specific activity of the blood for [^{3}H] and [^{14}C] was determined using the integral of the plasma 2-DG and [U-^{14}C] mannitol over the 15–60 min per glucose molecule. The extracellular space was calculated using the total muscle ^{14}C DPM and its specific activity. The intramuscular accumulation of 2-DG was calculated by subtracting its extracellular space DPM from its total muscle DPM divided by its specific activity.

Tissue processing

Approximately 60 mg of muscle was homogenized (1:9) in an ice-cold buffer (pH 7.4) containing 20 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), 2 mM ethylene glycol tetraacetic acid (EGTA), 50 mM

sodium fluoride (NaF), 100 mM potassium chloride (KCl), 0.2 mM EDTA, 50 mM glycerolphosphate, 1 mM DL-dithiothreitol (DTT), 0.1 mM phenylmethanesulphonyl fluoride (PMSF), 1 mM Benzamidine, and 0.5 mM sodium vanadate (1 ml/100 mg muscle) 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. Thereafter aliquots of the supernatant were stored at −80 °C for later analysis. The protein concentration of the homogenate was determined using the Lowry method (Lowry et al. 1951).

Western blotting

The phosphorylation of protein kinase B (Akt/PKB), mTOR, Akt substrate of 160 kDa (AS160) and glycogen synthase (GS) were used as an indirect measurement of activity. Sample protein (100 μg for Akt/PKB, 70 μg mTOR and AS160 and 60 μg for GS) was combined with an equal amount (1:1) of Laemmli sample buffer (125 mM tris, 20 % glycerol, 2 % sodium dodecyl sulfate (SDS), 0.008 % bromophenol blue, pH 6.8) (Laemmli 1970) and boiled for 5 min. Next, sample proteins were subject to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the proteins separated on either an 8 % (mTOR and AS160) or 12 % (Akt/PKD and GS)polyacrylamide resolving gel for either 1 h (Akt/PKB) or 1.5 h (mTOR, AS160 and GS). The resolved proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane using a semidry transfer unit and blocked in 7 % nonfat dry milk in Tris–Tween–buffered saline (NFDM/TTTBS) for 1 h at room temperature. The membranes were then incubated with either affinity purified anti-phospho-Akt/PKB (Thr-308) (Cell Signaling Technology, Danvers, MA), anti-phospho-mTOR (Ser-2448) (Cell Signaling Technology), anti-phospho-AS160 (Thr-642) (Millipore, Billerica, MA) or anti-phospho-GS (Ser-641) (Cell Signaling Technology) overnight at 4 °C. These phosphorylation sites were chosen as an indirect measurement of activity because they represent the primary sites of phosphorylation of the respective protein under insulin-stimulated conditions (Fingar and Blenis 2004; Sano et al. 2003; Sarbassov et al. 2005; Skurat et al. 2000). The primary antibodies were diluted to either 1:500 (phospho-Akt/PKB), 1:800 (phospho-AS160) or 1:1,000 (phospho-mTOR and phospho-GS) in TTBS containing 2 % NFDM. Following the overnight incubation, the membranes were washed three times for 5-min washes in TTBS and then re-blocked in 7 % NFDM for an additional 15 min. Next, membranes were washed in TTBS and then incubated for either 1 h (Akt/PKB and GS) or 2 h (mTOR and AS160) at room temperature with the species-specific (anti-rabbit) immunoglobulin G (IgG) secondary antibodies (Cell Signaling Technology). The secondary antibodies were diluted to either 1:750 (Akt/PKB and AS160), 1:900 (mTOR) or 1:2,000 (GS) in TTBS containing 2 % NFDM. The membranes were washed for 5, 8-min washes with TTBS and antibody binding was visualized by enhanced chemiluminescence in accordance to the manufacture's instructions (Perkin Elmer, Boston, MA). Images were captured using a charge-coupled device camera in a ChemiDoc system (BioRad, Hercules, CA) and saved to a computer. Density of the bands quantified with Quantity One analysis software (BioRad) and expressed as a percentage of a standard run on each gel.

After the phosphorylation status of each protein had been determined, the primary phospho-antibody was stripped from the membrane to determine the total protein concentration. 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 3, 15-min washes with TTBS to remove the stripping solution. The primary and secondary antibody concentrations, incubation times, washing and quantification were the same as that described for the determination of phosphorylation status.

Statistics

A two-way analysis of variance (ANOVA) was performed on the blood data (treatment × time). A one-way ANOVA was performed for the muscle tissue analysis. When a significant *F* ratio was obtained, a Fisher's least significant difference (LSD) post hoc test was performed to identify statistically significant differences ($P < 0.05$) between means. All statistical analyses were completed using SPSS software (SPSS Inc., Chicago, IL) and all values expressed as means ± standard error (SE).

Results

Animal characteristics

For Exp-1, there were no significant differences in body mass among treatment groups (OB-CHO 336.6 ± 9.3; OB-AA-1 344.9 ± 6.5; OB-AA-2 338.2 ± 8.7; OB-PLA 333.5 ± 10.0) on the day of testing. For Exp-2, there was no difference in body mass between the obese Zucker rat treatment groups (OB-CHO 338.7 ± 5 g; OB-AA-1 340.3 ± 13.2 g; OB-PLA 329 ± 15.9 g) on the day of testing. However, the lean Zucker rats were significantly smaller (185 ± 6.9 g) than all obese Zucker treatment groups.

OGTT glucose and insulin

For both experiments, glucose and insulin concentrations were determined following a 12-h fast and during the OGTT. For Exp-1, there were no significant differences in fasting glucose or insulin levels among treatment groups. There was little change in the blood glucose response for OB-PLA throughout the OGTT during Exp-1. This would indicate minimal stress on the animals throughout the OGTT. In Exp-1, blood glucose was significantly lower for OB-AA-1 and OB-AA-2 compared to OB-CHO at 15, 30, 60 and 120 min post-supplementation (Fig. 1a). The glucose area under the curve (AUC) was also lower for both the low and high amino acid treatments compared to carbohydrate alone in the obese Zucker rats (Fig. 1b). Although there were no statistically significant differences in plasma insulin at any time point between OB-CHO, OB-AA-1 or OB-AA-2 (Fig. 2a), both amino acid treatment groups had a greater insulin AUC compared to the carbohydrate only treatment (Fig. 2b).

