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Abundance of amino acid transporters involved in mTORC1 activation in skeletal muscle of neonatal pigs is developmentally regulated

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Abstract

Previously we demonstrated that the insulin- and amino acid-induced activation of the mammalian target of rapamycin complex 1 (mTORC1) is developmentally regulated in neonatal pigs. Recent studies have indicated that members of the System A transporter (SNAT2), the System N transporter (SNAT3), the System L transporters (LAT1 and LAT2) and the proton-assisted amino-acid transporters (PAT1 and PAT2) have crucial roles in the activation of mTORC1 and that the abundance of amino acid transporters is positively correlated with their activation. This study aimed to determine the effect of the post-prandial rise in insulin and amino acids on the abundance or activation of SNAT2, SNAT3, LAT1, LAT2, PAT1, and PAT2 and whether the response is modified by development. Overnight fasted 6- and 26-day-old pigs were infused for 2 h with saline (Control) or with insulin or amino acids to achieve fed levels while amino acids or insulin, respectively, as well as glucose were maintained at fasting levels. The abundance of SNAT2, SNAT3, LAT1, LAT2, PAT1 and PAT2 was higher in muscle of 6- compared to 26-day-old pigs. The abundance of the PAT2-mTOR complex was greater in 6- than in 26-day-old pigs, consistent with the higher activation of mTORC1. Neither insulin nor amino acids altered amino acid transporter or PAT2-mTOR complex abundance. In conclusion, the amino acid transporters, SNAT 2/3, LAT 1/2, and PAT1/2, likely have important roles in the enhanced amino acid-induced activation of mTORC1 in skeletal muscle of the neonate.

Keywords

protein synthesis; amino acid transceptor; nutrient sensing; amino acid signaling

Introduction

The neonatal period is characterized by rapid growth that is due to a high rate of protein synthesis (Davis et al. 1989). In previous studies, we demonstrated that protein deposition is elevated in neonatal pigs, especially in skeletal muscle (Davis et al. 1996). This rapid gain in protein mass is in part due to their ability to increase protein synthesis in response to feeding, a response that declines markedly with development. The feeding-induced stimulation of protein synthesis in skeletal muscle of neonatal pigs is independently modulated by the rise in insulin and amino acids, especially leucine (O'Connor et al. 2003; Escobar et al. 2005). Furthermore, the activation of the insulin and amino acid signaling pathways leading to protein synthesis is high in neonatal pigs and decreases with development (Suryawan et al. 2007).

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Both insulin- and amino acid-induced protein synthesis is partly facilitated by mammalian target of rapamycin (mTOR), a major kinase that is crucial for the well-being of individual cells (Wang and Proud 2011; Zoncu et al. 2011). mTOR exist in two distinct protein complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (Zoncu et al. 2011). Studies suggest that amino acids only activate mTORC1 while insulin activates both complexes (Wang and Proud 2011). Biochemical and genetic approaches have been used to search for upstream signaling components that modulate the activation of mTORC1 leading to protein synthesis. During recent years, much progress has been made in dissecting mTORC1 modulators, including the finding that amino acid transporters are crucial for mTORC1 activation (Nicklin et al. 2009). The molecular mechanism by which amino acid transporters alter mTORC1 activation has been studied largely using cell culture systems. Since cell culture or *in vitro* systems often have weaknesses in interpreting biological outcomes, the role of amino acid transporters in mTORC1 activation *in vivo* warrants investigation.

It is intriguing that beyond the classical function of transferring amino acids into or out of cells, amino acid transporters have been implicated in regulating mTORC1 activation and protein synthesis (Nicklin et al. 2009; Kurayama et al. 2011; Pinilla et al. 2011). To this end, there are three major functions of amino acid transporters in the regulation of mTORC1 activation. First, amino acid transporters synchronize the transport of essential amino acids, such as leucine, which is important for mTORC1 activation (Nicklin et al. 2009). For example, glutamine transporters, such as the System A transporter (SNAT2) and the System N transporter (SNAT3), preserve the glutamine gradient across the plasma membrane, resulting in the transport of leucine by the System L transporters (LAT1 or LAT2). Second, amino acid transporters act as 'transceptors' which are capable of sensing and signaling amino acid availability to the mTOR pathway (Pinilla et al. 2011). SNAT2 is also considered a transceptor. Third, amino acid transporters participate in the activation of mTORC1 by directly binding to members of the mTORC1 protein complex (Reynolds et al. 2007). An example of this category is the proton assisted-amino acid transporters, PAT1 and PAT2. Recent studies also indicate that PAT1 and PAT2 possess the characteristics of a transceptor (Heublein et al. 2010).

