Intestinal Threonine Utilization for Protein and Mucin Synthesis Is Decreased in Formula-Fed Preterm Pigs

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3 Abbreviations used: ASR, absolute synthesis rate; COL, colostrum; FORM, formula; FSR, fractional synthesis rate; NEC, necrotizing enterocolitis; TPN, total parenteral nutrition.
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Abstract

Threonine is an essential amino acid necessary for synthesis of intestinal (glyco)proteins such as mucin MUC2 to maintain adequate gut barrier function. In premature infants, reduced barrier function may contribute to the development of necrotizing enterocolitis (NEC). Human milk protects against NEC compared with infant formula. Therefore, we hypothesized that formula feeding decreases the MUC2 synthesis rate concomitant with a decrease in intestinal first-pass threonine utilization, predisposing the preterm neonate to NEC. Preterm pigs were delivered by caesarian section and received enteral feeding with formula (FORM; n = 13) or bovine colostrum (COL; n = 6) for 2 d following 48 h of total parenteral nutrition. Pigs received a dual stable isotope tracer infusion of threonine to determine intestinal threonine kinetics. NEC developed in 38% of the FORM pigs, whereas none of the COL pigs were affected (P = 0.13). Intestinal fractional first-pass threonine utilization was lower in FORM pigs (49 ± 2%) than in COL pigs (60 ± 4%) (P = 0.02). In FORM pigs compared with COL pigs, protein synthesis (369 ± 31 mg·kg⁻¹·d⁻¹ vs. 615 ± 54 mg·kg⁻¹·d⁻¹; P = 0.003) and MUC2 synthesis (121 ± 17%/d vs. 184 ± 15%/d; P = 0.02) were lower in the distal small intestine (SI). Our results suggest that formula feeding compared with colostrum feeding in preterm piglets reduces mucosal growth with a concomitant decrease in first-pass splanchnic threonine utilization, protein synthesis, and MUC2 synthesis in the distal SI. Hence, decreased intestinal threonine metabolism and subsequently impaired gut barrier function may predispose the formula-fed infant to developing NEC. J. Nutr. 141: 1306–1311, 2011.

Introduction

Necrotizing enterocolitis (NEC)10 is the most common gastrointestinal disorder that affects preterm neonates (1). Because of the rising incidence of preterm births and improved survival rates of very low–birth weight babies, NEC still remains a challenge in neonatal intensive care. Treatment is limited and reported mortality rates are as high as 50% for infants requiring surgery (2). Infants who recover from NEC have an increased risk for complications, such as short bowel syndrome (3) and impaired neurodevelopment (4,5). Despite extensive research, the pathogenesis of NEC remains poorly understood. Major risk factors identified for the development of NEC are immaturity, enteral (formula) feeding, and bacterial colonization (6). Therefore, the responses of the immature gut to enteral feeding and bacterial colonization require further investigation.

Feeding preterm infants formula increases NEC incidence compared with their own mother’s milk or donor human milk (7–9). Human milk, especially colostrum, contains various growth factors and Ig that may reduce the NEC incidence. In preterm piglets, NEC incidence is greatly increased with formula feeding compared with bovine or porcine colostrum feeding (10). Formula feeding in preterm pigs decreases digestive capacity, induces mucosal atrophy and disruption, causes microbial overgrowth, and increases gut permeability (10–12). This negatively affects the gut barrier function that is necessary for epithelial protection.

An important feature for gut barrier function is the mucus layer that overlies the gut epithelium. Goblet cells synthesize and secrete large gel-forming glycoproteins, called mucins. MUC2 is
the predominant secretory mucin in the human intestinal tract (13,14). The mucus layer provides protection against luminal pathogens and toxic substances, and disruption of the mucus layer causes intestinal inflammation and mucosal erosion (15,16). Decreased gut barrier function caused by a diminished mucus layer may facilitate bacterial translocation and, in combination with an immature immune system, render the preterm infant at risk for the development of intestinal inflammation, sepsis, and NEC (17).