For Exp-2, there was no difference in fasting blood glucose levels (among the obese treatment groups (Fig. 3a). However, the fasting blood glucose of the lean rats was significantly lower compared to each obese treatment group. The blood glucose response for OB-PLA was crucial to this study as this measurement indicated that the rats were properly familiarized and had recovered from the surgical procedure. The small increase in blood glucose measured for OB-PLA was similar to the changes observed in Exp-1, suggesting that the rats had recovered from surgery and that stress was minimal throughout the OGTT procedure. The blood glucose response for LN-CHO was significantly lower compared to OB-CHO and OB-AA-1 at all time points post-supplementation. Blood glucose was also significantly reduced for OB-AA-1 compared to OB-CHO at 27, 37, 47 and 60 min. The glucose AUC displayed a similar trend (Fig. 3b). OB-PLA was lower than all other treatment groups, and the glucose AUC was reduced in LN-CHO compared to the obese Zucker rat treatment groups. In addition, the glucose AUC was significantly lower in OB-AA-1 compared to OB-CHO.

There was no difference in fasting plasma insulin among the obese rat treatment groups in Exp-2 (Fig. 4a). However, fasting insulin was significantly lower in the lean rats compared to obese rats. The insulin for LN-CHO was lower than all obese treatments at each time point during the OGTT. OB-PLA, which was gavaged with distilled water, had little change in insulin over the 60 min OGTT, and their insulin was significantly lower than OB-CHO and OB-AA-1 at all time points. There was no difference in plasma insulin detected between OB-CHO and OB-AA-1

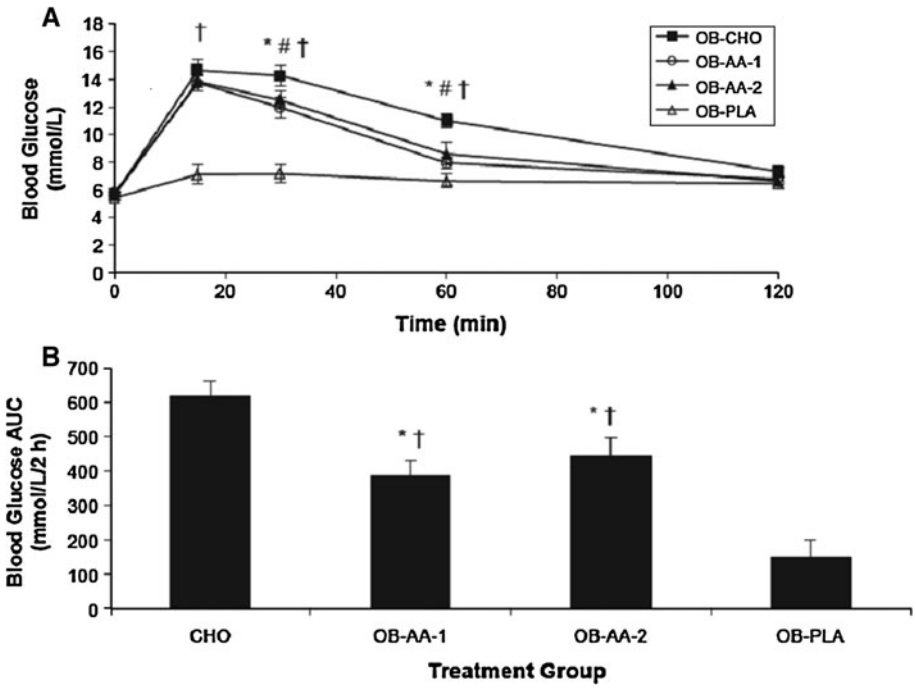

Fig. 1 **a** Exp-1 blood glucose during the OGTT. Obese Zucker rats were orally gavaged 8 ml/kg with either carbohydrate (OB-CHO, $n = 9$), carbohydrate plus a 5-amino acid mixture (OB-AA-1, $n = 9$), carbohydrate plus a 5-amino acid mixture with increased leucine concentration (OB-AA-2, $n = 9$) or placebo (OB-PLA, $n = 8$). Blood was taken from the tail immediately before the gavage and 15, 30, 60 and 120 min after. A drop of blood was used to measure blood glucose with a portable glucose analyzer. Values are means ± SE. $^{*}P < 0.05$ OB-CHO vs. OB-AA-1; $^{\#}P < 0.05$ OB-CHO vs. OB-AA-2; $^{\dagger}P < 0.05$ OB-PLA vs. all other treatments. **b** Exp-1 blood glucose AUC during the OGTT. Values are means ± SE. $^{*}P < 0.05$ vs. OB-CHO; $^{\dagger}P < 0.05$ OB-PLA. Fasting blood glucose levels were OB-CHO = 5.4 ± 0.3 mmol/L; OB-AA-1 = 5.8 ± 0.2 mmol/L; OB-AA-2 = 5.6 ± 0.1 mmol/L; OB-PLA = 5.4 ± 0.4 mmol/L