The expression of amino acid transporters in different cell types is varied depending on the physiological functions of the cell (Palacín et al. 1998). In skeletal muscle, the majority of bidirectional transmembrane glutamine movement is carried out by System N transporters (SNAT3 and SNAT5) (Mackenzie and Erickson 2004). Branched-chain amino acids (leucine, isoleucine and valine) make up about 18% of total skeletal muscle protein (Riazi et al. 2003), therefore the transport of branched-chain amino acids into and out of skeletal muscle is critical. Recent studies suggest that branched-chain amino acid movement in skeletal muscle is facilitated by the System L transporters, which include LAT1 and LAT2 (Drummond et al. 2010, 2012; Hamdi and Mutungi 2011). PAT1 and PAT2 are responsible for transport of a variety of small neutral amino acids (Goberdhan et al. 2005). The important role of these transporters was recognized after genetic studies in the drosophila fly demonstrated that deletion of these transporters retards overall growth (Goberdhan et al. 2005).

Despite this wealth of new information, little is known about the physiological role of the amino acid transporters *in vivo* during the neonatal period. Since our previous studies suggest that the abundance and activation of positive regulators of mTORC1 are higher in the neonate and decrease with development in parallel with the developmental decline in the activation of mTORC1 (Suryawan and Davis 2010) and rates of protein synthesis (Suryawan et al. 2007), it is critical to examine the effect of development on the amino acid transporters that are involved in mTORC1 activation. Therefore, the aim of this study was to determine

the effect of insulin, amino acids, and age on the activation and protein abundance of the amino acid transporters in skeletal muscle of neonatal pigs.

Material and methods

Animals and experimental protocols

Multiparous cross-bred (Landrace × Yorkshire × Duroc × Hampshire) pregnant sows (Agriculture Headquarters, Texas Dept. of Criminal Justice, Huntsville, TX) were housed in lactation crates in individual, environmentally controlled rooms prior to farrowing. Sows consumed a commercial diet (no. 5084; PMI Feeds, Richmond, IN) and drank water ad libitum. After farrowing, piglets remained with the sow but were not allowed access to the sow's diet. Piglets were anesthetized for sterile catheter insertion into a jugular vein and carotid artery 3 days before study. Piglets from four litters were studied at 6 (1.9 ± 0.3 kg) and 26 days of age (5.2 ± 0.8 kg), following a 12-h fast. The protocol, previously described in Suryawan et al. (2010), was approved by the Animal Care and Use Committee of Baylor College of Medicine and was conducted in accordance with the National Research Council's *Guide for the Care and Use of Laboratory Animal*.

We randomly assigned piglets within each litter to one of three treatment groups ($n = 4-6$ per age group per treatment group): 1) euinsulinemic-euglycemic-euaminoacidemic conditions (control), 2) euinsulinemic-euglycemic-hyperaminoacidemic clamp (AA), and 3) hyperinsulinemic-euglycemic-euaminoacidemic clamp (INS), as previously described (Suryawan et al. 2010). Blood samples were obtained and immediately analyzed for glucose (YSI 2300 STAT Plus; Yellow Springs Instruments, Yellow Springs, OH) and total branched-chain amino acids (rapid enzymatic kinetic assay) to establish the basal concentration of blood glucose and plasma branched-chain amino acids to be used during the clamp procedures. Clamps were initiated with a primed, constant (12 ml/h) infusion of insulin (Eli Lilly, Indianapolis, IN) at 0 or 100 ng·kg^{-0.66}·min⁻¹ given to achieve plasma insulin concentrations of 3 (fasting insulin level) or 30 μU/ml (fed insulin level). To clamp glucose and amino acids at fasting levels, venous blood samples were acquired every 5 min and immediately analyzed for glucose and branched-chain amino acids concentrations. The dextrose (Baxter Healthcare, Deerfield, IL) infusion rate was adjusted to maintain blood glucose concentrations within $\pm 10\%$ of the basal fasting concentrations. Euaminoacidemia was obtained by adjusting the infusion rate of a balanced amino acid mixture to maintain plasma branched-chain amino acids within 10% of fasting levels. Hyperaminoacidemia was obtained by infusion of a balanced amino acid mixture to raise plasma branched-chain amino acid concentrations by two-fold the fasting level to reproduce the level of amino acids present in the fed state. Blood samples also were taken at intervals for later determination of circulating insulin concentration.