The peptide backbone of MUC2 is particularly rich in the essential amino acid threonine, which constitutes ~30% of the total amino acids in this protein (18–22). Threonine availability affects protein mucosal synthesis and mucin synthesis in pigs and rats (23–26). Using a dual stable isotope tracer method, which allows the determination of dietary first-pass threonine utilization, we showed that in preterm infants, the splanchnic tissues extract 70–82% of dietary threonine, which indicates a high need for threonine (27). Because very little of the sequestered threonine in the gut is oxidized, a majority is used for gut protein and glycoprotein synthesis (28,29). However, the effect of colostrum and formula feeding on dietary threonine utilization, protein, and mucin synthesis has not yet been investigated.

We hypothesize that formula feeding predisposes the preterm neonate to developing NEC by a mechanism of a decreased Muc2 synthesis rate, which is accompanied by a decrease in the first-pass intestinal threonine utilization that is necessary for protein and MUC2 synthesis. Thus, the aim of the present study was to determine differences in NEC incidence and first-pass threonine utilization, measured by dual stable isotope tracer technique, in preterm piglets that were fed either formula or colostrum. Furthermore, we aimed to determine differences in gut barrier function in preterm piglets that were fed formula or colostrum by measuring intestinal proteins and MUC2 synthesis.

Materials and Methods

Experimental design

Nineteen preterm pigs (Danish landrace × Yorkshire) from 3 sows were delivered via cesarean section at d 105–107 of gestation, as previously described in detail (10). Animal protocols and procedures were approved by the Danish National Committee on Animal Experimentation.

Diet

Total parenteral nutrition (TPN) was administered for the first 2 d to mimic the clinical setting in a neonatal intensive care unit, where most preterm infants initially receive TPN. In addition, TPN administration in preterm piglets predisposes them to develop NEC when enteral nutrition is commenced (10). The parenteral nutrition solution was prepared intravenously and was based on the infusion product Nutriflex Lipid Plus (B. Braun). The nutrient composition of the TPN solution (glucose, 72 g/L; lipid, 31.1 g/L; amino acids, 45 g/L; solution was provided at a rate of 4–6 mL·kg⁻¹·h⁻¹) was identical to that which was previously described in detail (10). After 48 h, TPN was discontinued and the pigs received either human milk formula (FORM; n = 13) or bovine colostrum feeding (COI; n = 6) via an orogastric tube (at a rate of 15 mL·kg⁻¹·h⁻¹) for 2 d. The milk formula consisted of a mix of 3 different commercial formulas for human infants (80 g/L; peptide; 70 g/L; maiprop; 75 mL/L). Liiquigen-MCT, all products kindly donated by SHS International) to meet protein and energy requirements. The nutrient composition of the formula mix was calculated from the specifications of the commercial formulas (Table 1). Bovine colostrum was obtained from the first milking of Holstein-Friesian cows and irradiated (1 × kGy) before use. Energy, lactose, and fat concentrations of colostrum were calculated from reported measurements (30–33). An aliquot of the diluted colostrum was assayed for protein content using the Pierce assay (BCA, Protein Assay, Thermo Scientific). To make the diets isocaloric, the colostrum was diluted 2:1 with water (Table 1). The threonine concentration of both formula and colostrum was determined using GC-MS. An aliquot of colostrum and formula was hydrolyzed for 24 h at 110°C in 6 mmol/L HCl and dried (Speedvac Savant, ThermoFisher). Samples were then esterified, derivatized, and analyzed using the same method used for plasma threonine concentrations.

