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Intermittent Bolus Compared With Continuous Feeding Enhances Insulin and Amino Acid Signaling to Translation Initiation in Skeletal Muscle of Neonatal Pigs

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ABSTRACT

Background: Nutrition administered as intermittent bolus feeds rather than continuously promotes greater protein synthesis rates in skeletal muscle and enhances lean growth in a neonatal piglet model. The molecular mechanisms responsible remain unclear.

Objectives: We aimed to identify the insulin- and/or amino acid-signaling components involved in the enhanced stimulation of skeletal muscle by intermittent bolus compared to continuous feeding in neonatal pigs born at term.

Methods: Term piglets (2–3 days old) were fed equal amounts of sow milk replacer [12.8 g protein and 155 kcal/(kg body weight · d)] by orogastric tube as intermittent bolus meals every 4 hours (INT) or by continuous infusion (CTS). After 21 days, gastrocnemius muscle samples were collected from CTS, INT-0 (before a meal), and INT-60 (60 minutes after a meal) groups ($n = 6$/group). Insulin- and amino acid-signaling components relevant to mechanistic target of rapamycin complex (mTORC) 1 activation and protein translation were measured.

Results: Phosphorylation of the insulin receptor, IRS-1, PDK1, mTORC2, pan-Akt, Akt1, Akt2, and TSC2 was 106% to 273% higher in the skeletal muscle of INT-60 piglets than in INT-0 and CTS piglets ($P < 0.05$), but phosphorylation of PTEN, PP2A, Akt3, ERK1/2, and AMPK did not differ among groups, nor did abundances of PHLPP, SHIP2, and Ubl4A. The association of GATOR2 with Sestrin1/2, but not CASTOR1, was 51% to 52% lower in INT-60 piglets than in INT-0 and CTS piglets ($P < 0.05$), but the abundances of SLC7A5/LAT1, SLC38A2/SNAT2, SLC38A9, Lamtor1/2, and V-ATPase did not differ. Associations of mTOR with RagA, RagC, and Rheb and phosphorylation of S6K1 and 4EBP1, but not eIF2α and eEF2, were 101% to 176% higher in INT-60 piglets than in INT-0 and CTS piglets ($P < 0.05$).

Conclusions: The enhanced rates of muscle protein synthesis and growth with intermittent bolus compared to continuous feeding in a neonatal piglet model can be explained by enhanced activation of both the insulin- and amino acid-signaling pathways that regulate translation initiation. *J Nutr* 2021;151:2636–2645.

Keywords: neonate, insulin, amino acids, skeletal muscle, translation initiation

Introduction

Optimal nutritional support for infants is vital for their growth and development (1). Infants who cannot suck, swallow, or breathe normally are fed human milk or formula by orogastric tube either continuously or as intermittent bolus feeds (2). However, the effectiveness of these nutritional support methods is contentious (3). Recently, using the neonatal pig as a model for the human infant, we showed that bolus feeding enhances lean growth more than continuous feeding, and that the higher muscle mass was attained via enhanced rates of protein synthesis (4).

During the neonatal period, the feeding-induced stimulation of skeletal muscle protein synthesis is enhanced and contributes to rapid lean accretion (5). This enhanced rate of protein synthesis is mediated by the heightened activation of signaling pathways upstream and downstream of mechanistic target of rapamycin complex (mTORC) 1 that regulate the mRNA binding step in translation initiation (6). The activation of mTORC1 by feeding is mediated by the postprandial rise in circulating insulin and amino acids that stimulates the intracellular insulin- and amino acid-signaling pathways (7).

Insulin mediates its biological effects by autoactivation of its receptor, followed by phosphorylation of insulin receptor substrate 1 (IRS-1), activation of PI3-Kinase, and Akt phosphorylation (**Figure 1**) (8, 9). The Akt kinase family is comprised of 3 homologous isoforms: Akt1, Akt2, and Akt3 (10). Their activity is controlled by positive regulators, such as 3-phosphoinositide-dependent protein kinase-1 (PDK1) (11),

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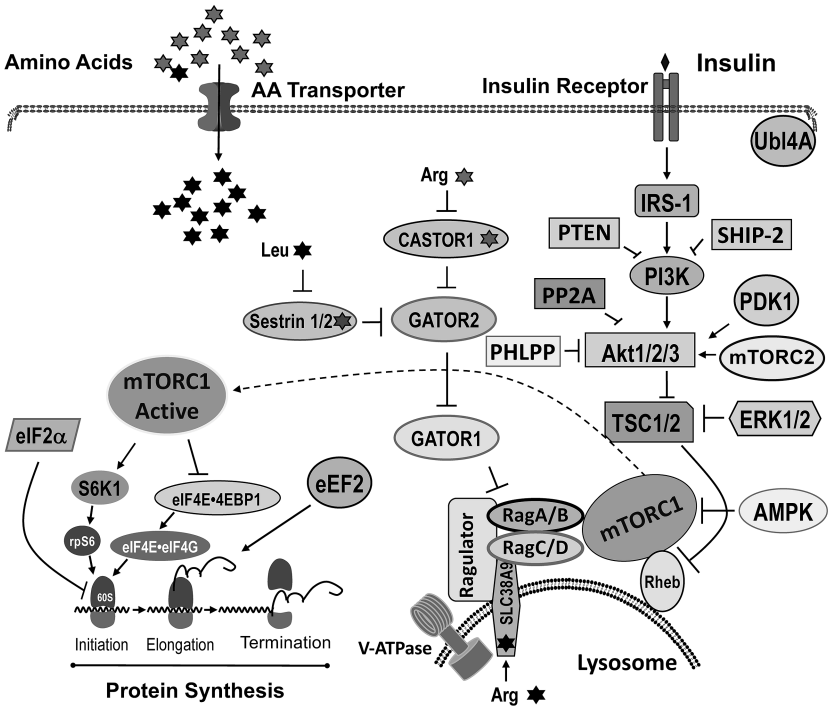

