Anabolic Signaling and Protein Deposition Are Enhanced by Intermittent as Compared with Continuous Feeding in Skeletal Muscle of Neonates

Running title: Intermittent feeding increases muscle protein deposition

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Orogastric tube feeding is indicated for neonates with impaired ability to ingest, and can be administered by intermittent bolus or continuous schedule. Our aim was to determine whether feeding modalities affect muscle protein deposition and to identify mechanisms involved. Neonatal pigs were overnight fasted (FAS) or fed the same amount of food continuously (CON) or intermittently (INT; 7 x 4 h meals) for 29 h. For 8 h, between hour 20 and 28, pigs were infused with [\(^{2}\text{H}_5\)]phenylalanine and [\(^{2}\text{H}_2\)]tyrosine and amino acid (AA) net balances were measured across the hindquarters. Insulin, branched-chain AA, phenylalanine and tyrosine arterial concentrations, and whole-body phenylalanine and tyrosine fluxes were greater for INT after the meal than for CON or FAS. The activation of signaling proteins leading to initiation of mRNA translation including eukaryotic initiation factor (eIF) 4E•eIF4G complex formation in muscle was enhanced by INT compared to CON feeding or FAS. Signaling proteins of protein degradation were not affected by feeding modalities except for microtubule-associated protein light chain 3-II (LC3-II) which was highest in the FAS. Across the hindquarters, AA net removal increased for INT but not for CON or FAS, with protein deposition greater for INT. This was because protein synthesis increased following feeding for INT but remained unchanged for CON and FAS, while there was no change in protein degradation across any dietary treatment. These results suggest that muscle protein accretion in neonates is enhanced with intermittent bolus to a greater extent than continuous feeding, mainly by increased protein synthesis.

Keywords: protein degradation, amino acid, protein turnover, stable isotope, translation initiation
INTRODUCTION

Orogastric tube feeding is indicated in neonates with impaired ability for normal ingestion and can be administered by an intermittent bolus or continuous schedule. Evidence suggests that feeding frequency can affect feeding outcome, but whether intermittent or continuous feeding is more beneficial remains inconclusive (48). Intermittent feeding is considered more physiologic based on a more natural feed-fast cycle, and promotes gastrointestinal hormone secretions (3, 41). Intermittent feeding also stimulates intestinal growth and development in newborn piglets by increasing mucosal and intestinal protein mass compared with continuous feeding (54). Most comparisons between intermittent and continuous feeding have focused on clinical outcomes, including days to full feed, time to regain birth weight, time to discharge, feeding intolerance and weight gain (22, 53), but with little attention given to macronutrient retention (55). Although intermittent feeding may increase diet-induced thermogenesis as compared with continuous feeding (30), it remains unclear whether this also involves greater amino acid oxidation.

High rates of protein turnover and deposition are characteristics of growth during the neonatal period (17, 19, 29, 38). Studies in pigs and rats have shown that neonates are efficient at depositing ingested amino acids into proteins, although this efficiency decreases with age (7, 8, 10, 15, 16). Such responses to feeding are common to all growing animals, including humans (21). Accordingly, we have used the piglet as a model for the human neonate to study early postnatal muscle growth and metabolism.

It is well-recognized that insulin plays an important role in protein accretion in skeletal muscle of the neonate (11). Although the insulin-specific effect on muscle continues to play a
role in protein deposition during growth, the sensitivity greatly diminishes with age (68). In contrast, amino acids exert a positive effect on muscle protein synthesis throughout life (11, 21, 64). The signaling pathways for both insulin and amino acids are upregulated in muscle during early life. While the postprandial rise in insulin and the subsequent activation of the insulin signaling cascade is well-defined, amino acid signaling upstream of the translation initiation pathway is less well understood.

Insulin, through protein kinase B (PKB), and amino acids independently activate mammalian target of rapamycin (mTOR) which induces an increase in phosphorylation of 4E-binding protein 1 (4EBP-1) and ribosomal protein S6K1 (S6K1) (39, 56). The phosphorylation of 4EBP-1 results in dissociation from eukaryotic initiation factor 4E (eIF4E) and subsequent formation of the eIF4E·eIF4G complex, a key component in the regulation of mRNA binding to the 43S pre-initiation complex (39, 56). We have previously shown that the fractional rate of protein synthesis concomitantly increases with the rise in insulin and amino acids following a complete meal in the neonate (67). Consequently, protein synthesis has both a short latency and a transient response, returning to fasting levels between 2 and 4 h after a meal.