Isotope infusion protocol

FORM piglets (n = 7, randomly assigned) and COL piglets (n = 6) were both subjected to the dual stable isotope tracer infusion protocol. The stable isotope infusion was started 9 h prior to euthanization to measure intestinal threonine utilization, protein synthesis, and MUC2 synthesis. A primed (25 μmol·kg⁻¹, continuous (25 μmol·kg⁻¹·h⁻¹) infusion of [U-13C]threonine (99.47 atom%, Cambridge Isotope Laboratories) was administered through an arterial catheter. Simultaneously, a primed (25 μmol·kg⁻¹·h⁻¹) hourly bolus (25 μmol·kg⁻¹·h⁻¹) of [13C]threonine (98 atom%, Cambridge Isotope Laboratories) was administered via an orogastric tube. During the infusion protocol, piglets were switched from 3-h feeding intervals to 1-h feeding intervals. Blood samples were taken at 0, 6, 8.5, and 9 h after the start of the tracer infusion for MS analyses. Blood samples were centrifuged immediately after collection to separate plasma and cells. The plasma was stored at −80°C until further analysis. After the 9-h infusion protocol, piglets were euthanized with an overdose of pentobarbital (200 mg/kg i.v.; University Pharmacy, University of Copenhagen).

Tissue collection

Immediately after the piglet was euthanized, the entire small intestine (SI) and colon were removed, weighed, and sampled for protein analysis and histology as previously described (10). From each SI segment, the ratio of mucosa:total intestine was determined after drying both the mucosa and the underlying tissues. Mucosal scrapings of the last cm of the distal SI was frozen in liquid nitrogen for mucin analysis (below). The lungs, liver, spleen, heart, kidneys, and stomach were removed and wet weights were recorded.

NEC evaluation and histology

The piglets were evaluated for clinical symptoms of NEC every 3 h as previously described (10). If any suffering was observed from NEC prior to the end of the study protocol, the piglets were immediately euthanized and tissue collected. Upon removal of the gut, the proximal, middle, and distal SI segments and colon were evaluated for NEC lesions and scored from 1 (no or minimal inflammation) to 6 (severe extensive hemorrhage and necrosis), as described previously (10). A score ≥ 3 was indicative of NEC.

Distal small intestinal and colonic tissue sections (5 μm) were stained with Alcian blue–periodic acid Schiff to study morphological changes of the mucosa and identify the presence of neutral and acidic mucins in goblet cells. Goblet cell numbers were analyzed using a Visiopharm integrator system in a blinded manner (Visiopharm). The number of goblet cells and the total amount of epithelial cells were counted using a specially constructed counting grid, which moved around randomly in the tissue section and analyzed ~25% of the section. In total, 5 tissue sections/piglet were analyzed. The number of goblet cells was expressed as a percentage of total epithelial cells per crypt or villous.

Protein content

Intestinal tissue samples were pulverized in liquid nitrogen and homogenized in ice-cold homogenization buffer (50 mmol/L Tris/HCl (pH 7.5),

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Macronutrient composition of COL and FORM</th>
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<tr>
<td></td>
<td>COL</td>
</tr>
<tr>
<td>Energy, MJ/L</td>
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<tr>
<td>Protein, g/L</td>
<td>86</td>
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<tr>
<td>Carbohydrate, g/L</td>
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<tr>
<td>Lactose, g/L</td>
<td>18–20</td>
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<tr>
<td>Fat, g/L</td>
<td>30–44</td>
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5 mmol/L EDTA (pH 8), 1% triton, 10 mmol/L iodacetamide, SBTI (100 mg/L), pepstatin A (10 mg/L), leupeptin (10 mg/L), aprotinin (1%), and 1 mmol/L Phenylmethylsulfonylfluoride at a concentration of 100 g/L. Samples were assayed for protein concentration using the Pierce assay.

**MUC2 isolation**

In the distal SI and colon, MUC2 was isolated using a CsCl density gradient ultracentrifugation method combined with gravity gel filtration chromatography, as previously described for human MUC2 (14,34,35).