Immunoblotting and immunoprecipitation

Frozen longissimus dorsi muscle samples were homogenized and centrifuged at 10,000 *g* for 10 min at 4°C. The protein concentration was determined in the supernatant by the Bradford method (Suryawan et al. 2010). Equal amounts (50 μg) of extracted protein were electrophoretically separated in polyacrylamide gels and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA), which was incubated with appropriate primary antibodies followed by appropriate secondary antibodies as previously described (Suryawan et al. 2010). Blots were developed using an enhanced chemiluminescence kit (Amersham), visualized, and analyzed using a ChemiDoc-It Imaging System (UVP, Upland, CA). The protein abundance of each transporter was normalized with actin abundance in the samples. Primary antibodies that were used in the immunoblotting were LAT2, PAT1/2, SNAT3, Actin (Santa Cruz Biotechnology, Santa Cruz, CA), LAT1,

mTOR, and raptor (Cell Signaling Technology, Danvers, MA), and SNAT2 (Aviva System Biology, San Diego, CA).

To determine the association between mTOR and PAT1 or PAT2, muscle samples were homogenized in CHAPS buffer as previously described (Suryawan et al. 2007). Briefly, the CHAPS buffer (40 mM HEPES, pH 7.5, 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM β -glycerolphosphate, 40 mM NaF, 1.5 mM sodium vanadate, 0.3% CHAPS, 0.1 mM PMSF, 1 mM benzamidine, and 1 mM DTT) was used for tissue homogenization. The homogenate was mixed on a platform rocker for 30 min at 4°C and then centrifuged at 1,000 *g* for 3 min (4°C). The supernatant containing 500 μ g of protein was combined with 2 μ l of anti-mTOR antibody (Cell Signaling Technology) and mixed on a platform rocker overnight at 4°C. Following the incubation, the immune complexes were isolated with a anti-rabbit BioMag IgG (PerSeptive Diagnostics, Cambridge, MA) bead slurry. The magnetic bead complexes were collected using a magnetic stand, washed twice with CHAPS buffer, and once in CHAPS buffer containing 200 mM instead of 120 mM NaCl and 60 mM instead of 40 mM HEPES. The precipitates were rinsed with 100 μ l of 1 $\times$ SDS sample buffer and then boiled for 5 min and centrifuged to collect the supernatant. The samples were subjected to SDS-PAGE followed by immunoblotting with anti PAT-1, PAT2 and raptor. The protein-protein complexes were normalized by the mTOR abundance in the precipitates. We have optimized the efficiency of the mTOR immunoprecipitation. The above mTOR antibody concentration was sufficient to maximally precipitate mTOR protein from 500 μ g of protein homogenate. Furthermore, we also have determined that the majority of PAT2 interacted with mTOR. The mTOR-raptor complex was used as a positive control.

Statistical analysis

Two-way ANOVA was used to determine the effects of insulin, amino acids, age, and their interaction on the abundance of amino acid transporters. When significant interactions were detected, the value in each treatment group for each age was compared with the control value by use of *t*-tests. Probability values of $P < 0.05$ were considered statistically significant. Data are presented as mean $\pm$ SEM.

Results

In order to study the independent effects of insulin and amino acids on the activation and abundance of the amino acid transporters, the clamp technique was performed. For the purpose of clarifying the overall aim of this study, we herein report previously published data on substrate analysis (Table 1) and the activation of mTORC1 and protein synthesis (Table 2) (Suryawan et al. 2007) for reference. As indicated in Table 1, circulating glucose levels were maintained at baseline (fasting levels) in all treatments. Plasma amino acid levels (branched-chain amino acids concentrations were used as indicators) were increased more than two-fold to fed levels only in the amino acid group ($P < 0.05$). Similarly, plasma insulin levels were maintained at the fed level only in the insulin group ($P < 0.05$). Thus, the clamp technique was successful in achieving the desired substrate and hormone concentrations.