The ubiquitin-proteasome and autophagy-lysosome systems are thought to be the two most important proteolytic pathways in skeletal muscle (52, 63). High rates of proteolysis are necessary to provide amino acids for ongoing tissue modeling and rapid growth (29, 38, 47) as well as to provide amino acids to other organs when dietary supplies are insufficient (63). Acute parenteral amino acid infusion reduces whole-body proteolysis in neonates, but this suppression is lost after prolonged (> 24 h) treatment (32, 45). Although pharmacological levels of insulin decrease whole-body proteolysis in neonates (1, 47), concentrations within the physiological range appear to exert little effect on protein catabolism (20, 31).
Recently, we showed that protein synthesis, measured 90 min following a meal, was enhanced by intermittent bolus compared to continuous feeding (28). However, whether feeding frequency affects protein degradation and protein deposition were not determined. This study examined how feeding frequency affects amino acid and protein metabolism in both muscle and the whole body of neonates over a 4 h complete feeding cycle, 24 h after initiation of intermittent bolus or continuous feeding. The objectives of this study were to: 1) compare the effect of continuous vs. intermittent bolus feeding on amino acid net balance across the hindquarters of the neonatal pig, 2) determine whether any difference in protein deposition between continuous and intermittent bolus feeding is due to altered protein synthesis and/or degradation, and 3) identify the signaling mechanisms by which feeding frequency modulates protein deposition. We hypothesized that intermittent feeding increases muscle protein synthesis while decreasing protein degradation as compared with continuous feeding in neonates.

**MATERIALS AND METHODS**

**Animals and Surgeries.** Pigs (n=6/group) 2-3 d of age and weighing approximately 2 kg were surgically fitted under general anesthesia using sterile techniques with indwelling catheters in the carotid artery, jugular vein, and inferior vena cava and a blood flow probe was placed around the caudal aorta. Pigs were allowed to recover for 3 d post-surgery and studied at 9 d of age weighing approximately 2.5 kg. The Animal Care and Use Committee of Baylor College of Medicine approved all experimental procedures. This study was conducted in accordance with the National Research Council’s *Guide for the Care and Use of Laboratory Animals*.

**Diet.** Throughout the pre-experimental and recovery periods, pigs were allowed free access to sow milk replacer (Soweena litter life, Merrik’s, Middleton, WI). Following recovery, pigs were
divided into three groups; two were fed the same amount of sow milk replacer (240 ml•kg\(^{-1}\)•d\(^{-1}\); 10 g CP•kg\(^{-1}\)•d\(^{-1}\) and 175 kcal•kg\(^{-1}\)•d\(^{-1}\)) either continuously (CON; 10 ml•kg\(^{-1}\)•h\(^{-1}\)) or intermittently (INT; 7 equal meals given over 15 min at 4 h intervals; 40 ml•kg\(^{-1}\)•bolus\(^{-1}\)) for 29 h (Table 1). The milk replacer was supplied via an orogastric feeding tube. The third group was fasted overnight (FAS).

**Tracer infusion and blood sampling.** To assess amino acid kinetics, a primed continuous infusion of \(^{[2\text{H}_5]}\)phenylalanine and \(^{[2\text{H}_2]}\)tyrosine (Cambridge Isotope Laboratories, Andover, MA) were given (each primed at 10 ml•kg\(^{-1}\) followed by infusion at 10 µmol•kg\(^{-1}\)•h\(^{-1}\)) into the jugular vein for 8 h between 20 h and 28 h of the 29 h feeding period (CON and INT) or the last 8 h of the fasting period (FAS). During the final 4 h of the tracer infusion, blood samples were taken every 15 min from 0 to 90 min and every 30 min thereafter (Fig. 1). Blood flow was measured using ultrasonic flow probes (Transonic Systems Inc., Ithaca, NY). The concentration and enrichment of amino acids were determined gravimetrically using \([\text{U-}^{13}\text{C}, ^{15}\text{N}]\)amino acids added to plasma samples. Plasma samples were stored at -20º C until analysis. N-heptafluorobutyryl derivatives of amino acids were prepared by esterification with \(n\)-propanol followed by derivatization with heptafluorobutyric anhydride (43). Amino acid enrichment and concentration were measured by gas chromatography/ mass spectrometry (GC 6890 with 5973 mass selective detector, Agilent Technologies, Wilmington, DE) under negative chemical ionization.

**Hindquarters mass balance.** All calculations assume that there is no change in the size of the muscle free phenylalanine pool during treatments. As phenylalanine is neither catabolized nor synthesized in muscle, then phenylalanine net removal by the hindquarters occurs when protein synthesis exceeds protein degradation, with the converse true if there is net release. Thus:
Arterial Phe inflow = \([A] \cdot PF\)

Venous Phe outflow = \([V] \cdot PF\)