**MS**

**Threonine plasma concentration and enrichment analyses.** Plasma samples were prepared to determine threonine concentration and enrichment by GC-MS, as described previously (28,35), with minor modifications. [2,3,4,4-D₄]N-threonine was used as an internal standard and an additional derivatization step was performed to block the free hydroxy group of threonine by adding 20 mL of pyridine and 50 mL of acetic anhydride to the dried ethyl chloroformate derivatives. The samples were briefly vortexed and incubated for 60 min at 60°C. After cooling, the samples were dried under a gentle nitrogen flow at room temperature and resuspended in 50 μL of ethyl acetate. Standard curves were prepared by mixing aqueous solutions of natural and labeled threonine for both enrichment and concentration determination. GC-MS analyses were performed in selective ion-monitoring mode after electron impact ionization with a MSD 5975C Agilent GCMS (Agilent Technologies). Selective ion-monitoring mode was carried out at m/z of 146.1, 147.1, 149.1, and 152.1. Separation was achieved on a VF17MS (30-m × 0.25-mm i.d., 0.25-μm film thickness) fused silica capillary column (Varian). Helium was used as a carrier gas at a constant flow of 1.2 mL/min. The column was held at 55°C for 1 min, after which the program was altered to 160°C at 30°C/ min, then to 200°C at 5°C/min, and finally to 300°C at 10°C/min, with an 8-min hold time. Threonine enrichment was expressed in molar percent excess. Threonine concentration was expressed in μmol/L.

**Intestinal free amino acids and protein-bound amino acids.** Intestinal tissues from the middle SI, distal SI, and colon were homogenized with ice-cold water to achieve a 100-g/L concentration. The protein fraction was isolated by precipitating protein-bound tissue pool, hydrolyzed samples were derivatized to form cation exchange separation, as described above for the plasma amino acid fraction. To measure the enrichment of [U-13C]threonine in the acid-free tissue pool was expressed in mole percent excess. Threonine enrichment was expressed in molar percent excess. Threonine concentration was expressed in μmol/L.

**Intestinal Score**

Intestinal scores were determined by a blinded endpoint panel. This scoring system was modified from previous studies (1,12). Minitab statistical software (Minitab) was used for statistical analysis. The NEC incidence between COL and FORM was analyzed using the chi-square test. The NEC severity score was normally distributed (KS normality test) and therefore a t test was used for the analysis. Data were analyzed for a correlation with NEC score using the Pearson test. Differences between FORM and COL pigs regarding tissue measurements, histology, and MS were analyzed by 1-way ANOVA General Linear Model. Data are presented as the mean ± SEM and P < 0.05 was considered significant.

**Results**

**NEC development and intestinal evaluation.** Before the completion of the study, 4 pigs were euthanized due to suffering from severe clinical symptoms of NEC. The incidence of NEC, defined as a score > 3, tended to be greater in FORM (38%) than in COL piglets (0%) (P = 0.13) as did NEC severity based on the intestinal score, which was 1.5 ± 0.2 in the COL group and 2.8 ± 0.5 in the FORM group (P = 0.08).

Birth weight did not differ between the COL and FORM piglets (data not shown). Weight gain was lower in FORM pigs (5.9 ± 3.8 g · kg⁻¹ · d⁻¹) compared with COL pigs (20.0 ± 1.7 g · kg⁻¹ · d⁻¹) (P = 0.02). There was a negative correlation between weight gain and NEC score (r = −0.69; P = 0.001).

The wet weights of the SI and colon did not differ between FORM and COL piglets (data not shown). However, the proportion of mucosa and dry matter (mucosa percentage) was higher in COL pigs (72 ± 1.2%) than in FORM pigs (65 ± 1.9%) (P = 0.046). Wet weights of the heart, lungs, liver, stomach, kidneys, and spleen did not differ between the groups (data not shown).