The abundance of SNAT2, a well known glutamine transporter and transceptor, was significantly higher in skeletal muscle of 6-day-old pigs when compared to 26-day-old pigs ($P < 0.05$). However, raising either insulin or amino acids to the fed level had no effect on the abundance of SNAT2 (Fig. 1a). In this study, we used SNAT3 to represent other glutamine transporters. As shown in Fig. 1b, 6-day-old pigs had higher SNAT3 abundance compared to their older counterparts ($P < 0.05$), but neither insulin nor amino acids had any affect on SNAT3 abundance. As representative of the system L transporters, we determined the protein abundance of LAT-1 and LAT-2. Although increased age resulted in a reduction

in LAT-1 and LAT-2 abundance ($P < 0.05$) (Fig. 2), insulin and amino acids failed to modulate the protein abundance of these transporters. To study members of the mammalian proton-assisted transporters, we analyzed the abundance of PAT-1 and PAT-2. As shown in Fig. 3, the protein abundance of both PAT-1 and PAT-2 was higher in skeletal muscle of 6-day-old compared to 26-day-old pigs ($P < 0.05$). Similar to system L transporters, there was no effect of insulin or amino acids on the protein abundance of PAT1 and PAT2. Since measurement of protein-protein interactions among members of mTORC1 is considered to be an indirect method for monitoring mTORC1 activation, the association between mTOR and PAT1 as well as PAT2 was examined (Fig. 4). We were not able to detect an interaction between mTOR and PAT1. The abundance of the mTOR-PAT2 complex was higher in skeletal muscle of 6-day-old compared to 26-day-old pigs ($P < 0.05$). On the other hand, raising insulin or amino acids to the fed levels did not modify mTOR-PAT2 interaction.

Discussion

One of our research interests is focused on understanding the molecular mechanisms by which anabolic agents induce protein synthesis in skeletal muscle during periods of high metabolic activity such as during early postnatal life. We have previously shown that the activation of the insulin as well as amino acid signaling pathways leading to stimulation of muscle protein synthesis is considerably higher in neonatal pigs compared to their older counterparts (Suryawan and Davis 2010). Although these findings are encouraging, questions still remain regarding the exact mechanism by which amino acids stimulate muscle protein synthesis through the nutrient signaling pathway *in vivo*. While progress has been made, the amino acid signaling pathway is not completely understood (Wang and Proud 2011). Recent studies indicate that amino acid transporters not only serve in their classical function as nutrient transporters, but can also act as nutrient signaling components responsible for activation of mTORC1 leading to activation of protein translation (Nicklin et al. 2009; Heublein et al. 2010; Pinilla et al. 2011). However, *in vivo* studies on these important issues are limited. Therefore, we conducted the current study to evaluate the effect of insulin, amino acids, as well as age on the abundance and activation of the amino acid transporters that are known to be crucial for mTORC1 activation.

To maintain growth, cells require a steady supply of energy and nutrients, including amino acids (Tennant et al. 2009; Wu 2012). As the primary source of cellular nitrogen, amino acids are used as the building blocks for protein synthesis, as well as sources for nucleotide, glutathione, and ATP synthesis (Tennant et al. 2009). Amino acid transporters play pivotal roles in maintaining intercellular amino acids in response to cellular needs. For example, human cancer cells, which are extremely dependent on nutrient supply to sustain a high proliferation rate, express high levels of amino acid transporters (Fuchs et al. 2005). Curiously, there are two major types of amino acid transporters that are markedly elevated in human cancer cells: transporters for glutamate and transporters for essential amino acids, including leucine (Sidoryk et al. 2004; Fuchs et al. 2005; Nawashiro et al. 2006). The higher expression of these transporters is consistent with the over-activated mTORC1 signaling that is crucial for cancer cell growth (Tennant et al. 2009; Kaira et al. 2011).

An important role of glutamine receptors in regulating mTORC1 signaling has been demonstrated by Nicklin et al. (2009). They found that a bidirectional glutamine transporter, SLC1A5, regulates the simultaneous efflux of glutamine out of cells and transport of leucine into cells, resulting in the activation of mTORC1 signaling. Since the SLC1A5 transporter is mainly expressed in rapidly growing epithelial cells and tumor cells (McGivan and Bungard 2007), we did not detect this transporter in skeletal muscle of neonatal pigs (data not shown). Several studies indicate that in skeletal muscle, one of the transporters responsible for glutamine uptake and release is SNAT3 (System N) (Onan et al. 2005; McGivan and

Bungard 2007). Interestingly, in this study we found that the abundance of SNAT3 is markedly elevated in skeletal muscle of younger piglets compared to older pigs. If the mechanistic models proposed by Nicklin et al. (2009) apply to all glutamine transporters, our findings support the notion that the higher abundance of SNAT3 in neonatal as compared to older pigs may be in part responsible for the higher activation of mTORC1 signaling.