Phe net balance = \(((A) - (V)) \cdot PF\)

where \([A]\) is arterial and \([V]\) is venous amino acid concentration (\(\mu\text{mol}\cdot\text{ml}^{-1}\)), and \(PF\) is plasma flow (\(\text{ml} \cdot \text{h}^{-1}\)). Similarly, phenylalanine tracer kinetics were calculated using the following equations:

\[
\text{Arterial } [^2\text{H}_5]\text{Phe inflow} = \left[A[^2\text{H}_5]\text{phe}\right] \cdot PF
\]

\[
\text{Venous } [^2\text{H}_5]\text{Phe outflow} = \left[V[^2\text{H}_5]\text{phe}\right] \cdot PF
\]

\[
[^2\text{H}_5]\text{Phe net balance} = \left[A[^2\text{H}_5]\text{phe}\right] - \left[V[^2\text{H}_5]\text{phe}\right] \cdot PF
\]

\[
\text{Fractional utilization} = \left[A[^2\text{H}_5]\text{phe}\right] - \left[V[^2\text{H}_5]\text{phe}\right] / \left[A[^2\text{H}_5]\text{phe}\right]
\]

\[
\text{Amount of Phe used} = \left[A[^2\text{H}_5]\text{phe}\right] - \left[V[^2\text{H}_5]\text{phe}\right] / \left[A[^2\text{H}_5]\text{phe}\right] \cdot [A_{Phe}] \cdot PF
\]

where \(\left[A[^2\text{H}_5]\text{phe}\right]\) and \(\left[V[^2\text{H}_5]\text{phe}\right]\) are the arterial and venous \([^2\text{H}_5]\text{phenylalanine tracer}\) concentrations, and \([A_{Phe}]\) is the phenylalanine tracee arterial concentration.

**Systemic phenylalanine flux.** Whole-body flux of phenylalanine and tyrosine (\(\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{ml}^{-1}\)) were calculated according to the Steele equation (57) as modified by Proietto (49) for non-steady state conditions (5, 6):
\[ \text{Whole - body flux} = \left( IR - (pV \times C(t) \times \frac{dE}{dt}) \right) / E(t) \]

where \( IR \) is the infusion rate of the \(^2\text{H}_5\)phenylalanine or \(^2\text{H}_2\)tyrosine tracers (\(\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}\)), \( p \) is the pool fraction for instant mixing \((p = 0.25)\), \( V \) is the volume of distribution \((V = 0.5 \text{ l}\cdot\text{kg}^{-1})\), \( C(t) \) is the mean arterial phenylalanine or tyrosine concentration \((\mu\text{mol}\cdot\text{ml}^{-1})\), \( \frac{dE}{dt} \) is the variation in phenylalanine or tyrosine enrichment (atom percent excess), and \( E(t) \) is the mean arterial phenylalanine or tyrosine enrichment between two time points. When steady state conditions were achieved, whole-body flux was calculated using the following equation:

\[ \text{Whole - body flux} = \left( E_A / E_I - 1 \right) \times IR \]

where \( E_A \) and \( E_I \) are the arterial and infusate phenylalanine or tyrosine enrichments, and \( IR \) is the \(^2\text{H}_5\)phenylalanine or \(^2\text{H}_2\)tyrosine tracer infusion rates (\(\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}\)).

**Hindquarters protein synthesis and degradation.** Synthesis and degradation (\(\mu\text{mol}\cdot\text{h}^{-1}\)) were calculated using the following equations, with venous phenylalanine enrichments assumed similar to the true precursor pool (27):

\[
\text{Protein synthesis} = PF \times \left( ([A_{\text{Phe}]} \times E_A / E_V) - [V_{\text{Phe}}] \right)
\]

\[
\text{Protein degradation} = PF \times \left( ([A_{\text{Phe}]} \times E_A / E_V) - 1 \right)
\]

and,

\[
\text{Protein deposition} = \text{Synthesis} - \text{Degradation}
\]

where \([A_{\text{Phe}}]\), \(E_A\), are arterial and \([V_{\text{Phe}}]\) \(E_V\) are venous phenylalanine concentrations (\(\mu\text{mol}\cdot\text{ml}^{-1}\)) and enrichments, and \(PF\) is plasma flow (\(\text{ml}\cdot\text{h}^{-1}\)).
**Whole-body phenylalanine hydroxylation to tyrosine.** Phenylalanine oxidation was estimated from the rate of phenylalanine hydroxylation to tyrosine, according to the following equation (9):

\[
\text{Phenylalanine hydroxylation} = Q_{Tyrosine} \times E_{d4\ Tyrosine} / E_{d5\ Phenylalanine}
\]

where \(Q_{Tyrosine}\) is the whole-body flux of tyrosine (\(\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}\)), \(E_{d4\ Tyrosine}\) and \(E_{d5\ Phenylalanine}\) are the enrichments of [ring-d4]tyrosine and [ring-d5]phenylalanine in plasma.

**Glucose and insulin.** Plasma samples were analyzed for glucose (Yellow Springs Instrument, model 2300, Yellow Springs, OH). Plasma radioimmunoreactive insulin concentrations were measured using porcine insulin radioimmunoassay kits (Millipore, St. Charles, MO).