FIGURE 1 Current models of insulin- and amino acid-induced mTORC1 activation leading to protein synthesis. Akt1/2/3, RAC-alpha serine/threonine-protein kinase 1/2/3; AMPK, AMP-activated protein kinase; Arg, arginine; CASTOR1, cytosolic arginine sensor for mTORC1 subunit 1; eEF2, eukaryotic elongation factor 2; eIF2α, eukaryotic translation initiation factor 2α; eIF4E, eukaryotic translation initiation factor 4E; eIF4G, eukaryotic translation initiation factor 4G; ERK1/2, extracellular signal-regulated kinases 1 and 2; GATOR, GAP activity toward Rags; IRS-1, insulin receptor substrate 1; Leu, leucine; mTORC, mechanistic target of rapamycin complex; PDK1, 3-phosphoinositide-dependent protein kinase-1; PI3K, phosphatidylinositol-3-kinase; PHLPP, PH domain leucine-rich repeat protein phosphatase; PP2A, protein phosphatase 2A; PTEN, phosphatase and tensin homolog, deleted on chromosome 10; Rag, Ras-related GTP-binding protein; Ragulator, late endosomal/lysosomal adaptor and mitogen-activated protein kinase and mechanistic target of rapamycin activator; Rheb, Ras homolog enriched in brain; S6K1, p70 ribosomal protein S6 kinase 1; Sestrin1/2, stress response protein 1/2; SHIP2, SH2 domain-containing inositol phosphatase; TSC, tuberous sclerosis complex; SLC38A9, solute carrier family 38 member 9; Ubl4A, ubiquitin-like protein 4A; V-ATPase, vacuolar H+-ATPase; 4EBP1, eukaryotic translation initiation factor 4E–binding protein 1.

mTORC2 (12), and ubiquitin-like protein 4A (Ubl4A) (13), as well as by inhibitors including phosphatase and tensin homolog, deleted on chromosome 10 (PTEN); Src homology domain 2 (SH2)-containing inositol phosphatase (SHIP2); protein phosphatase 2A (PP2A); and PH domain leucine-rich repeat protein phosphatase (PHLPP) (14–17). Insulin- or growth factor-induced Akt activation stimulates the rapid phosphorylation of tuberous sclerosis complex 2 (TSC2), an inhibitor of mTORC1 that enables Ras homolog enriched in brain (Rheb) to activate mTORC1 (9).

Unlike the insulin-signaling networks, the amino acid-signaling pathways that activate mTORC1 are less well understood. In addition to being building blocks for protein synthesis, amino acids such as leucine and arginine, through their transporters [L-type amino acid transporter 1 (SLC7A5/LAT1); sodium-coupled neutral amino acid transporters, member 2 (SLC38A2/SNAT2); and solute carrier family 38 member 9 (SLC38A9)] (18–20), can act as anabolic agents that activate mTORC1 via nutrient-sensing components localized in the cytosol or lysosomal membrane (Figure 1) (21). Current in vitro studies indicate that when amino acids are in

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Supplemental Table 1 is available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents available on the table of contents at https://academic.oup.com/jn.
Address correspondence to TAD (e-mail: tdavis@bcm.edu).
Abbreviations used: AMPK, AMP-activated protein kinase; BCAA, branched-chain amino acid; BW, body weight; CASTOR1, cytosolic arginine sensor for mechanistic target of rapamycin complex 1 subunit 1; CTS, continuous infusion; eEF2, eukaryotic elongation factor 2; eIF2α, eukaryotic translation initiation factor 2α; eIF4E, eukaryotic translation initiation factor 4E; eIF4G, eukaryotic translation initiation factor 4G; ERK, extracellular signal-regulated kinases; GATOR, GTPase activating protein (GAP) activity toward Ras-related GTP binding proteins (Rag); INT, intermittent bolus feeding; IR, insulin receptor; IRS-1, insulin receptor substrate 1; Lamtor, late endosomal/lysosomal adaptor, mitogen-activated protein kinase and mechanistic target of rapamycin activator; mTOR, mechanistic target of rapamycin; mTORC, mechanistic target of rapamycin complex; PDK1, 3-phosphoinositide-dependent protein kinase-1; PHLPP, pleckstrin homology (PH) domain leucine-rich repeat protein phosphatase; PP2A, protein phosphatase 2A; PTEN, phosphatase and tensin homolog, deleted on chromosome 10; Rag A/B C/D, Ras-related GTP-binding protein A/B C/D; Rheb, Ras homolog enriched in brain; S6K1, p70 ribosomal protein S6 kinase 1; SAM, S-adenosylmethionine; Sestrin, stress response protein; SHIP2, Src homology domain 2 (SH2)-containing inositol phosphatase; SLC38A2/SNAT2, sodium-coupled neutral amino acid transporters, member 2; SLC38A9, solute carrier family 38 member 9; SLC7A5/LAT1, L-type amino acid transporter 1; TSC, tuberous sclerosis complex; Ubl4A, ubiquitin-like protein 4A; V-ATPase, vacuolar H+-ATPase; 4EBP1, eukaryotic translation initiation factor 4E–binding protein 1.

sufficient supply, mTORC1 is recruited to the lysosome surface through interactions with Ras-related GTP-binding proteins (Rag) and the Ragulator complex, a complex encoded by the *MAPKSP1*, *ROBLD3*, and *c11orf59* genes (21). In the cytosol, leucine and arginine bind to their respective sensors, stress response proteins (Sestrins) and cytosolic arginine sensor for mechanistic target of rapamycin complex 1 subunit 1 (CASTOR1) dimers, respectively, causing the release of these sensors from GAP activity toward Rags (GATOR) 2 (21). The action of GATOR2, along with the binding of Rheb with mechanistic target of rapamycin (mTOR), is necessary for the full activation of mTORC1 on the lysosome (Figure 1) (21). The regulation of signaling by insulin and amino acids downstream of mTORC1 is similar (22).

In a recent 3-week feeding study in neonatal pigs, we showed that intermittent bolus feeding induces higher rates of mTORC1-dependent protein synthesis in skeletal muscle compared to continuous feeding, resulting in enhanced lean growth (4, 23). However, the relative contributions of the insulin- and amino acid-signaling pathways that mediate the effects of these different feeding modalities on protein synthesis and degradation have not been fully elucidated. Therefore, this study aimed to compare the effects of continuous and intermittent bolus feeding on protein abundances, protein phosphorylation, and protein-protein interactions of signaling components that are responsible for insulin- and amino acid-dependent activation of mTORC1 in the skeletal muscle of neonatal pigs.