SNAT2 (System A) is another glutamine transporter (Mackenzie and Erickson 2004) that is expressed in skeletal muscle cell lines (Evans et al. 2007) and adult human skeletal muscle (Drummond et al. 2010). Similar to SNAT3, we found that the abundance of SNAT2 was high in skeletal muscle of neonatal pigs and decreased with development. It also has been reported that SNAT2 can act as a transceptor, a transporter that can carry out the function of nutrient sensor (Pinilla et al. 2011). This concept is supported by several studies including a study using MCF7 cells which showed that SNAT2 may be able to signal directly to mTOR (Taylor 2009). Although the mechanism for this transceptor remains unknown, it is tempting to speculate that SNAT2 is one of the key players for activation of mTORC1 in skeletal muscle of neonatal pigs.

L-type amino acid transporters, LAT1 and LAT2, have been shown to be expressed in adult human skeletal muscle (Drummond et al. 2010). Since these transporters are responsible for active transport of essential amino acids (including leucine) across the plasma membrane, it is reasonable to assert that these transporters are important for mTORC1 activation. The current study showed that the abundance of both LAT1 and LAT2 was higher in younger pigs as compared with their older counterparts. Interestingly, a recent study demonstrated that dihydrotestosterone stimulates mouse skeletal muscle protein synthesis partly by up-regulating the expression of LAT2 (Hamdi and Mutungi 2011). Furthermore, LAT2 also has been shown to have a pivotal role in the activation of the mTORC1 pathway in glomerulus epithelial cells (Kurayama et al. 2011). In another study, Liu et al. (2004) demonstrated that LAT1 is crucial for vascular smooth muscle growth induced by platelet-derived growth factor. Taken together, our findings supports a role for LAT in regulating mTORC1 activation.

Another group of amino acid transporters that have the capability of being transceptors is PAT (Reynolds et al. 2007; Goberdhan 2010). Although studies on PAT are very limited, PAT1 and PAT2 mRNA have been detected in adult human skeletal muscle (Drummond et al. 2010). Similar to other amino acid transporters determined in the current study, PAT1 and PAT2 abundance was high in skeletal muscle of neonatal pigs and declined with age.

A recent study using cancer cell lines demonstrated that PAT is one of the key regulators of cell growth and it does so in an mTORC1-dependent fashion (Heublein et al. 2010). The binding of PAT with mTOR complex proteins is one of proposed mechanisms by which mTORC1 is activated by PAT (Heublein et al. 2010). Since protein-protein interactions are likely to be important for signal transduction, this proposed mechanism places PAT as a strong candidate for a transceptor. Moreover, our findings, which indicate that the abundance of the PAT2-mTOR complex was higher in younger pigs and decreased with development in parallel with the developmental decrease in mTORC1 activation, fits the notion that PAT may be one of the important nutrient sensing components of the mTORC1 pathway in skeletal muscle. Although we could not detect the interaction between PAT1 and mTOR, a recent study by Zoncu et al. (2011) indicates that PAT1 is a partner of mTOR that resides in the lysosomal membrane. The discrepancy between our results and the above study (Zoncu et al. 2100) is probably due to differences in cell type or experimental protocol.

Although we found developmental regulation of amino acid transporter abundance, we did not detect an effect of either insulin or amino acids on the parameters measured in this study. One possible explanation for the lack of treatment effects was the relatively short infusion time. Interestingly, in a study using cultured human myotubes, Gran and Cameron-Smith (2011) found that incubation with either leucine or insulin up to 24 h had no effect on SNAT2 and LAT2 mRNA abundance. Conversely, Drummond et al. (2010) demonstrated that 1 h ingestion of essential amino acids is sufficient to stimulate the mRNA abundance of SNAT2, LAT2, and LAT1 in adult human skeletal muscle. Unfortunately, the protein abundance of these transporters was not measured in that study. Considering that the protein abundance of the amino acid transporters reflects their activity (Taylor 2009), more studies are needed to evaluate the effect of both amino acids and insulin in the regulation of amino acid transporter protein abundance. Nonetheless, the results of the current study do not support the notion that amino acid transporter abundance or activity modulates the activation of mTORC1 in response to the post-prandial rise in insulin and amino acids.