**Protein immunoblot analysis.** Pigs were euthanized with pentobarbital 1 h following the last meal for the INT group, and at the same time for CON (i.e., 29 h after the initiation of feeding for both groups) and FAS groups. Muscle samples were removed and frozen at -70°C until analysis. Proteins from longissimus dorsi and gastrocnemius muscles homogenates were separated on polyacrylamide gels (PAGE). For each assay, all samples were run at the same time on triple-wide gels (CBS Scientific C, Del Mar, CA) to eliminate inter-assay variation. Proteins were transferred electrophoretically to polyvinylidene difluoride transfer membranes (Pall, Pensicola, FL), incubated with appropriate primary antibodies, washed, and exposed to an appropriate secondary antibody, as previously described (61). Immunoblotting was performed using the following primary antibodies: PKB (Ser 473; Cell Signaling Technology, Beverly, MA), S6K1 (Thr 389, Millipore, Bedford, MA), 4EBP-1 (Thr 46, Invitrogen, Carlsbad, CA), AMPK\(\alpha\) (Thr 172, Cell Signaling Technology), eIF2\(\alpha\) (Ser 51, Cell Signaling Technology), eEF2 (Thr 56, Cell Signaling Technology), microtubule-associated protein 1 light chain 3 (LC3; Cell Signaling Technology), muscle RING finger-1 (MURF-1; R & D Systems, Inc.,
Minneapolis, MN), F-box protein atrogin-1/MAFbx (Atrogin-1; ECM Biosciences, Versailles, KY), forkhead transcription factor-1 (Fox01; Ser 256, Cell Signaling Technology), and forkhead transcription factor-4 (Fox04; Ser 262, Cell Signaling Technology) and β-actin (Cell Signaling Technology). Blots were developed using an enhanced chemiluminescence kit (GE Health Sciences, Buckinghamshire, UK), visualized, and analyzed using a ChemiDoc-It Imaging System (UVP, Upland, CA).

Quantification of eIF4E•eIF4G complex. The complex were immunoprecipitated using an anti-eIF4E monoclonal antibody (gift of Dr. Leonard Jefferson, Penn State University College of Medicine, Hershey, PA) from aliquots of fresh tissue homogenates (24). Immunoprecipitates were recovered with goat anti-mouse IgG magnetic beads (Polysciences, Warrington, PA), washed, re-suspended in sample buffer as described elsewhere (33), and immediately subjected to protein immunoblot analysis using rabbit anti-eIF4G (Novus Biologicals, Littleton, CO). Amounts of eIF4G were corrected by the eIF4E (Cell Signaling Technology) recovered from the immunoprecipitate.

Statistical analysis. Treatments were assigned to experimental units using Complete Randomized Design. To investigate temporal effects, all samples were considered in a repeated measures analysis. Various auto-correlation structures were investigated based on BIC. Compound Symmetry gave the best fit and this structure was then used for all repeated measure analyses. When a significant effect was determined by ANOVA, means were compared using Fisher’s protected least significant differences post hoc test. Data are presented as least square means ± SEM and differences considered significant at $P \leq 0.05$. 

RESULTS
Glucose and insulin. There was a modest increase \((P < 0.05)\) in plasma glucose concentration (Fig. 2) following the meal for INT compared with CON or FAS pigs, but plasma glucose concentrations returned to baseline by 27.5 h (210 min after the bolus meal). Plasma insulin concentrations was highest at 24.5 h (30 min following the meal) for the INT fed pigs \((P < 0.001)\), while plasma insulin levels for the CON and FAS groups remained unchanged throughout the sampling period.

Amino acid concentration. Level and frequency of feeding impacted plasma amino acid concentrations (Fig. 3). Plasma branched-chain amino acids (i.e. isoleucine, leucine, and valine), phenylalanine, and tyrosine (data not shown) increased after the bolus meal \((P < 0.001)\) and reached the highest concentrations between 24.75 and 25.5 h (45 and 90 min after the meal) but returned to baseline by 26.5 h (150 min after the meal). The temporal concentrations of all these amino acids were unaffected during either continuous feeding or fasting.

Whole-body flux of phenylalanine and tyrosine, and phenylalanine hydroxylation. Feeding pigs intermittently increased whole-body flux of phenylalanine and tyrosine (Fig. 4). During this period there was also a numerical increase in phenylalanine hydroxylation, a measure of oxidation, which over the 4 h sampling period, was higher for the INT compared with the CON and FAS groups \((P < 0.05)\).

Hindquarters amino acid net balance. The net balance of the BCAA, phenylalanine (Fig. 5), and tyrosine (data not shown) increased \((P \leq 0.05)\) in the INT group between 24.5 and 25.25 h (30-75 min after the bolus meal). There were no temporal changes in the net removal of these amino acids by the hindquarters for either the CON or FAS groups.
**Hindquarters protein turnover.** Protein synthesis (Fig. 6), estimated based on venous phenylalanine as indicative of the true precursor enrichment, increased \((P < 0.001)\) in the INT group between 24.25 and 26.5 h (15 and 150 min following the meal). In contrast, there were no temporal changes in protein synthesis for either the CON or FAS pigs. Furthermore, protein degradation also remained constant across all groups. These differences in protein turnover did result, however, in increased protein deposition in the INT pigs compared with CON \((P < 0.001)\), with both INT and CON greater than for the FAS pigs. This pattern of net retention was similar to that determined from the mass balance.