Methods

Animals and surgeries

The research protocol was approved by the Animal Care and Use Committee of the Baylor College of Medicine and was conducted in accordance with the National Research Council's *Guide for the Care and Use of Laboratory Animals*. The protocol was previously described in El-Kadi et al (4, 23) and is briefly described herein. At 2 days of age, female crossbred piglets (Yorkshire x Landrace x Hampshire x Duroc) weighing ~2 kg ($n = 18$) underwent surgery to place indwelling catheters into their carotid artery and jugular vein, and a gastrostomy tube for diet delivery as previously described (24). Piglets were housed individually in stainless-steel cages equipped with a swivel and tether system to administer the diet. During the 3-day recovery period, piglets were fed a commercial sow-milk replacer (Soweena Litter Life; Merrick's) and then randomly assigned to 1 of 3 treatment groups.

Diet and sample collection

Piglets were fed the same amount [240 mL/(kg body weight [BW] · d)] of the experimental diet to provide 12.8 g crude protein/(kg BW · d) and 155 kcal/(kg BW · d) (4). Via a gastrostomy tube, diets were administered either through continuous infusion [CTS; $n = 6$; 10 mL/(kg BW · h)] or intermittently [INT; $n = 12$; 40 mL/(kg BW · bolus delivered over 15 minutes every 4 hours)] for 21 days. Previous studies demonstrated that ingestion of a meal rapidly activates the mTORC1-signaling pathways that stimulate translation initiation, peaking at 1 hour and returning to baseline by 4 hours (25). Therefore, at the end of the study, the INT group was euthanized either immediately before a meal (INT-0; $n = 6$) or 60 minutes after a meal (INT-60; $n = 6$) and the CTS group ($n = 6$) was euthanized without interruption of continuous feeding. Gastrocnemius muscles were sampled immediately, frozen in liquid nitrogen, and stored at -80°C until analysis.

Western blotting and immunoprecipitation assay

Muscle samples were homogenized and centrifuged at 10,000 g for 10 minutes at 4°C as previously described (26). An equal amount of protein homogenate from each animal was subjected to SDS-PAGE followed by electrophoretic transfer to polyvinylidene difluoride membrane (Pall Corporation). The blots were then incubated with primary antibodies, washed, and exposed to horseradish peroxidase–tagged secondary antibodies. To normalize the phosphorylated target protein, blots were stripped in stripping buffer (Pierce Biotechnology) and reprobed with antibodies against the nonphosphorylated form of the protein. GAPDH was used to normalize for differences in total protein loading among samples.

Blots were developed with an enhanced chemiluminescence kit (Bio-Rad), and bands were visualized and analyzed by a ChemiDoc-It Imaging System (UVP). Antibodies that were used in the Western blot analyses were primary antibodies against phosphorylated (p-) pan-/Akt (Ser472/473/474, no. ab192623; Abcam), total pan-Akt (no. 9272), total Akt2 (no. 3063), total Akt3 (no. 14293), total and p–extracellular signal-regulated kinases (ERK) 1/2 (Thr202/Tyr204, no. 4695 and no. 4370), total and p-AMP-activated protein kinase (AMPK; Thr172, no. 2532 and no. 2535), total and p-TSC2 (Thr1462, no. 4308 and no. 3617), total and p–p70 ribosomal protein S6 kinase 1 (S6K1; Thr398, no. 9202 and no. 9206), p–eukaryotic translation initiation factor 4E–binding protein 1 (4EBP1; Thr70, no. 9455), total and p–elongation factor 2 (eEF2; Thr56, no. 2332 and no. 2331), total and p–eukaryotic translation initiation factor 2α (eIF2α; Ser52, no. 9722 and no. 9721), Rheb (no. 13879), Mios (GATOR2, no. 13557), RagA/C (no. 4357 and no. 3360), total mTOR (no. 2972), total and p-PTEN (Ser380, no. 9552 and no. 9551), total PP2A (no. 2259), total and p-PDK1 (Ser241, no. 3062 and no. 3061; Cell Signaling Technology); p-PP2A (Tyr307, no. AF3989-SP; R&D Systems); Sestrin1 (no. 55010-1-AP), Sestrin2 (no. 66297-1-lg), PHLPP (no. 22789-1-AP), Ubl4A (no. 14253-1-AP; Proteintech), p–insulin receptor (IR; Tyr1185, no. orb5550; Biorbyt LCC), total IR (no. SC-57342; Santa Cruz Biotechnology), total 4EBP1 (no. A300-501A) and SHIP2 (A300-574A; Bethyl Laboratories), SLC38A9 (no. AA559532C; Antibody Verify), CASTOR1/GATSL3 (no. A13309; Boster Biological Technology), p-mTOR (Ser2481, no. 651701; BioLegend), SLC7A5/LAT1 (no. BMP011; MBL International), SLC38A2/SNAT2 (no. ARP33059_P050; Aviva Systems Biology), and GAPDH (no. AP7873a; Abgent).

Protein-protein complex interactions were determined by immunoprecipitation assay as previously described (26) using Mios (GATOR2; Cell Signaling Technology), Akt1 (no. LS-C-351854; LSBio), Akt2 (no. 3063), Akt3 (no. 14293), IRS-1 (no. 2382), and Raptor (no. 2280; Cell Signaling Technology), followed by Western blots analysis using phospho-Akt-1/2/3 Ser472/473/474 (Abcam), RagA, RagC, Rheb, total mTOR, and phospho-tyrosine antibody (no. 9411; Cell Signaling Technology); CASTOR1/GATSL3 (Boster Biological Technology); and Sestrin1 and Sestrin2 (Proteintech).

Statistical analysis

Our statistical power calculations showed that 6 piglets per treatment group were necessary to detect a 30% difference with a between-animal variation of 20%, a type 1 error of 0.05, and a power of 0.80. Treatments were assigned to experimental units using a Complete Randomized Block design. Data were analyzed using a 1-way ANOVA followed by a Tukey post hoc multiple comparison test (Graph Pad Prism version 5, Graph Pad Software). Probability values of $P < 0.05$ were considered statistically significant.