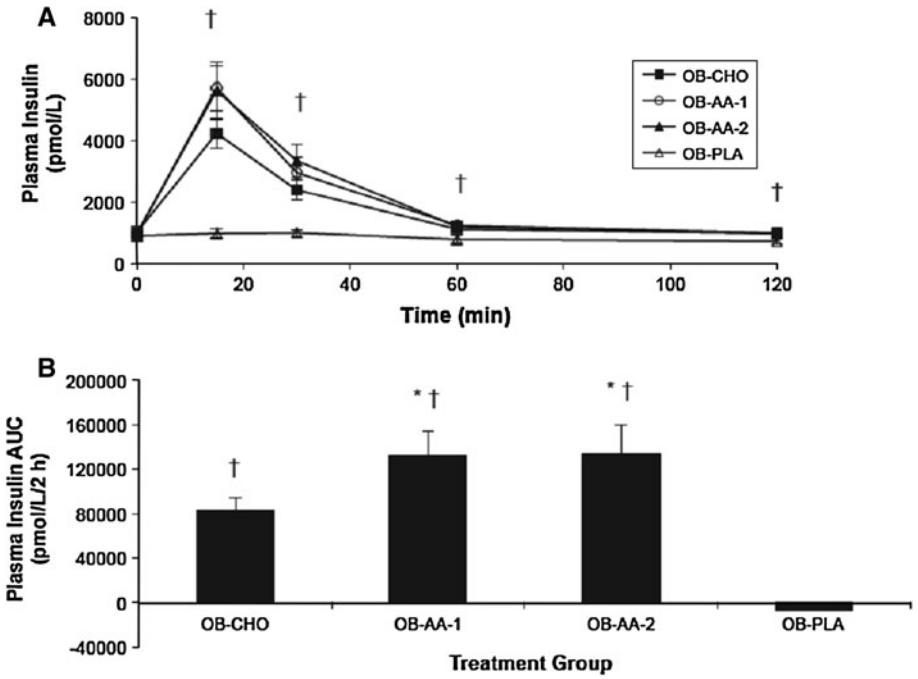

Fig. 2 **a** Exp-1 plasma insulin during the OGTT. Obese Zucker rats were orally gavaged 8 ml/kg with either carbohydrate (OB-CHO, $n = 9$), carbohydrate plus a 5-amino acid mixture (OB-AA-1, $n = 9$), carbohydrate plus a 5-amino acid mixture with increased leucine concentration (OB-AA-2, $n = 9$) or placebo (OB-PLA, $n = 8$). Blood was taken from the tail immediately before the gavage and 15, 30, 60 and 120 min after. Plasma insulin was determined using a radioimmunoassay kit. Values are means ± SE. $^{\dagger}P < 0.05$ OB-PLA vs. all other treatments. **b** Exp-1 plasma insulin AUC during the OGTT. Values are means ± SE. $^{*}P < 0.05$ vs. OB-CHO. $^{\dagger}P < 0.05$ vs. OB-PLA. Fasting plasma insulin levels were OB-CHO = 1,010.8 ± 110.1 pmol/L; OB-AA-1 = 933.8 ± 91.4 pmol/L; OB-AA-2 = 977.2 ± 73.1 pmol/L; OB-PLA = 902.1 ± 134.6 pmol/L

at 0, 17, 27, 47 or 60 min. When the insulin AUC was calculated there was no difference between OB-CHO and OB-AA-1 (Fig. 4b). The insulin AUC was significantly lower for LN-CHO compared to OB-CHO and OB-AA-1, but not different from OB-PLA.

Muscle glucose uptake

Rates of skeletal muscle 2-DG uptake for Exp-2 are shown in Fig. 5a, b. Glucose uptake was significantly increased in all treatment groups compared to OB-PLA in the red gastrocnemius. Glucose uptake for OB-AA-1 and LN-CHO treatment groups were significantly increased compared to OB-CHO in the red gastrocnemius. Similar to that observed in the red muscle, glucose uptake was increased for all treatment groups compared to OB-PLA in the white gastrocnemius. However, there were no differences detected for rates of glucose uptake among OB-CHO, OB-AA-1 and LN-CHO treatment groups in the white gastrocnemius.

Protein concentration and phosphorylation status of signaling proteins

There was no difference between treatment groups in the total protein concentration for Akt/PKB, mTOR, GS or AS160 detected following acute supplementation in either the red or white gastrocnemius in Exp-2. The phosphorylation status for these proteins was then assessed as an indirect measurement of activity. There was also no difference between treatment groups for the phosphorylation of Akt/PKB (Fig. 6a, b), mTOR (Fig. 7a, b), or GS (Fig. 8a, b) in both red and white gastrocnemius. However, a treatment difference did exist in the red gastrocnemius for AS160 phosphorylation (Fig. 9a). AS160 phosphorylation was significantly increased in OB-AA-1 compared to OB-CHO, and was similar to that in LN-CHO, while all treatment groups were significantly elevated above OB-PLA. In the white gastrocnemius, there was no difference in AS160 phosphorylation among OB-CHO, OB-AA-1 and LN-CHO but each was increased compared to OB-PLA (Fig. 9b).

Discussion

The primary finding of the present investigation was that an amino acid mixture consisting of isoleucine, leucine, cysteine, methionine and valine improved glucose tolerance in insulin resistant obese Zucker rats on an acute basis. This effect does not appear to be due to an elevated plasma