**Translation initiation and degradation signaling.** In the gastrocnemius muscle from the hindquarters (Fig 7), INT feeding stimulated PKB phosphorylation compared with FAS \((P < 0.05)\) but was intermediate for the CON group. Phosphorylation of S6K1 and 4EBP-1 and formation of active eIF4E·eIF4G complex in the hindquarters was also enhanced in response to INT feeding as compared to FAS \((P < 0.05)\). For the CON group, while S6K1 phosphorylation was intermediate (NS from either INT or FAS), formation of eIF4E·eIF4G was lower than that of the INT group \((P < 0.05)\).

In the gastrocnemius muscle, MURF-1 and atrogin-1 abundance, and Fox01 phosphorylation (Fig. 8) were unaffected by treatment. Nonetheless, the ratio of LC3-II to total LC3 was lower \((P < 0.05)\) in the INT than in either the FAS or CON groups. Elongation factor 2 (eEF2), eukaryotic initiation factor-2-\(\alpha\) (eIF2\(\alpha\)), and AMP-activated protein kinase (AMPK\(\alpha\)) phosphorylation did not differ between the three groups (Fig. 9).

Protein synthesis and degradation pathways in the longissimus dorsi were also examined to check the representativeness of the results from the gastrocnemius muscle. In longissimus
dorsi muscle, PKB, S6K1, and 4EBP-1 phosphorylation (Fig. 10) were all stimulated in the INT group compared with both the CON and FAS groups ($P < 0.05$). INT feeding also increased the formation of active eIF4E-eIF4G complex compared with FAS ($P < 0.05$), with the CON group intermediate. The abundances of MURF-1 and atrogin-1 and the phosphorylation of Fox01 in the longissimus dorsi (Fig. 11) were not affected by either feeding status or frequency. In contrast, the ratio of microtubule-associated protein light chain 3 (LC3)-II (LC3-II) to total LC3 (sum LC3-II and LC3-I) in the longissimus dorsi was greater in the FAS ($P < 0.001$) compared with both the CON and INT groups.

DISCUSSION

Experimental aspects. In the current study, a combination of protein expression and stable isotope dilution methods in a multi-catheterized piglet model were used to assess how feeding modalities affect protein accretion in skeletal muscle of the neonate. Due to their invasive nature, these techniques preclude using human neonates, but the pig has already been proved as an excellent surrogate (50). Although it is well established that the simultaneous postprandial increases in insulin and amino acids activate a cascade of intracellular signaling events leading to increased protein synthesis (11, 13, 14), much less attention has been focused on the regulation of protein degradation. The approaches adopted here allow dynamic measurement of both protein synthesis and breakdown with these coupled to state-of-the-art analysis of appropriate signaling cascades. This provides integrated information, both metabolic and molecular, and helps address key issues related to how best to feed the neonate under clinical conditions.
Feeding modalities and protein deposition. Whether intermittent or continuous feeding is more beneficial remains contentious. This stems, in part, from limited data and confounding factors, including selection bias, protocol violations, pathological and clinical complications and the provision of nutrient parenterally for different periods of time (48). In addition, the type of food and/or the mode of nutrient delivery can have adverse metabolic consequences for the neonate (58). Most comparative studies have focused mainly on clinical outcomes, and the effect of intermittent and continuous feeding on muscle protein accretion has not been elucidated. We recently demonstrated that protein synthesis measured 90 min after a meal was enhanced by intermittent bolus feeding as compared to continuous feeding (28), however, the effects of feeding frequency on protein degradation and deposition were not determined. In the current study, protein deposition was greater in pigs fed intermittently compared with those fed continuously. This contrasts with one study where human neonates fed continuously showed faster linear growth (based on lower leg length) compared with those fed intermittently, although weight gains were similar (23). However, most studies show that intermittent feeding results in equal (2, 40, 55) or better (53) weight gains than continuous feeding. Although muscle growth is fastest in the neonatal period, weight gain may not necessarily be a good indicator of muscle protein deposition. Indeed, data from a study where macronutrient retention was determined by net balance suggest that nitrogen retention is similar between infants fed either continuously or intermittently (55). In practice, nitrogen balance may not be ideal to monitor small changes in retention due to measurement errors associated with both intake and losses (4, 51, 62). A limitation of the current study is that pigs were fed for only 29 h, short in comparison to long periods of hospitalization. Thus, further studies are needed to evaluate whether the enhanced protein deposition observed in this study persists over longer treatment periods.
Protein synthesis. In the current study, skeletal muscle protein synthesis was greater in intermittent as compared to continuous feeding. We have previously shown, using pancreatic-substrate clamps, that insulin and amino acids can independently stimulate protein synthesis in a dose-dependent manner in neonates (44) and this explains the responses observed in response to the bolus meal. In addition, earlier studies showed that meal feeding stimulates signals of translation initiation, with changes in mTOR activation leading to increased fractional protein synthesis rates in muscle (25, 26, 67). This rapid process is sustained for at least 2 h following a meal, mirroring that of insulin and amino acids, in particular leucine (66), and this is supported by the current findings.