Results

Hormones and substrates

Circulating insulin and amino acid concentrations during the last day of feeding were reported previously (4, 23) but are reported here for context. Plasma insulin and amino acid concentrations were constant in the CTS group [insulin = 5.6 ± 0.4 μU/mL; branched-chain amino acid (BCAA) = 1333 ± 94 μmol/L]. In the INT group, plasma insulin and amino acid levels rose from baseline (insulin = 4.0 ± 3.0

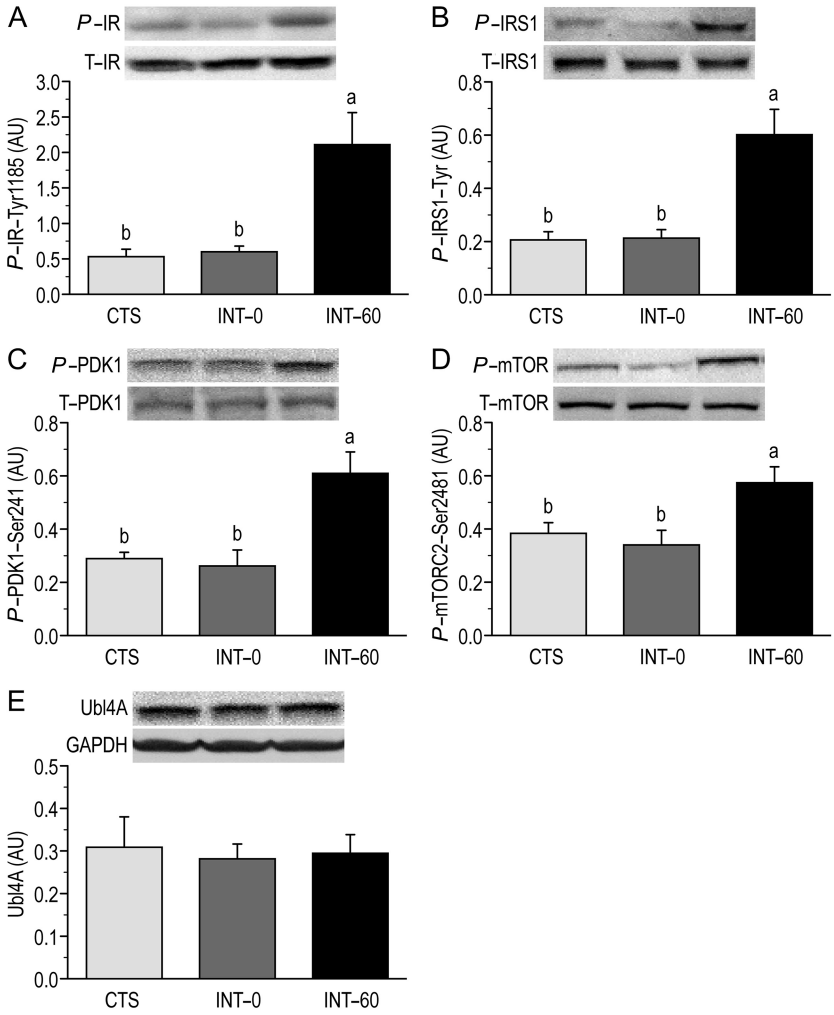

FIGURE 2 Phosphorylation and abundance of positive regulators of the early steps in the insulin signaling pathway in the gastrocnemius muscle of neonatal pigs fed for 21 days continuously (CTS; $n = 6$) or intermittently with measurements made just before (INT-0; $n = 6$) or 60 minutes after (INT-60; $n = 6$) a meal. (A) Phosphorylation of IR at Tyr1185, (B) phosphorylation of IRS-1 at tyrosine residues, (C) phosphorylation of PDK1 at Ser241, (D) phosphorylation of mTORC2 at Ser2481, and (E) Ubl4A abundance. Phosphorylation values were corrected by their protein abundance in the samples. Representative blots are shown. Values are means ± SEMs. Means with uncommon letters are significantly different at a P value <0.05. AU, arbitrary units; CTS, continuously fed; INT-0, intermittently fed at 0 minutes, just before a meal; INT-60, intermittently fed at 60 minutes after a meal; IR, insulin receptor; IRS-1, insulin receptor substrate 1; mTORC, mechanistic target of rapamycin complex; PDK1, 3-phosphoinositide-dependent protein kinase-1; Ubl4A, ubiquitin-like protein 4A.

μU/mL; BCAA = 970 ± 94 μmol/L) to peak at 30 minutes (insulin = 48.4 ± 3.0 μU/mL; BCAA = 1333 ± 94 μmol/L) and 60 minutes (insulin = 34.4 ± 3.0 μU/mL; BCAA = 1763 ± 94 μmol/L), respectively ($P < 0.001$) after the meal, and returned to pre-feeding levels before 4 hours.

Insulin signaling

To examine the effects of feeding modality on the activation of the insulin- and amino acid-signaling pathways in the gastrocnemius muscle, immunoprecipitation and Western blot assays were performed. In the initial steps of the insulin-signaling pathway, the protein abundances of IR, IRS-1, pan-Akt, Akt1, Akt2, and Akt3 did not differ between groups (**Supplemental Table 1**). The phosphorylation of the IR at Tyr1185 and IRS-1 at Tyr residues were more highly activated in the INT-60 group than in INT-0 and CTS groups ($P < 0.05$; **Figure 2**A and B). The phosphorylation of PDK1 at Ser241 and mTORC2 at Ser2481, but not the abundance of Ubl4A, all positive regulators of Akt activation, were greater in the INT-60 group than in INT-0 and CTS groups ($P < 0.05$; Figure 2C–E). However, the phosphorylation of negative regulators of Akt activation—that is, PTEN at Ser380 and PP2A at Tyr307—as well as the abundance of SHIP2 and PHLPP, were unaffected by treatment (**Figure 3**A–D). The phosphorylation of the downstream effectors, pan-Akt at Ser472/473/474, Akt1 at Ser472, Akt2 at Ser473, and TSC2 at Thr1462, but not Akt3 at 474, were greater in the INT-60 group than in INT-0 and CTS groups ($P < 0.05$; **Figure 4**A–D). However, the phosphorylation of ERK1/2 at Thr202/Tyr204 and AMPK at Thr172 were similar among groups (Figure 4E and F).