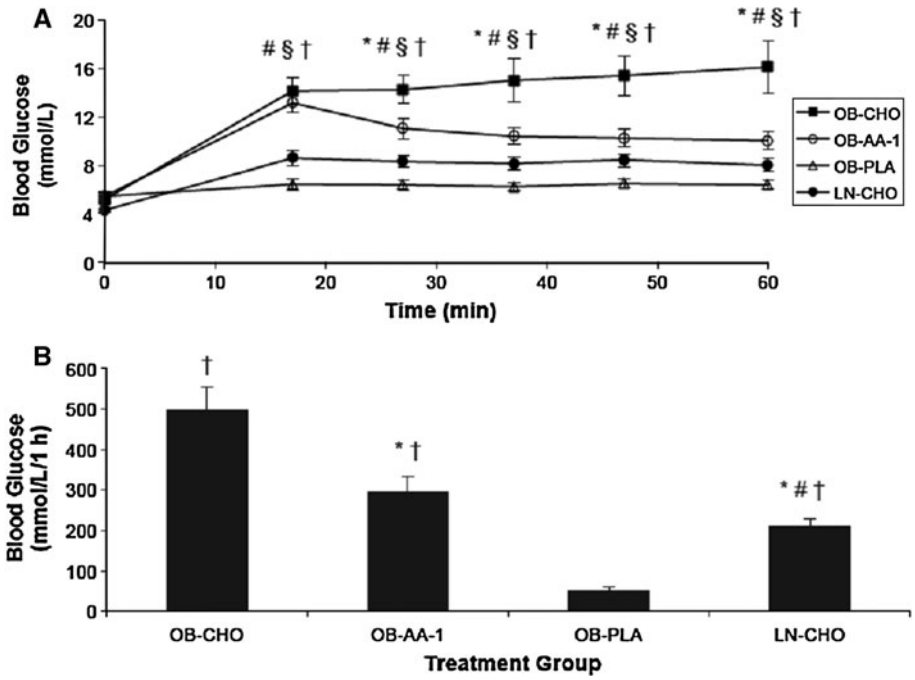

Fig. 3 **a** Exp-2 blood glucose during the OGTT. Obese Zucker rats were orally gavaged 8 ml/kg with either carbohydrate (OB-CHO, $n = 6$), carbohydrate plus a 5-amino acid mixture (OB-AA-1, $n = 7$) or placebo (OB-PLA, $n = 5$). Lean Zucker rats were gavaged with carbohydrate (LN-CHO, $n = 6$). Blood was taken from the tail immediately before the gavage and 17, 27, 37, 47 and 60 min after. A drop of blood was used to measure blood glucose with a portable glucose analyzer. Values are means ± SE. $^{*}P < 0.05$ OB-CHO vs. OB-AA-1; $^{\#}P < 0.05$ OB-CHO vs. LN-CHO; §OB-AA-1 vs. LN-CHO; $^{\dagger}P < 0.05$ OB-PLA vs. all other treatments. **b** Exp-2 blood glucose AUC during the OGTT in Zucker rats. Values are means ± SE. $^{*}P < 0.05$ vs. OB-CHO; $^{\#}$vs. OB-AA-1; $^{\dagger}P < 0.05$ OB-PLA. Fasting blood glucose levels were OB-CHO = 5.2 ± 0.3 mmol/L; OB-AA-1 = 5.5 ± 0.2 mmol/L; OB-PLA = 5.4 ± 0.4 mmol/L; LN-CHO = 4.3 ± 0.1 mmol/L

insulin concentration. Rather, the improved glucose tolerance appears due to increased skeletal muscle glucose uptake and enhanced AS160 phosphorylation. Previously, our laboratory reported that the addition of an amino acid mixture significantly lowered the blood glucose response to an oral glucose challenge in Sprague–Dawley rats. Using the radioactive glucose analog 2-DG, we then determined that the attenuated glucose response was due to enhanced skeletal muscle glucose clearance with no differences in the plasma insulin response between amino acid and carbohydrate treatment groups (Bernard et al. 2011). Thus, the present investigation adds to what we previously reported in healthy, non-insulin resistant tissue and demonstrates that the enhanced amino acid-induced glucose uptake can be applied to an insulin resistant model as well.

Insulin is the primary regulator of glucose uptake during the postabsorptive state. In general, the greater the insulin response, the greater insulin-stimulated glucose uptake. Therefore, ways to increase the insulin response have been investigated as a mean to increase the amount of glucose cleared by the muscle. Ingesting carbohydrates elicit rapid insulin secretion from the pancreas, however, co-ingesting carbohydrate with proteins and/or amino acids has been shown to further elevate the insulin response (Kalogeropoulou et al. 2008; Nuttall et al. 1984; van Loon et al. 2000, 2003; Zawadzki et al. 1992). The BCAA leucine in particular is known to stimulate in vivo insulin secretion (Floyd et al. 1966; Kalogeropoulou et al. 2008). With this in mind, for Exp-1, we increased the leucine concentration of the amino acid mixture from 0.13 to 1 mg/ml. Despite increasing the leucine concentration of the amino acid mixture, we did not observe a further increase in plasma insulin, nor did we observe an additional effect of the added leucine on blood glucose levels, which was in agreement with our previous investigation using Sprague–Dawley rats (Bernard et al. 2011). Therefore, we proceeded to use the amino acid mixture containing the low leucine concentration for our second experiment. Possible reasons we were unable to elicit a greater insulin response by adding more leucine to the amino acid mixture were the relatively low amount of leucine in the mixture, the rate at which amino acids was released into the gut or possibly because the insulin levels were already in excess in the obese Zucker rat. These findings are similar to what we reported previously in healthy non-insulin resistant tissue (Bernard et al. 2011) and suggest that the lower blood glucose response to the carbohydrate plus amino acid mixture was not due to increased insulin secretion for the amino acid treatment groups.

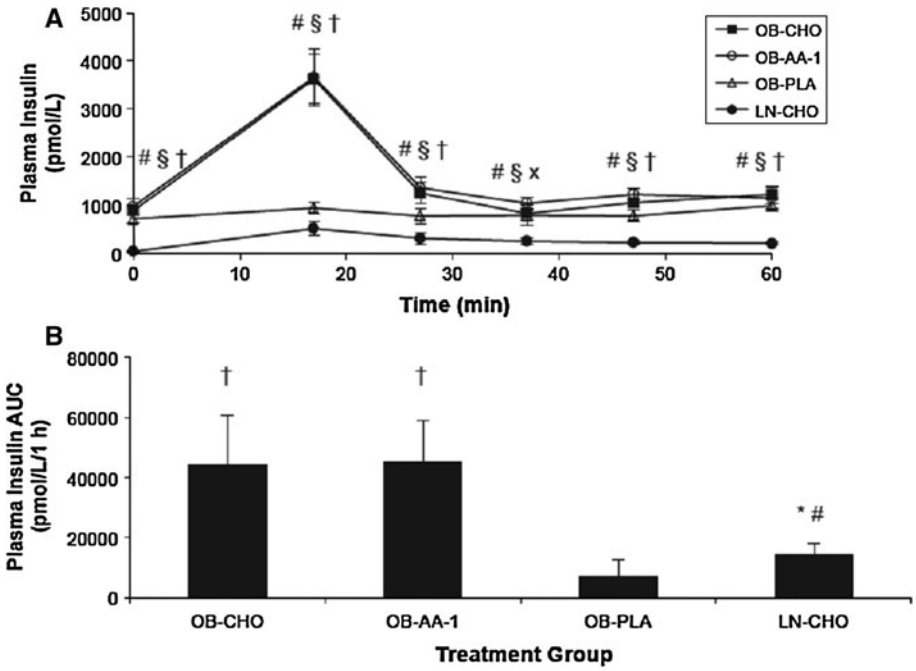

Fig. 4 **a** Exp-2 Plasma insulin during the OGTT in Zucker rats. Obese Zucker rats were orally gavaged 8 ml/kg with either carbohydrate (OB-CHO, $n = 6$), carbohydrate plus a 5-amino acid mixture (OB-AA-1, $n = 7$) or placebo (OB-PLA, $n = 5$). Lean Zucker rats were gavaged with carbohydrate (LN-CHO, $n = 6$). Blood was taken from the tail immediately before the gavage and 17, 27, 37, 47 and 60 min after. Plasma insulin was determined using a radioimmunoassay kit. Values are means ± SE. $^{*}P < 0.05$ OB-CHO vs. OB-AA-1; $^{\#}P < 0.05$ OB-CHO vs. LN-CHO; §OB-AA-1 vs. LN-CHO; $^{\times}P < 0.05$ OB-PLA vs LN-CHO; $^{\dagger}P < 0.05$ OB-PLA vs. all other treatments. **b** Exp-2 plasma insulin during the OGTT in Zucker rats. Values are means ± SE. $^{*}P < 0.05$ vs. OB-CHO; $^{\#}$vs. OB-AA-1; $^{\dagger}P < 0.05$ OB-PLA. Fasting plasma insulin levels were OB-CHO = 885.9 ± 243.9 pmol/L; OB-AA-1 = 976.1 ± 171.9 pmol/L; OB-PLA = 819.1 ± 185.8 pmol/L; LN-CHO = 45.9 ± 9.8 pmol/L