Changes in the energy status of the cell can influence the mTOR pathway; for example, an increase in AMP levels activates AMPK, with resultant activation of the TSC1/2 complex, an allosteric inhibitor of mTOR (46). Previously, we have shown that insulin and amino acids have no effect on AMPK phosphorylation (28, 59, 61, 66). Similarly, in the current study, AMPK phosphorylation was not affected by changes in energy supply through either fasting or feeding. Together these data suggest that, under such normal physiologic conditions, AMPK activity has little impact on the mTOR signaling pathway.

Muscle PKB phosphorylation did increase in response to feeding and this was greatest for the INT group. We have previously shown that PKB phosphorylation increases after a meal, with this due to insulin and not amino acids (60), and returns to pre-feeding values by 4 h after the meal (28, 67). PKB phosphorylation inactivates tuberous sclerosis complex 1/2 (TSC1/2), thereby activating mTOR (12, 28) with subsequent phosphorylation of the downstream target proteins, S6K1 and 4EBP-1, activation of ribosomal subunit 6 (rpS6), the dissociation of
eIF4E·4EBP-1, and the formation of eIF4E·eIF4G, leading to enhanced translation initiation (39).

Translation initiation is controlled by two regulatory processes. The first involves binding of methionyl-tRNA to the 40S ribosomal subunit forming the 43S pre-initiation complex, a step mediated by eIF2α (39, 56). The second is through binding of the active mRNA to the 43S preinitiation complex, facilitated by eukaryotic initiation factor 4E and eukaryotic initiation factor 4G (eIF4E·eIF4G) complex. In this study, eIF2α phosphorylation was not affected by either feeding status or frequency and supports previous findings that the response to feeding is independent of changes in eIF2α phosphorylation (28, 61). Both insulin and amino acids (44) can activate mTOR, with effects on the downstream regulators of protein synthesis. While it is not possible in this study to separate the effects of insulin and amino acids, when both change in response to a bolus feed (as in INT), the mTOR pathway is stimulated above the value observed for an equivalent amount of food supplied over a longer period. Therefore over relatively short duration (29 h), regulatory mechanisms do not involve integration of total intake but rather are designed to follow the metabolic patterns created by meal-feeding.

The regulation of signaling components by amino acids upstream of mTOR is not completely understood, but has been shown to be independent of PKB (60). Amino acids activate mTOR, which leads to the phosphorylation of S6K1 and 4EBP-1 and the formation of the active eIF4E·eIF4G complex (44). The current study shows that activation of downstream effectors of mTOR was enhanced with intermittent feeding compared with continuous feeding. It also showed that the enhanced activation of the intracellular signaling proteins that regulate mTOR-dependant translation initiation resulted in an increased protein deposition when measured by stable isotope balance, despite a slight rise in amino acid oxidation. Thus, the increased
activation of downstream effectors of mTOR is likely due to the simultaneous rise in insulin and amino acids.

Although studies in vitro have shown that insulin accelerates peptide elongation through inactivation of eEF2 kinase, a regulator of eEF2 activity (65), studies in vivo indicated that neither amino acids nor insulin affect eEF2 phosphorylation (28, 61). Combined, these data suggest that binding of the 43S ribosomal subunit responds to feeding status and frequency and that peptide elongation is not a rate limiting step in protein synthesis under normal physiologic conditions.

**Protein degradation.** Skeletal muscle is an important source of amino acids under conditions where dietary supply is insufficient to meet critical organ requirements or where extra glucose precursors are required (63). Although a number of proteolytic pathways exist, only two are thought to play important roles in skeletal muscle: the ubiquitin-proteasome (63) and autophagy-lysosome (52) systems. In mammals, polyubiquitination by ubiquitin-ligases is the rate limiting step of the ubiquitin-proteasome system (52). The two most important muscle-specific ubiquitin-ligases are Atrogin-1 and MURF-1 (37, 63). Reduced activity of PKB decreases phosphorylation of the forkhead family of transcription factors (FOX01, 3, and 4) causing enhanced expression of Atrogin-1 and MURF-1 and a resultant increase in protein degradation (37). In addition, FOX01 induces muscle atrophy in vitro though increased expression of Ub-ligases, Atrogin-1 and MURF-1 (42). In the current study, although PKB phosphorylation increased in muscle of both continuously and intermittently fed pigs compared with those that were fasted, there was no effect on FOX01 activation or on the expression of Atrogin-1 and MURF-1, and neither were there observed differences in protein degradation. These findings agree with data from human studies that suggest, unlike catabolic and cachectic
diseased states, 40 h fasting was not long enough to induce changes in the expression of Atrogin-1 and MURF-1 genes (36). Similarly, in rodents only long (51 h) fasting periods (18) or severe amino acid starvation (35) led to an increase in Atrogin-1 and MURF-1 expression.