Amino acid sensing mechanisms

The protein abundances and protein-protein interactions in the amino acid-sensing network control the activation of the amino acid-signaling pathway (21). The associations of both Sestrin1

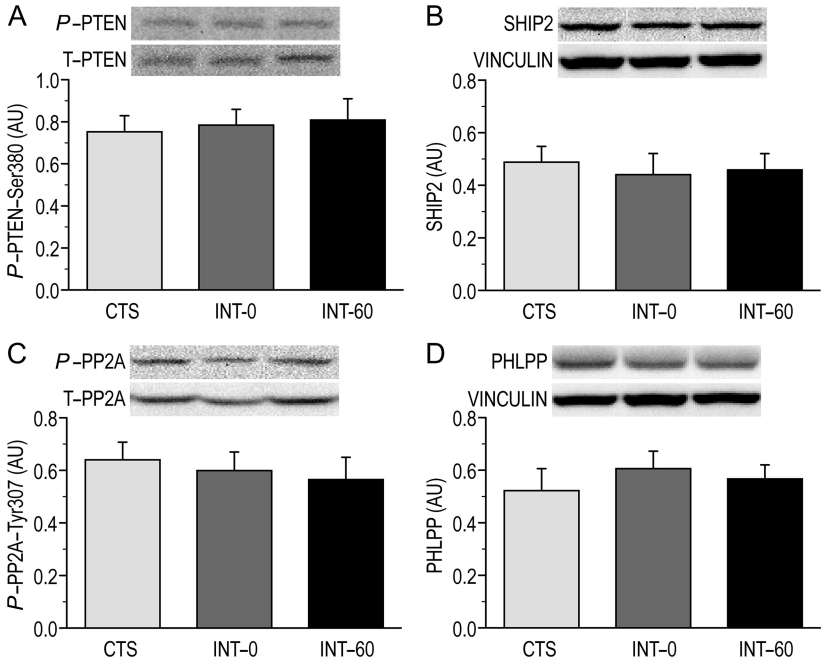

FIGURE 3 Phosphorylation and abundance of negative regulators of Akt activation in the gastrocnemius muscles of neonatal pigs fed for 21 days continuously (CTS; $n = 6$) or intermittently with measurements made just before (INT-0; $n = 6$) or 60 minutes after (INT-60; $n = 6$) a meal. (A) Phosphorylation of PTEN at Ser380, (B) abundance of SHIP2, (C) phosphorylation of PP2A at Tyr307, and (D) abundance of PHLPP. Phosphorylation values were corrected by their protein abundance in the samples. SHIP2 and PHLPP abundance was normalized with vinculin. Representative blots are shown. Values are means ± SEM. AU, arbitrary units; CTS, continuously fed; INT-0, intermittently fed at 0 minutes, just before a meal; INT-60, intermittently fed at 60 minutes after a meal; PHLPP, PH domain leucine-rich repeat protein phosphatase; PP2A, protein phosphatase 2A; PTEN, phosphatase and tensin homolog, deleted on chromosome 10; SHIP2, SH2 domain-containing inositol phosphatase.

and Sestrin2 with GATOR2 were significantly lower in the INT-60 group than in INT-0 and CTS groups ($P < 0.05$; **Figure 5**A and B). However, there were no treatment effects on the interaction of CASTOR1 with GATOR2 (Figure 5C). The associations of RagA, RagC, and Rheb with mTOR were greater in the INT-60 group than in the INT-0 and CTS groups ($P < 0.05$; Figure 5D–F). The protein abundances of Sestrin1, Sestrin2, CASTOR1, GATOR2, RagA, RagC, and Rheb did not differ between feeding treatments (Supplemental Table 1). The protein abundances of the amino acid transporters, SLC7A5/LAT1 and SLC38A2/SNAT2, and the amino acid-sensing components that reside on the lysosome—that is, late endosomal/lysosomal adaptor, mitogen-activated protein kinase and mTOR activator (Lamtor) 1; Lamtor2; SLC38A9; and vacuolar H+-ATPase (V-ATPase)—were not affected by feeding modality (Supplemental Table 1).

Translation initiation signaling

Downstream of mTORC1, the phosphorylation of S6K1 at Thr398 and 4EBP1 at Thr70 were significantly higher in INT-60 than in INT-0 and CTS groups ($P < 0.05$; **Figure 6**A and B). However, the phosphorylation of eIF2α at Ser52 and eEF2 at Thr56 were similar among the groups (Figure 6C and D).

Discussion

Previously, we reported that intermittent bolus feeding induces higher rates of protein synthesis in skeletal muscle than continuous feeding in a neonatal piglet model (4, 23), resulting in an increase in lean growth (4). These results are supported by the findings that intermittent bolus feeding elicits cyclical hormone and substrate patterns in neonatal pigs (24) and preterm human infants (27), and suggest that the insulin and amino acid surges induce greater anabolic activation of the signaling networks that regulate protein synthesis (4, 23). However, the molecular mechanisms by which intermittent feeding promotes greater lean growth than continuous feeding have not been fully delineated.

In the current study, we report that the activation of both insulin- and amino acid-signaling components toward translation initiation was greater in the gastrocnemius muscle of neonatal pigs receiving long-term intermittent bolus feeding compared to those receiving continuous feeding. Unlike in our pancreatic-substrate clamp studies, where we can independently differentiate between the effects of insulin and amino acids (28, 29), in this study it is not possible to distinguish between the relative contributions of insulin and amino acids in promoting muscle protein synthesis. However, since both anabolic agents utilize different signaling components upstream of mTORC1, we could separately characterize the activation of individual signaling components in these pathways in response to different feeding modalities.