Although we are unable to completely rule out the involvement of insulin for amino acid-stimulated glucose uptake using our in vivo model, it does appear that insulin may not play a significant role in this process. It is noteworthy that the primary ingredient of the amino acid mixture is the BCAA isoleucine, which has been shown to increase glucose uptake in vivo without a synergistic increase in insulin secretion in rats (Bernard et al. 2011; Doi et al. 2003) and in human subjects (Nuttall et al. 2008). Lowered blood glucose response with amino acids, then, if not due to increased insulin concentrations, may be associated with improved intracellular signaling. Using specific inhibitors, previous studies suggest that enhanced amino acid-induced muscle glucose uptake requires both phosphatidylinositol 3-kinase (PI 3-kinase) (Doi et al. 2003; Nishitani et al. 2002) and protein kinase C (PKC) (Nishitani et al. 2002), but is independent of mTOR (Doi et al. 2003; Nishitani et al. 2002). However, we recently reported that amino acids stimulate glucose uptake independently of insulin (Kleinert et al. 2011) and without activation of Akt and PI 3-kinase, (Bernard et al. 2012). Accounting for the increase in glucose uptake, however, was an increased AS160 phosphorylation and GLUT4 translocation (Bernard et al. 2012). Although the permissive role of PI 3-kinase cannot be ignored, these results suggest that amino acids activate skeletal muscle glucose uptake in healthy, non-insulin resistant tissue by increasing AS160 phosphorylation, and that this increase in phosphorylation is not predicated on activation of proteins prior to Akt in the insulin signaling pathway.

The insulin resistance of the obese Zucker rat is associated with defects early in the insulin signaling pathway of skeletal muscle (Christ et al. 2002). After demonstrating that amino acid supplementation improved glucose tolerance in obese Zucker rats similar to non-insulin resistant rats, we investigated if the amino acid mixture would have a similar effect on muscle glucose uptake and cell signaling in insulin resistant and non-insulin resistant rats when challenged with an oral glucose load.

We first evaluated the effect of insulin resistance on muscle glucose uptake and the associated insulin signaling proteins after an oral glucose challenge. We found that the glucose uptake in the obese Zucker rat was reduced by 36 % across the red gastrocnemius compared to the lean Zucker rat when treated with the carbohydrate supplement. This is in agreement with previous reports, with the insulin resistance of obese Zucker rat due to a combination of insulin receptor and early post-receptor defects (Brozinick et al.

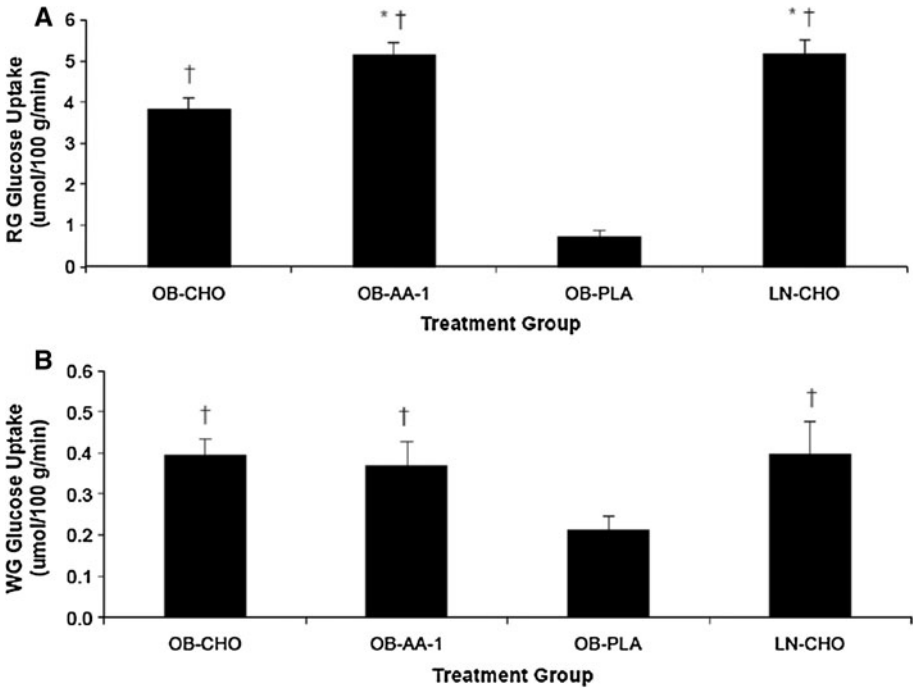

Fig. 5 Exp-2 glucose uptake in the (**a**) red gastrocnemius and (**b**) white gastrocnemius during the OGTT in Zucker rats. Obese Zucker rats were orally gavaged 8 ml/kg with either carbohydrate (OB-CHO, $n = 6$), carbohydrate plus a 5-amino acid mixture (OB-AA-1, $n = 7$) or placebo (OB-PLA, $n = 5$). Lean Zucker rats were gavaged with carbohydrate (LN-CHO, $n = 6$). Blood was taken from the tail immediately before the gavage and 17, 27, 37, 47 and 60 min after. Fifteen minutes after the gavage, a bolus containing [^{3}H]-2 DG and [U-^{14}C] mannitol was injected by syringe via the tail vein. Immediately after the 60 min blood sample, rats were anesthetized at which time the gastrocnemius was excised, sectioned into red and white portions, freeze clamped in liquid nitrogen and stored at −80 °C. Values are means ± SE. *$P < 0.05$ vs. OB-CHO; †$P < 0.05$ OB-PLA (color figure online)