A reduction in PKB phosphorylation has been proposed as a mechanism to induce autophagy (52). Autophagy-lysosomal protein degradation requires activation of the Ub-like molecule LC3 (LC3-II) followed by the transfer and conjugation to phosphatidylethanolamine and the formation of autophagosomes (34, 52). Autophagosome fusion with lysosomes to form the mature autolysosomes is the final and rate limiting step leading to autophagy. Efforts to relate autophagy markers to protein degradation are confounded because accumulation of autophagosomes may reflect increased degradation or decreased autophagosome turnover (i.e., failure to fuse with lysosomes) (34). The increased LC3-II lipidation observed here suggests accumulation of autophagosomes is the probable cause because no increase in protein degradation was detected by isotope dilution. Alternatively, the upregulation of the autophagy-lysosome pathway may precede muscle loss (52), and although the piglets were fasted overnight, the duration may have been insufficient to fully induce the pathway. Nonetheless, under the current experimental conditions, protein degradation signaling appeared more sensitive to dietary changes via the autophagy-lysosome rather than the ubiquitin-proteasome system, but further investigation is required.

In conclusion, the feeding-induced stimulation of protein synthesis is greater in intermittently than continuously fed neonatal pigs and this was the driving force for the improved protein deposition as protein degradation was insensitive to feeding frequency. Moreover, the results suggest the increased protein synthesis during intermittent feeding was via elevated initiation of mTOR-dependent translation. Furthermore, under the current experimental
condition, neither the ubiquitin-proteasome nor autophagy-lysosomal systems were activated, despite a rise in LC3-II levels.

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S.W.E., G.E.L and T.A.D. designed the research; S.W.E., A.S., M.C.G., N.S., R.A.O., and H.V.N. conducted the research; S.W.E. A.S., H.V.N., and T.A.D. analyzed the data; S.W.E., G.E.L., and T.A.D wrote the paper; and T.A.D. had primary responsibility for the final content. All authors read and approved the final manuscript.


43. Motil KJ, Opekun AR, Montandon CM, Berthold HK, Davis TA, Klein PD, and Reeds PJ. Leucine oxidation changes rapidly after dietary protein intake is altered in adult women but lysine flux is unchanged as is lysine incorporation into VLDL-apolipoprotein B-100. *Journal of Nutrition* 124: 41-51, 1994.


Figure legends

Fig. 1. Schematic representation of tracer infusions and blood sampling in 10-d-old pigs. Feeding was initiated after overnight fasting and continued for 29 h for intermittently (INT) and continuously (CON) fed pigs. Tracer infusion was initiated after overnight fasting for the FAS group. At 20 h, a primed continuous infusion of \[^2\text{H}_5\]phenylalanine (10 µmol/ kg at 10 µmol/kg•h⁻¹), \[^2\text{H}_2\]Tyrosine (10 µmol/ kg at 10 µmol/kg•h⁻¹) were given (8 h) in the jugular vein and blood sampled during the last 4 h of the tracer infusion. Arrows indicate collection of blood samples.

Fig. 2. Arterial plasma glucose (A) and insulin (B) concentrations in pigs either fasted overnight (FAS) or fed continuously (CON) or intermittently (INT; 7 × 4 h meals) for 29 h. Values are means ± SEM, \(n = 6\).

Fig. 3. Arterial plasma isoleucine (A), leucine (B), phenylalanine (C), and valine (D) concentrations in pigs either fasted overnight (FAS) or fed continuously (CON) or intermittently (INT; 7 × 4 h meals) for 29 h. Values are means ± SEM, \(n = 6\).

Fig. 4. Whole body phenylalanine (A) and tyrosine (B) flux and phenylalanine hydroxylation to tyrosine (C) in pigs either fasted overnight (FAS) or fed continuously (CON) or intermittently (INT; 7 × 4 h meals) for 29 h. Values are means ± SEM, \(n = 6\).

Fig. 5. Hindquarters net balance of isoleucine (A), leucine (B), phenylalanine (C), and valine (D) in pigs either fasted overnight (FAS) or fed continuously (CON) or intermittently (INT; 7 × 4 h meals) for 29 h. Positive values denote net removal and negative values net release. Values are means ± SEM, \(n = 6\).
Fig. 6. Hindquarters protein synthesis (A), degradation (B), and deposition (C) in pigs either fasted overnight (FAS) or fed continuously (CON) or intermittently (INT; 7 × 4 h meals) for 29 h. Values are means ± SEM, \( n = 6 \).