In neonates, insulin plays a crucial role in the regulation of the feeding-induced stimulation of muscle protein synthesis (5). It is well documented (9) that insulin stimulates muscle protein synthesis via activation of mTORC1 (Figure 1). Consistent with our previous data in the longissimus dorsi muscle (4, 24), in the current study, we found that the activation of the early steps of the insulin-signaling pathway leading to pan-Akt activation—namely the phosphorylation of the IR and IRS-1 in the gastrocnemius muscle—was greater in intermittent bolus–fed pigs after a meal compared to those continuously fed. In the current study, we sought to identify additional signaling

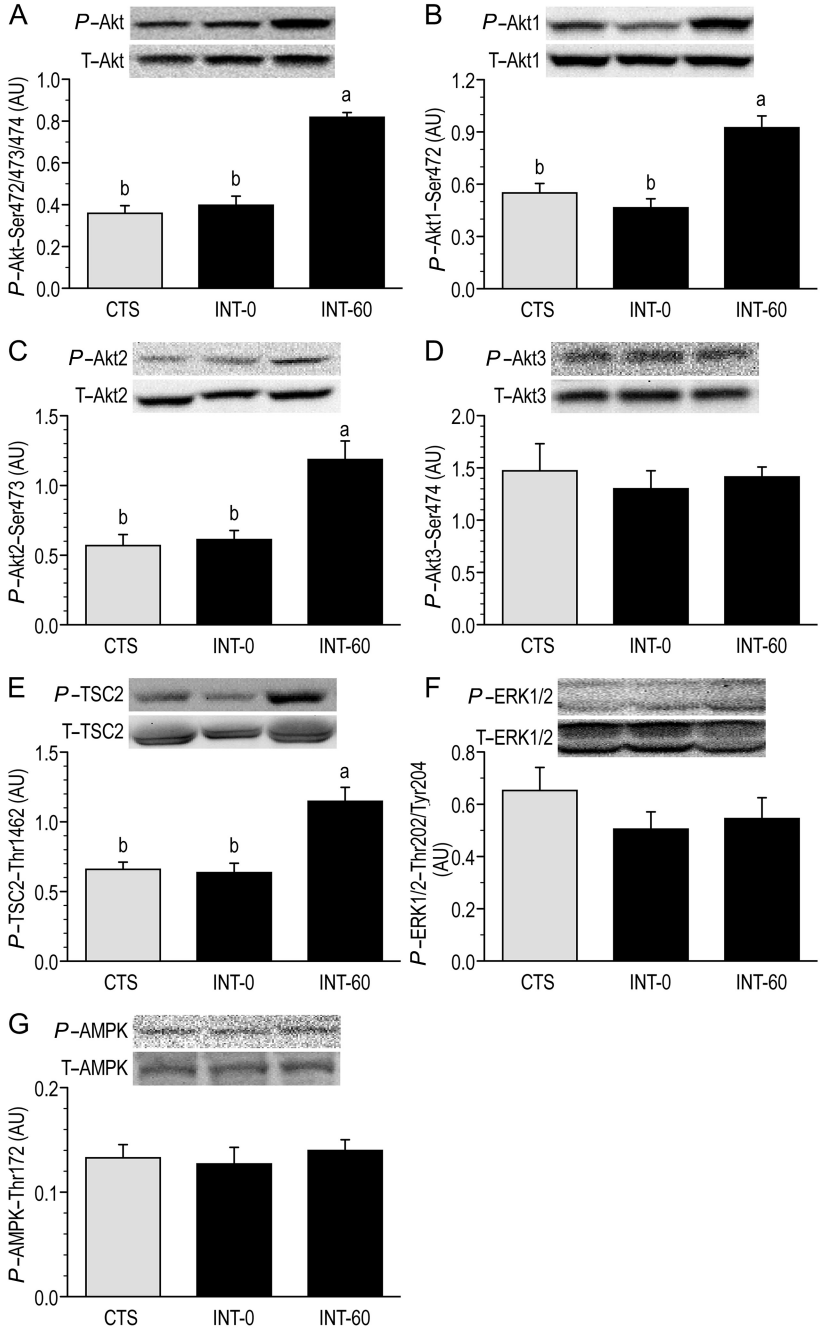

FIGURE 4 Phosphorylation of Akt and downstream effectors in the gastrocnemius muscle of neonatal pigs fed for 21 days continuously (CTS; $n = 6$) or intermittently with measurements made just before (INT-0; $n = 6$) or 60 minutes after (INT-60; $n = 6$) a meal. Phosphorylation of (A) pan-Akt at Ser472/473/474, (B) Akt1 at Ser472, (C) Akt2 at Ser473, (D) Akt3 at Ser474, (D) TSC2 at Thr1462, (F) ERK1/2 at Thr202/Tyr204, and (G) AMPK at Thr172. Phosphorylation values were corrected by their protein abundance in the samples. Representative blots are shown. Values are means ± SEM. Means with uncommon letters are significantly different at a P value <0.05. AMPK, AMP-activated protein kinase; AU, arbitrary units; ERK, extracellular signal-regulated kinases; TSC, tuberous sclerosis complex.

components that also may be involved. We found that the phosphorylation of kinases such as PDK1 and mTORC2, which positively regulate Akt phosphorylation, also were higher in intermittent bolus-fed pigs compared with those continuously fed. As the phosphorylation of PDK1 (30) and mTORC2 (31) serve as markers of their activation, our results support the notion that insulin- or growth factor-induced activation of PDK1 and mTORC2 is required for full activation of Akt (11, 12). Although Ubl4A seems to be required for Akt membrane translocation, which is a critical step in Akt activation (13), in this study, the Ubl4A abundance was not affected by the feeding modality. The activation of phosphatases (PTEN, SHIP2, PP2A, and PHLPP) plays an essential role in dampening the activation of Akt (14–17). Our results showed that the activation of these phosphatases was unaffected by the feeding modality, suggesting that the activation of these phosphatases is not a limiting factor for Akt activation in response to the pulsatile rise in insulin after a meal.