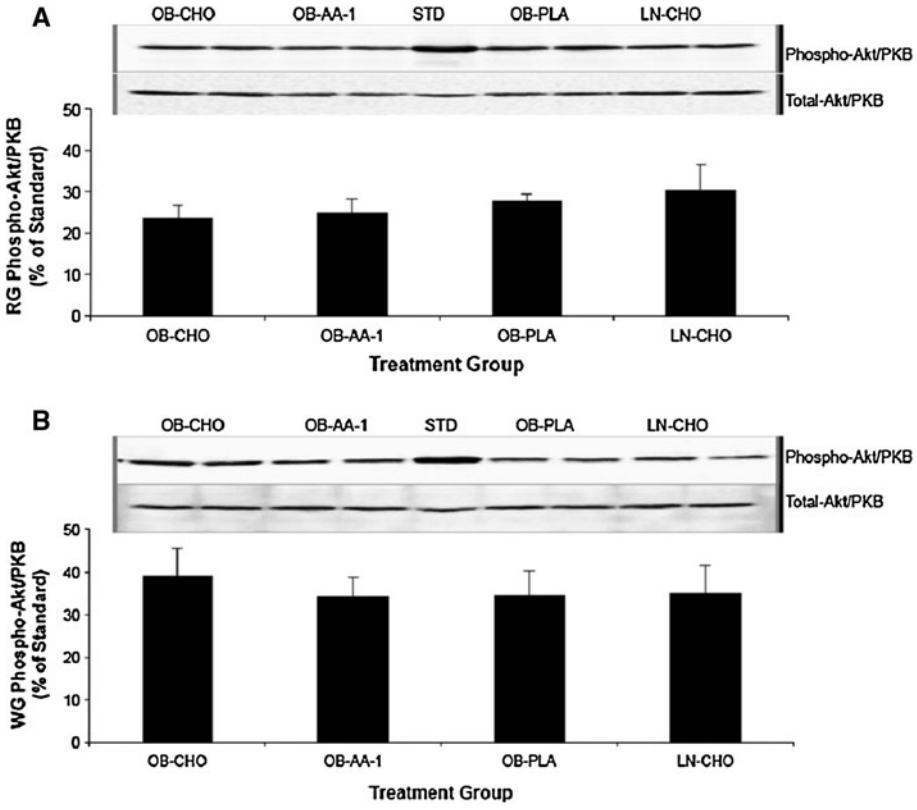

Fig. 6 Exp-2 Akt Thr-308 phosphorylation following the OGTT in Zucker rats from the (**a**) red gastrocnemius and (**b**) white gastrocnemius muscles. Obese Zucker rats were orally gavaged 8 ml/kg with either carbohydrate (OB-CHO, $n = 6$), carbohydrate plus a 5-amino acid mixture (OB-AA-1, $n = 7$) or placebo (OB-PLA, $n = 5$). Lean Zucker rats were gavaged with carbohydrate (LN-CHO, $n = 6$). Immediately after the 60 min blood sample, rats were anesthetized at which time the gastrocnemius was excised, sectioned into red and white portions, freeze clamped in liquid nitrogen and stored at −80 °C. Samples were then subjected to Western blot analysis. Values are means ± SE (color figure online)

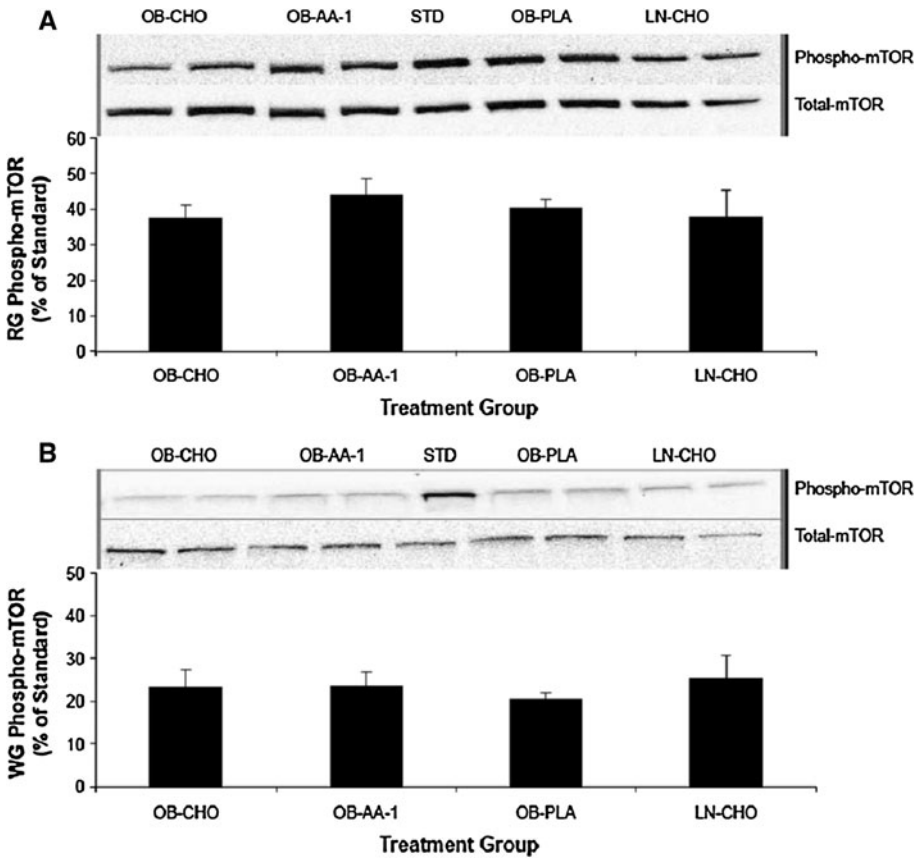

Fig. 7 Exp-2 mTOR Ser-2448 phosphorylation following the OGTT in Zucker rats from the (**a**) red gastrocnemius and (**b**) white gastrocnemius muscles. Obese Zucker rats were orally gavaged 8 ml/kg with either carbohydrate (OB-CHO, $n = 6$), carbohydrate plus a 5-amino acid mixture (OB-AA-1, $n = 7$) or placebo (OB-PLA, $n = 5$). Lean Zucker rats were gavaged with carbohydrate (LN-CHO, $n = 6$). Immediately after the 60 min blood sample, rats were anesthetized at which time the gastrocnemius was excised, sectioned into red and white portions, freeze clamped in liquid nitrogen and stored at −80 °C. Samples were then subjected to Western blot analysis. Values are means ± SE (color figure online)