Fig. 7. Protein kinase B (PKB; A), ribosomal protein S6 kinase 1 (S6K1; B), and eukaryotic initiation factor (eIF) 4B-binding protein-1 (4EBP-1; C) phosphorylation, and association of the eIF4E·eIF4G complex (D) in the gastrocnemius muscle of pigs either fasted overnight (FAS) or fed continuously (CON) or intermittently (INT; 7 × 4 h meals) for 29 h. Values are means ± SEM, \( n = 6 \). Values with different superscripts differ significantly \( (P < 0.05) \).

Fig. 8. Muscle RING-Finger Protein-1 (MURF-1; A), and atrophy F-box (atrogin-1; B) abundance, forkhead box-containing protein-01 (Fox01; C) phosphorylation, and microtubule-associated protein light chain 3 (LC3) II to total LC3 ratio (D) in the gastrocnemius muscle of pigs either fasted overnight (FAS) or fed continuously (CON) or intermittently (INT; 7 × 4 h meals) for 29 h. Values are means ± SEM, \( n = 6 \). Values with different superscripts differ significantly \( (P < 0.05) \).

Fig. 9. AMP-activated protein kinase (AMPK\( \alpha \); A), eukaryotic initiation factor-2-\( \alpha \) (eIF2\( \alpha \); B), and elongation factor 2 (eEF2; C) phosphorylation in the gastrocnemius muscle of pigs either fasted overnight (FAS) or fed continuously (CON) or intermittently (INT; 7 × 4 h meals) for 29 h. Values are means ± SEM, \( n = 6 \). Control blots in this figure appeared previously in figure 8.

Fig. 10. Protein kinase B (PKB; A), ribosomal protein S6 kinase 1 (S6K1; B), and eukaryotic initiation factor (eIF) 4B-binding protein-1 (4EBP-1; C) phosphorylation and association of the eIF4E·eIF4G complex (D) in the longissimus dorsi muscle of pigs either fasted overnight (FAS) or fed continuously (CON) or intermittently (INT; 7 × 4 h meals) for 29 h. Values are means ±
SEM, n = 6. Values with different superscripts differ significantly ($P < 0.05$). Control blots in this figure appeared in more than one panel.

Fig. 11. Muscle RING-Finger Protein-1 (MURF-1; A), and atrophy F-box (atrogin-1; B) abundance, forkhead box-containing protein-01 (Fox01; C) phosphorylation, and microtubule-associated protein light chain 3 (LC3) II to total LC3 ratio (D) in the longissimus dorsi muscle of pigs either fasted overnight (FAS) or fed continuously (CON) or intermittently (INT; 7 × 4 h meals) for 29 h. Values are means ± SEM, n = 6. Values with different superscripts differ significantly ($P < 0.05$). Control blots in this figure appeared previously in figure 10.
TABLE 1. Ingredients and nutrient composition of the experimental diet

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g/kg as fed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whey protein concentrate (80% CP)</td>
<td>50.3</td>
</tr>
<tr>
<td>Lactose</td>
<td>9.0</td>
</tr>
<tr>
<td>FatPak 80(^2)</td>
<td>65.4</td>
</tr>
<tr>
<td>Water</td>
<td>862.4</td>
</tr>
<tr>
<td>Xanthan gum</td>
<td>2.0</td>
</tr>
<tr>
<td>Vitamin premix(^3)</td>
<td>2.0</td>
</tr>
<tr>
<td>Mineral premix(^3)</td>
<td>9.0</td>
</tr>
</tbody>
</table>

\(^1\) Hilmar Ingredients, Hilmar, CA.

\(^2\) Milk Specialties Global Animal Nutrition, Carpentersville, IL.

\(^3\) Dyets Inc., Bethlehem, PN. Vitamin premix provided g/kg: Thiamine HCl, 0.1; riboflavin, 0.375; pyridoxine HCl, 0.1; Niacin, 1; calcium pantothenate, 1.2; folic acid, 0.13; biotin, 0.02; cobalamin B12, 1.5; retinyl palmitate, 0.8; cholecalciferol, 0.05; tocopheryl acetate, 8.8; menadione sodium bisulfite, 0.08. Trace mineral premix provided g/kg: calcium phosphate, dibasic, 187; calcium carbonate, 279; sodium chloride, 85; potassium phosphate monobasic, 155; magnesium sulfate, anhydrous, 44; manganous carbonate, 0.93; ferric citrate, 10; Zinc carbonate, 1.84; cupric carbonate, 0.193; potassium iodate, 0.005; sodium selenite, 0.007.
Fig 1.
Fig. 2.
Fig. 3.
Fig. 4.
Fig. 5.
Fig. 6.
Fig 7.
Fig 8.
Fig. 9.
Fig. 10.
Fig. 11.