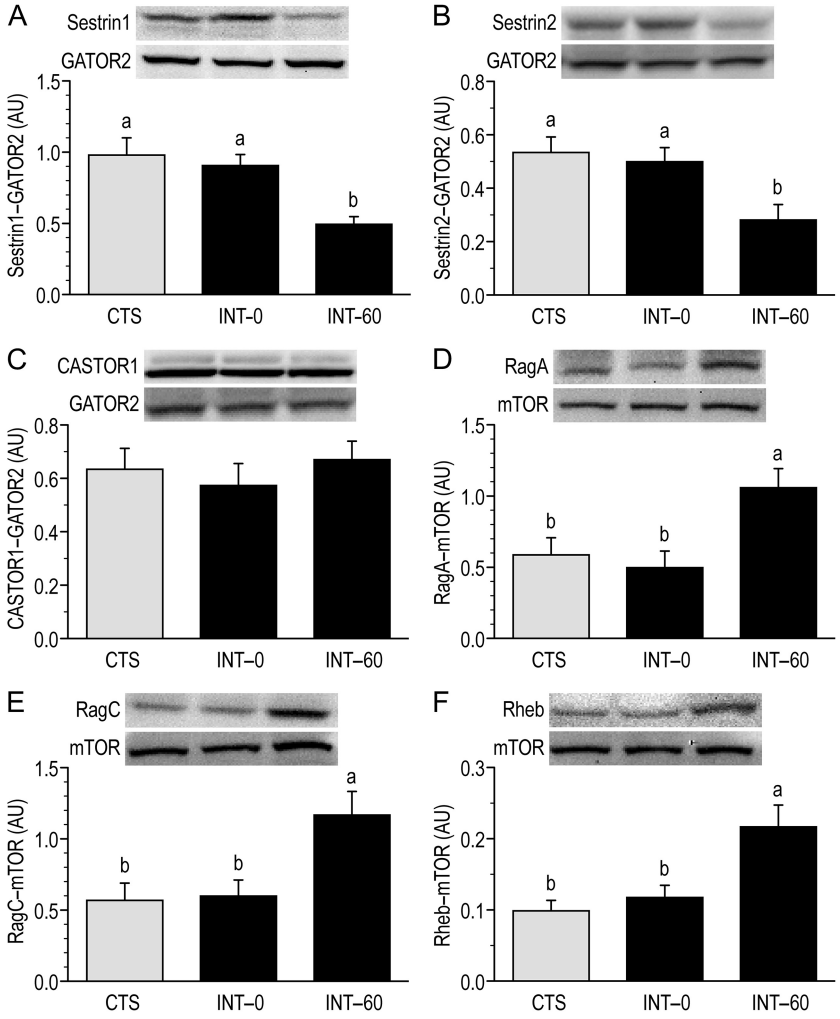

FIGURE 5 Abundance of amino acid-sensing components in the gastrocnemius muscles of neonatal pigs fed for 21 days continuously (CTS; $n = 6$) or intermittently with measurements made just before (INT-0; $n = 6$) or 60 minutes after (INT-60; $n = 6$) a meal. Abundance of complex (A) Sestrin1-GATOR2, (B) Sestrin2-GATOR2, (C) CASTOR1-GATOR2, (D) RagA-mTOR, (E) RagC-mTOR, and (F) Rheb-mTOR. Values were corrected by GATOR2 or mTOR protein abundances in the immunoprecipitation samples. Representative blots are shown. Values are means ± SEM; $n = 6$. Means with uncommon letters are significantly different at a P value <0.05. AU, arbitrary units; CASTOR1, cytosolic arginine sensor for mechanistic target of rapamycin complex 1 subunit 1; CTS, continuously fed; GATOR, GAP activity toward Rags; INT-0, intermittently fed at 0 minutes, just before a meal; INT-60, intermittently fed at 60 minutes after a meal; mTOR, mechanistic target of rapamycin; Rag, Ras-related GTP-binding protein; Rheb, Ras homolog enriched in brain; Sestrin, stress response protein.

Consistent with our previous short-term study (24), in the current long-term study, phosphorylation of both pan-Akt and TSC2 were higher in the gastrocnemius muscle after an intermittent bolus meal compared with continuous feeding. This enhanced pan-Akt phosphorylation was due to an increase in the phosphorylation of Akt1 and Akt2, but not Akt3. Our current results further support the notion that Akt1 and Akt2 are involved in cellular growth and glucose homeostasis (10). Among the Akt isoforms, Akt3 is less well characterized (10), although genetic studies suggest an important role of Akt3 in brain development (32). Our study revealed a robust protein expression of Akt3 in the skeletal muscle of neonatal pigs, although neither the abundance nor the phosphorylation of Akt3 was affected by the feeding modality.

Similar to our previous findings that indicated no effect of insulin or amino acids on the phosphorylation of AMPK (33), in the current study, there was no effect of feeding modality on the phosphorylation of AMPK in muscle. Although in vitro ERK 1/2 acts as a positive regulator of mTORC1 (34), the current data indicate that ERK1/2 phosphorylation was not affected by the feeding modality or status.

Unlike the insulin-signaling pathway, the amino acid-signaling pathway is complex and not completely elucidated (18, 35). We have previously shown that the association of the leucine sensor, Sestrin2 with GATOR2, is reduced in the skeletal muscle of the neonate with the rise in amino acids after a meal (26, 36). In this study, we demonstrated that intermittent bolus feeding reduced the associations of both Sestrin1 and Sestrin2 with GATOR2 in muscle, resulting in increases in the phosphorylation of S6K1 and 4EBP1, indicating mTORC1 activation (37, 38). Feeding is associated with increases in other amino acids in addition to leucine, including arginine (39). Cell culture studies indicate that arginine can act as a potent regulator of mTORC1 through different amino acid-sensing pathways (CASTOR1-GATOR2 complex), resulting in the stimulation of protein synthesis (18). In contrast to Sestrins, the abundance of the CASTOR1-GATOR2 complex was not