1993; Christ et al. 2002; Crettaz et al. 1980; Sherman et al. 1988). In the present investigation, amino acid supplementation resulted in a 35 % increase in glucose uptake in obese Zucker rats compared to obese Zuckers treated with carbohydrate only. Interestingly, we observed a similar increase in skeletal muscle glucose uptake for both the LN-CHO and OB-AA-1, suggesting that the amino acid mixture was able to restore glucose disposal in the insulin resistant rats, although it required a much greater insulin response. We did not, however, observe differences in the total protein expression between lean and obese Zucker rats for Akt, mTOR, GS or AS160. Therefore, the reduced glucose uptake in the obese Zucker rats does not appear due to reduced total protein expression of these specific insulin signaling proteins (Benton et al. 2010; Katta et al. 2009).

We also did not observe differences in the phosphorylation status between lean and obese Zucker rats for Akt, mTOR, or GS when treated with carbohydrate or the amino acid mixture. We did, however, find that the amino acid mixture significantly increased red gastrocnemius AS160 phosphorylation compared to OB-CHO. The phosphorylation and subsequent inhibition of AS160 by Akt are critical steps in GLUT4 translocation to the plasma membrane. Under basal conditions, AS160 maintains a Rab protein in its inactive GDP-bound state. Upon insulin stimulation, AS160 is phosphorylated, allowing the conversion of the Rab protein to its activated GTP-bound form, enabling GLUT4 translocation and tissue glucose uptake (Watson and Pessin 2006). Thus, the inability to attribute the increase in AS160 phosphorylation to increased Akt/PKB phosphorylation is noteworthy, which is in agreement with our findings in Sprague–Dawley rats (Bernard et al. 2011; Kleinert et al. 2011). In our previous study testing the amino acid mixture in Sprague–Dawley rats, we reported a 61 % increase in AS160 phosphorylation for rats gavaged with the amino acid mixture compared to carbohydrate alone (Bernard et al. 2011). Nevertheless, the 37 % increase in amino acid-stimulated AS160 phosphorylation translated to a 35 % increase in glucose uptake in the present investigation. The reasons why there was less effect of the amino acid mixture in obese Zuckers relative to Sprague–Dawley rats are not clear, but it is likely related to muscular and/or systemic impairments associated with the insulin resistant state.

It is noteworthy that we did not observe a difference in glucose uptake between lean and obese Zucker rats treated with carbohydrate in the white gastrocnemius, suggesting that the white muscle was not insulin resistant. However, the glucose uptake in the white muscle was very low in both the obese and lean Zucker rats relative to that observed in red muscle. The average glucose uptake in the white gastrocnemius for OB-CHO, OB-AA-1 and LN-CHO was

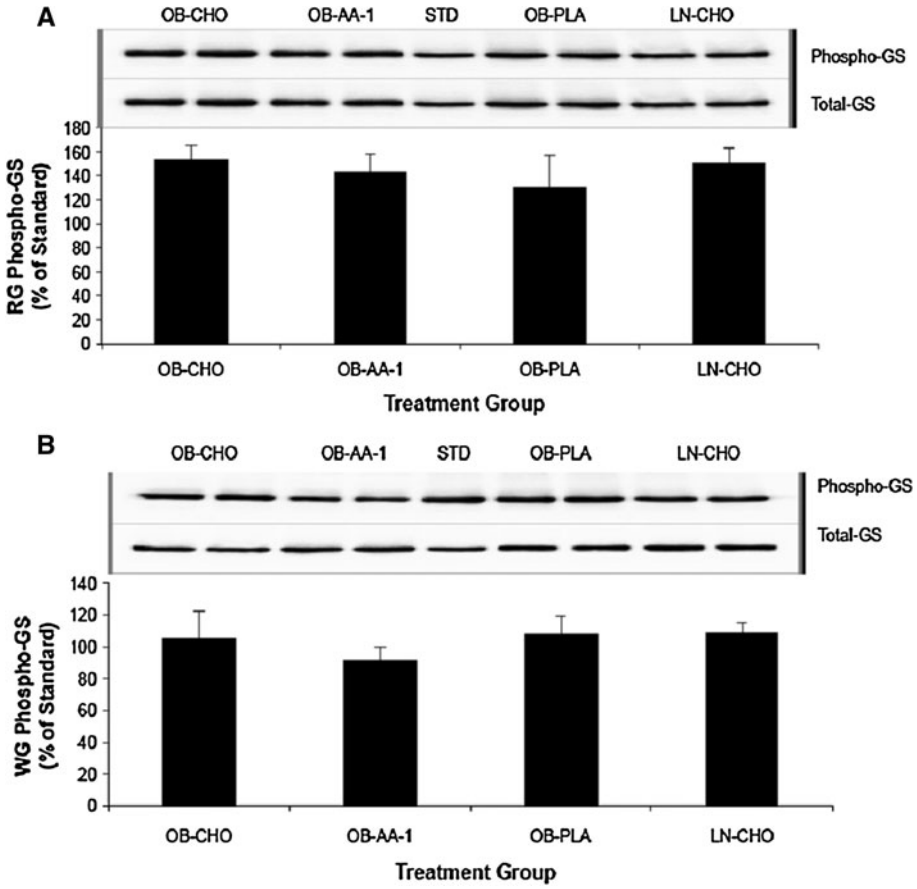

Fig. 8 Exp-2 glycogen synthase Ser-641 phosphorylation following the OGTT in Zucker rats from the (**a**) red gastrocnemius and (**b**) white gastrocnemius muscles. Obese Zucker rats were orally gavaged 8 ml/kg with either carbohydrate (OB-CHO, $n = 6$), carbohydrate plus a 5-amino acid mixture (OB-AA-1, $n = 7$) or placebo (OB-PLA, $n = 5$). Lean Zucker rats were gavaged with carbohydrate (LN-CHO, $n = 6$). Immediately after the 60 min blood sample, rats were anesthetized at which time the gastrocnemius was excised, sectioned into red and white portions, freeze clamped in liquid nitrogen and stored at −80 °C. Samples were then subjected to Western blot analysis. Values are means ± SE (color figure online)

0.387 µmol/100 g/min compared to 4.72 µmol/100 g/min, respectively, in the red gastrocnemius. Therefore, rates of glucose uptake in white muscle were approximately one-twelfth that seen in red muscle. Some possible reasons for this is that glycolytic white muscle has fewer capillaries surrounding the muscle fiber (Romanul 1965) and has significantly less GLUT4 (Banks et al. 1992; Friedman et al. 1990; Kern et al. 1990) compared to the more oxidative red muscle. As a result of these fiber-type differences, reduced blood flow and insulin-stimulated glucose uptake in the white gastrocnemius would be expected. Thus, it is also possible that the very low glucose uptake in the white muscle prevented us from detecting differences in uptake among OB-CHO, OB-AA-1 and LN-CHO.