Histology showed mucosal damage in the distal small intestinal and colonic tissue in FORM pigs, as previously described (37). In case of extensive damage of the mucosa, samples were excluded from further analysis (distal SI, n = 5; colon, n = 1). Intestinal epithelial cells that were stained with Alcian blue-periodic acid Schiff had the typical morphology of goblet cells in both the FORM- and COL-fed piglets. In the distal small intestinal crypts, blue-stained goblet cells, representing the presence of acidic sialylated mucins, were more abundant than in the villi. Cells showing only PAS stain, i.e. pink goblet cells containing neutral mucins, occasionally occurred at all levels of the crypt or villous. The majority of goblet cells were shades of purple, contained both acidic and neutral mucins, and occurred mainly between the tops of the crypts to the tops of the villi (Fig. 1A,B). A similar pattern was found in colon samples; more blue-stained goblet cells were present in the lower crypt, whereas pink- and purple-stained cells were found in the upper crypt and surface (Fig. 1C,D). Quantitative analysis of goblet cells expressed as a percentage of total epithelial cells in the distal SI (FORM, 5.1 ± 0.6%; COL, 6.6 ± 1.2%) and colon (FORM, 23.8 ± 1.7%; COL, 27.6 ± 1.9%) did not differ between the groups. In the colon, lower goblet cell counts correlated with a higher NEC score (r = −0.74; P < 0.001),
Threonine kinetics. One pig in the FORM group was excluded from isotopic analyses because of infusion failure of the tracer. All remaining threonine-infused pigs (FORM, n = 6; COL, n = 6) had a NEC score < 3. Plasma threonine concentrations were higher in FORM pigs compared with COL pigs, although their intake was lower (Table 2). Plasma threonine flux, based on the i.v.-infused [U-13C]threonine tracer, was higher in FORM pigs compared with COL pigs (Table 2). Plasma threonine flux, based on the i.g.-infused [15N]threonine tracer, did not differ between FORM and COL pigs (Table 2). Fractional first-pass utilization of threonine was lower in FORM pigs than in COL pigs (Table 2). When corrected for enteral threonine intake, the absolute first-pass utilization of threonine was much lower in FORM pigs compared with COL pigs, which corresponds to the difference in plasma threonine concentrations (Table 2).

Intestinal protein. FORM piglets had lower protein concentrations in the distal SI and colon compared with COL piglets (Table 3). Protein FSR in the middle SI (P = 0.06) and colon (P = 0.08) tended to be higher in FORM pigs than in COL pigs, whereas that in the distal SI did not differ between the groups (Table 3). Protein ASR in both groups was the highest in the middle SI, decreased toward the distal SI, and declined even further toward the colon (Table 3). However, protein ASR in the distal SI was lower in FORM pigs compared with COL pigs, whereas protein ASR in the middle SI and colon did not differ between the groups (Table 3).

Mucin MUC2 synthesis. Purified MUC2 isolates from distal SI and colon tissue were further analyzed to determine MUC2 FSR, which is an indication of the percentage of newly synthesized MUC2 per day. In the distal SI, the MUC2 FSR was high, and it which is an indication of the percentage of newly synthesized and colon tissue were further analyzed to determine MUC2 FSR, which is an indication of the percentage of newly synthesized MUC2 per day. In the distal SI, the MUC2 FSR was high, and it which is an indication of the percentage of newly synthesized

Discussion
Preterm infants who are fed formula have an increased risk of developing NEC compared with infants who are fed donor or their own mother’s milk (7,9). Similarly, formula feeding in preterm pigs increases NEC incidence and induces mucosal atrophy and intestinal dysfunction compared with colostrum feeding (10,12). Furthermore, formula-fed preterm pigs that developed NEC showed altered expression of the intestinal proteome (38). Colostrum, containing growth factors, Ig, and other immunostimulatory products, may directly stimulate gut barrier function by inducing proliferation and/or differentiation of intestinal epithelial cells and increasing nutrient absorption (10,37). However, colostrum may indirectly stimulate gut barrier function by activating different metabolic pathways, regulating protein expression, or enhancing colonization with beneficial bacteria-releasing products such as SCFA to stimulate MUC2 synthesis (10,12,39). We investigated the effect of formula compared with colostrum feeding on intestinal threonine metabolism, which is important for gut growth and barrier function. Our results showed that formula feeding reduced mucosal growth, first-pass threonine utilization, protein synthesis, and mucin synthesis compared with colostrum feeding. NEC incidence and intestinal NEC-scoring tended to be greater in formula-fed piglets. The differences in these clinical evaluation criteria were not significant as they were in previous studies (10,40), perhaps due to small sample size.