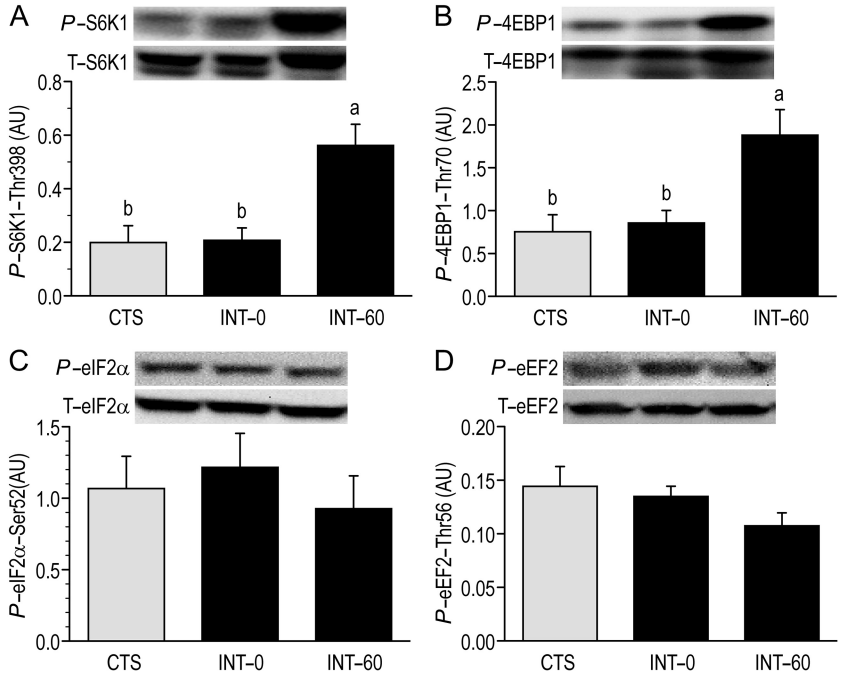

FIGURE 6 Phosphorylation of translation initiation factors in the gastrocnemius muscle of neonatal pigs fed for 21 days continuously (CTS; $n = 6$) or intermittently with measurements made just before (INT-0; $n = 6$) or 60 minutes after (INT-60; $n = 6$) after a meal. Phosphorylation of (A) S6K1 at Thr398, (B) 4EBP1 at Thr70 (B), (C) eIF2α at Ser52, and (D) eEF2 at Thr56. Phosphorylation values were corrected by their protein abundance in the samples. Representative blots are shown. Values are means ± SEM; $n = 6$. Means with uncommon letters are significantly different at a P value <0.05. AU, arbitrary units; CTS, continuously fed; eEF2, eukaryotic elongation factor 2; eIF2α, eukaryotic translation initiation factor 2α; INT-0, intermittently fed at 0 minutes, just before a meal; INT-60, intermittently fed at 60 minutes after a meal; S6K1, p70 ribosomal protein S6 kinase 1; 4EBP1, eukaryotic translation initiation factor 4E–binding protein 1.

affected by the feeding modality, indicating that arginine-sensing components may not be involved in the feeding-induced activation of mTORC1 in neonatal muscle. Yao et al. (40) reported that arginine supplementation enhanced mTORC1 activation and protein synthesis in the skeletal muscle of young swine, but amino acid-sensing components were not measured.

The interaction of leucine or arginine with their sensors promotes the binding of the Rag proteins with mTOR (21). In another branch of the pathway, insulin induces the binding of Rheb and mTOR (41, 42). Both events are essential for the full activation of mTORC1 (42). Consistent with our previous feeding studies (26, 36), the current results indicate that the abundances of RagA-mTOR, RagC-mTOR, and Rheb-mTOR complexes were greater in the gastrocnemius muscles of intermittent bolus-fed compared to continuously fed pigs. Furthermore, this finding is consistent with the report that the feeding-induced increase in myofibrillar protein synthesis is associated with Rheb-mTOR complex colocalization at the periphery of the myofibers (43).

Over- or underexpression of insulin- and amino acid-sensing molecules regulates the activation of mTORC1 (44). Commonly occurring in cancer cells, overexpression of Rheb (a positive regulator) or underexpression of nitrogen permease regulator 2-like protein (NPRL2)/GATOR1 (a negative regulator) enhances the activation of mTORC1 (44). In this study, we did not observe any effect of long-term feeding modalities on the protein expression of the insulin-signaling components (IRS-1, Akt, Akt1, Akt2, and Akt3) or the amino acid–signaling components (SLC7A5/LAT1, SLC38A2/SNAT2, Sestrin1, Sestrin2, CASTOR1, GATOR2, Lamtor1, Lamtor2, SLC38A9, and V-ATPase). This is consistent with a recent study showing that a 10-week dietary intervention with a high-protein diet in elderly men did not affect the mRNA expression of RagA, RagC, Mios/GATOR2, Sestrin2, and CASTOR1 (45).

The potential role of amino acids as anabolic agents that positively regulate muscle protein synthesis and protein deposition in whole animals is complex. In addition to leucine and arginine, glycine and glutamine also have been shown to stimulate muscle protein synthesis and growth in piglets (46, 47) via mechanisms that appear to be dependent on mTORC1 (46, 48). Likewise, methionine, via its metabolite S-adenosylmethionine (SAM), can act as an anabolic agent that promotes mTORC1-dependent protein synthesis (49) and is crucial for neonatal growth (50). Furthermore, glycine stimulates protein synthesis in muscle cell culture through the activation of Akt/mTOR and the inhibition of AMPK (48). Mechanistically, methionine induces the binding of SAM to its sensor, S-adenosylmethionine sensor upstream of mTORC1 (SAMTOR), which disturbs the SAMTOR-GATOR1 complex, a potent inhibitor of mTORC1 (49). In contrast to leucine, arginine, and methionine, glutamine also has been shown in cell cultures to activate mTORC1 via Rag GTPase-independent mechanisms (51). Although plasma glycine, glutamine, and methionine concentrations were not measured in this study, they may play important roles in the enhanced muscle deposition in pigs fed intermittently.

Taken together, these data suggest that the higher protein synthesis rate in the skeletal muscle of piglets receiving intermittent bolus feeding compared with those receiving continuous feeding is due to the pulsatile pattern of circulating insulin and amino acids that enhances the activation of insulin- and amino acid-signaling components that regulate mTORC1 signaling towards translation initiation and protein synthesis (4). Specifically, the positive regulators of the insulin-signaling

pathway, including IR, IRS-1, PDK1, mTORC2, pan-Akt, Akt1, and Akt2, appear to play an important role in the activation of translation initiation in response to the insulin surge after an intermittent bolus meal. However, only TSC2, but not other negative regulators of insulin signaling, including PTEN, PP2A, SHIP2, or PHLPP, were impacted by feeding modality. In the amino acid-signaling pathway, we further identified the amino acid sensors Sestrin1 and 2, but not CASTOR1, as being responsive to the amino acid pulse after a meal, as were Rag A/C and Rheb. Other potential regulators—that is, ERK1/2, AMPK, Ubl4A, SLC7A5/LAT1, SLC38A2/SNAT2, Lamtor1, Lamtor2, SLC38A9, V-ATPase, eIF2α, and eEF2—were not implicated. Thus, these findings confirm that intermittent bolus feeding stimulates a greater anabolic response in skeletal muscle than continuous feeding, and they provide further evidence that intermittent bolus feeding is more advantageous than continuous feeding in promoting lean body mass and growth of neonates.

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