Despite the positive results obtained in the present investigation using the amino acid mixture, some studies suggest that amino acid supplementation actually contributes to insulin resistance. It has been reported that circulating amino acids are elevated in obese individuals (Felig et al. 1969, 1974) and that diets high in protein are associated with impaired glucose metabolism (Linn et al. 1996, 2000). Some of these studies propose that amino acids attenuate glucose uptake via a negative feedback mechanism through mTOR (Linn et al. 2000; Tremblay et al. 2007). Accordingly, elevated amino acids activate the mTOR/p70 pathway and then inhibit PI 3-kinase through serine phosphorylation of insulin receptor substrate-1 (IRS-1) leading to skeletal muscle insulin resistance (Newgard et al. 2009; Tremblay and Marette 2001). Amino acids, especially the BCAA leucine, are strong activator of mTOR signaling. However, isoleucine is not believed to activate mTOR (Atherton et al. 2010) as confirmed by the present results (Fig. 7). Since the predominant ingredient of the amino acid mixture is isoleucine, rather than leucine, this may help explain the discrepancy among studies. Although leucine has been shown to increase glucose uptake, it is feasible that relatively low concentrations of leucine are beneficial for glucose metabolism, while high concentrations of leucine may impair this process (Nishitani et al. 2002). Nevertheless, future studies exploring the dose and temporal response relationship of leucine and glucose uptake are warranted.

In conclusion, we demonstrated that an amino acid mixture enhanced skeletal muscle glucose uptake in the obese Zucker rat during an oral glucose challenge. The effects of the amino acid mixture appear to be independent of insulin and due, at least in part, to increased AS160 phosphorylation by a process that does not involve activation of insulin signaling proteins known to be defective in the obese Zucker rat. These findings suggest that the acute ingestion of an

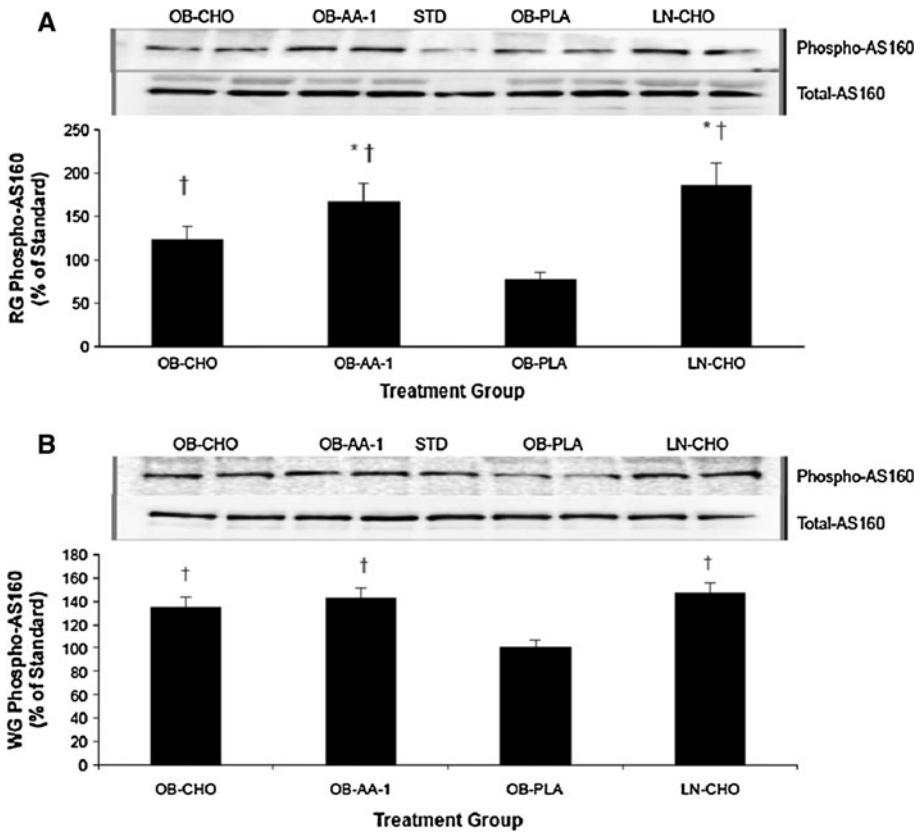

Fig. 9 Exp-2 AS160 Thr-642 phosphorylation following the OGTT in Zucker rats from the (**a**) red gastrocnemius and (**b**) white gastrocnemius muscles. Obese Zucker rats were orally gavaged 8 ml/kg with either carbohydrate (OB-CHO, $n = 6$), carbohydrate plus a 5-amino acid mixture (OB-AA-1, $n = 7$) or placebo (OB-PLA, $n = 5$). Lean Zucker rats were gavaged with carbohydrate (LN-CHO, $n = 6$). Immediately after the 60 min blood sample, rats were anesthetized at which time the gastrocnemius was excised, sectioned into red and white portions, freeze clamped in liquid nitrogen and stored at −80 °C. Samples were then subjected to Western blot analysis. Values are means ± SE. $^{*}P < 0.04$ vs. OB-CHO; $^{\dagger}P < 0.05$ OB-PLA (color figure online)

amino acid mixture consisting predominantly of isoleucine can improve insulin resistance. However, further research is needed to determine the long-term effects of amino acid supplementation on insulin resistance and whether the positive results found in the present investigation translate to humans and those with more severe insulin resistance such as that observed in type 2 diabetes.

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