Fractional and absolute first-pass splanchnic utilization of threonine were markedly lower in FORM pigs compared with COL pigs. The lower first-pass intestinal threonine utilization in FORM pigs corresponded well with the results obtained for protein and MUC2 synthesis. Both were lower in FORM piglets compared with COL piglets. The fractional first-pass threonine utilization in preterm pigs was lower than that found in 4-wk-old pigs and preterm infants (27,28). However, the preterm pigs in our study were only enterally fed for 72 h and still had a low gut mass/kg of body weight that likely accounted for the lower threonine utilization that we found.

[U-13C]-threonine enrichment was less and threonine flux or turnover was greater in FORM than in COL pigs. Because similar amounts of i.v. threonine were infused, the threonine tracer must have been diluted from either increased dietary threonine amounts passed on to the circulation and/or from endogenous threonine release from protein breakdown. Splanchnic utilization was lower

### Table 2: Threonine kinetics in preterm pigs fed COL or FORM for 2 d

<table>
<thead>
<tr>
<th></th>
<th>COL</th>
<th>FORM</th>
<th>P</th>
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<tbody>
<tr>
<td>Concentration in diet, g/L</td>
<td>4.8</td>
<td>4.5(^1)</td>
<td>0.063</td>
</tr>
<tr>
<td>Intake, mg/kg(^{-1})*d(^{-1})</td>
<td>576</td>
<td>533</td>
<td></td>
</tr>
<tr>
<td>Plasma concentration, mmol/L</td>
<td>304 ± 50</td>
<td>672 ± 82</td>
<td>0.003</td>
</tr>
<tr>
<td>Flux [U-13C]-threonine (i.v.) tracer, mmol/kg(^{-1})*h(^{-1})</td>
<td>160 ± 14</td>
<td>218 ± 24</td>
<td>0.08</td>
</tr>
<tr>
<td>Flux [15N]-threonine (i.g.) tracer, mmol/kg(^{-1})*h(^{-1})</td>
<td>404 ± 19</td>
<td>422 ± 39</td>
<td>0.66</td>
</tr>
<tr>
<td>First-pass splanchnic utilization, % of intake</td>
<td>60 ± 4</td>
<td>49 ± 2</td>
<td>0.02</td>
</tr>
<tr>
<td>First-pass splanchnic utilization, mg/kg(^{-1})*d(^{-1})</td>
<td>343 ± 20</td>
<td>250 ± 11</td>
<td>0.004</td>
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\(^1\) Values are mean ± SEM, n = 6.

\(^2\) The measured threonine concentration of the formula was in the same range as the calculated threonine concentration from the formula specifications (4.0 g/L).

Figure 1: Histology of the distal ileum (A, B) and colon (C, D) of preterm pigs fed COL (B, D) or FORM (A, C) for 2 d.
feeding increased amounts of mucin precursors, i.e. threonine, cysteine, and proline, enhanced mucin synthesis in a rat model of colitis, which emphasizes the importance of adequate nutrition during inflammation (23). Future studies will have to elucidate whether increasing amounts of protein and/or threonine in the formula may counteract the negative effect of formula feeding on intestinal threonine metabolism and gut barrier function in preterm piglets.