Studies on the amino acid transporters have allowed us to better understand the task of amino acid transporters beyond their classical function as simple transports for nutrients to move into and out of cells. However, most of mechanistic studies have been done in cell cultures, mainly by using cancer cell lines. Therefore, the exact physiological role of these transporters in altering cell growth *in vivo* is largely unknown. Due to the complexity of the nature of amino acid transporters (types and tissues specificity), many challenges still lie ahead of us to understand the role of amino acid transporters in the regulation of mTORC1 signaling. Nevertheless, our findings may offer a glimpse into the possible functions of SNAT 2/3, LAT 1/2, and PAT 1/2 as mediators for the amino acid-induced activation of mTORC1 pathways leading to protein synthesis in skeletal muscle. Our findings also support the notion that up-regulation of amino acid transporter expression or activity helps to amplify anabolic signals during periods of rapid cell growth (Taylor 2009).

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Fig 1.

The abundance of SNAT2 (a) and SNAT3 (b) in skeletal muscle of 6- and 26-day-old pigs after 2 h infusion of saline (C), amino acids (AA), or insulin (INS). The values were normalized by actin abundance. Values are means $\pm$ SEM; $n = 4-6$. Bars with different letters are significantly different at $P < 0.05$.

Fig 2.

The abundance of LAT1 (a) and LAT2 (b) in skeletal muscle of 6- and 26-day-old pigs after 2 h infusion of saline (C), amino acids (AA), or insulin (INS). The values were normalized by actin abundance. Values are means $\pm$ SEM; $n = 4-6$. Bars with different letters are significantly different at $P < 0.05$.

Fig 3.

The abundance of PAT1 (a) and PAT2 (b) in skeletal muscle of 6- and 26-day-old pigs after 2 h infusion of saline (C), amino acids (AA), or insulin (INS). The values were normalized by actin abundance. Values are means $\pm$ SEM; $n = 4-6$. Bars with different letters are significantly different at $P < 0.05$.

Fig 4.

The abundance of the PAT2-mTOR complex in skeletal muscle of 6- and 26-day-old pigs after 2 h infusion of saline (C), amino acids (AA), or insulin (INS). The values were normalized by mTOR abundance in precipitates. A representative blot of mTOR-raptor that was used as a positive control is shown. Values are means $\pm$ SEM; $n = 4-6$. Bars with different letters are significantly different at $P < 0.05$.

Table 1

Circulating glucose, branched-chain amino acids, and insulin concentrations in 6- and 26-day-old neonatal pigs (Suryawan et al. 2007)

	Baseline [*]	Control	AA	INS
Glucose (mg/dl)				
6-day-old	67.3 ± 5.9	62.5 ± 3.3	76.2 ± 3.8	72.7 ± 6.3
26-day-old	97.1 ± 5.8 ^b	96.8 ± 7.6 ^b	98.2 ± 3.9 ^b	83.1 ± 5.3 ^b
BCAA (nmol/ml)				
6-day-old	433 ± 100	470 ± 51	1,139 ± 47 ^a	549 ± 110
26-day-old	442 ± 46	487 ± 47	1,075 ± 50 ^a	477 ± 14
Insulin (μU/ml)				
6-day-old	2.65 ± 0.5	2.07 ± 0.7	2.26 ± 0.6	28.3 ± 7.6 ^a
26-day-old	3.97 ± 0.9 ^b	5.35 ± 0.8 ^b	4.45 ± 0.7 ^b	23.2 ± 2.5 ^a

Values are means ± SEM; *n* = 4–6. BCAA, branched-chain amino acids; Control, response to euinsulinemic -euglycemic-euaminoacidemic clamps; AA, response to euinsulinemic-euglycemic-hyperaminoacidemic clamps; INS, response to hyperinsulinemic-euglycemic-euaminoacidemic clamps.

^{*} The baseline values are the data collected at 0 min.

^a Differ at *P* < 0.05 compared with baseline.

^b Differ at *P* < 0.05 compared with 6-day-old pigs.