In this study, we did not investigate the effects of formula compared with colostrum feeding on whole body protein metabolism. A whole body protein kinetics model could provide additional data regarding whole body protein synthesis and proteolysis. Furthermore, because 4 piglets developed NEC and had to be euthanized, we were unable to study those animals. Therefore, threonine kinetics in an earlier stage of formula feeding may illuminate whether piglets developing NEC have a more affected intestinal threonine metabolism compared with formula-fed piglets that were not developing NEC. Additionally, we previously showed that TPN administration prior to colostrum feeding in preterm pigs diminished intestinal functions and increased NEC, although not to the same extent as formula feeding (10). Therefore, the differences between the COL- and FORM-fed piglets in intestinal threonine metabolism and protein and mucin MUC2 synthesis found in the present study may be even more pronounced with colostrum feeding compared with formula feeding starting from birth.

In conclusion, our results suggest that feeding preterm piglets formula decreases mucosal mass and first-pass splanchnic threonine utilization compared with colostrum feeding. This decrease in intestinal threonine metabolism is concomitant with a decrease in both protein and mucin MUC2 synthesis in the distal SI. Hence, decreased intestinal threonine metabolism and the subsequently impaired gut barrier function may explain why the formula-fed infant is more prone to develop NEC.

Acknowledgments
P.J.P., B.S., P.T.S., and J.B.v.G. designed the experiments; P.J.P., B.S., P.T.S., and J.B.v.G. performed the experiments; P.J.P., B.S., P.T.S., and J.B.v.G. analyzed the data; P.J.P., M.L.J., B.S., I.B.R., P.T.S., and J.B.v.G. interpreted the data; P.J.P., B.S., P.T.S., and J.B.v.G. drafted the manuscript; P.J.P., B.S., P.T.S., and J.B.v.G. read and approved the final manuscript; and D.B. provided advice and a critical review of the manuscript.

Literature Cited


| TABLE 3 | Protein content, FSR, and ASR in the SI and colon of preterm pigs fed COL or FORM for 2 d1 |
|----------------|-----------------|-----------------|-----------------|
|              | COL             | FORM            | P              |
| Middle SI    |                 |                 |
| Protein content, g/kg BW | 876 ± 69 | 786 ± 138 | 0.57 |
| FSR, %/d     | 55 ± 4.0        | 68 ± 5.0        | 0.06 |
| ASR, mg kg⁻¹·d⁻¹ | 487 ± 72 | 523 ± 78 | 0.75 |
| Distal SI    |                 |                 |
| Protein content, g/kg BW | 945 ± 42 | 678 ± 64 | 0.006 |
| FSR, %/d     | 65 ± 6.2        | 57 ± 6.8        | 0.36 |
| ASR, mg kg⁻¹·d⁻¹ | 615 ± 54 | 369 ± 31 | 0.003 |
| Colon        |                 |                 |
| Protein content, g/kg BW | 615 ± 44 | 448 ± 32 | 0.012 |
| FSR, %/d     | 30 ± 3.6        | 38 ± 1.9        | 0.08 |
| ASR, mg kg⁻¹·d⁻¹ | 191 ± 31 | 171 ± 14 | 0.58 |

1 Values are mean ± SEM, n = 6.
2 BW, body weight.

in FORM pigs, resulting in increased transport of threonine to the systemic circulation. However, proteolysis might have contributed to the increased threonine turnover and the high threonine plasma concentrations found in FORM pigs as well. Studies in rats and humans have shown that during sepsis, catabolism of muscle protein allowed the mobilization of amino acids required for increased synthesis of defensive proteins in the liver and intestine (41-43). Because first-pass threonine utilization was lower in FORM pigs, increased threonine supply for protein synthesis in the liver and gut may have been demanded from the systemic pool, most likely at the expense of muscle protein.

Adequate gut barrier function involves multiple intestinal mechanisms for the defense against NEC, such as synthesis of immune cells, defensins, tight junctions, and mucins, such as MUC2 (17). In FORM pigs, protein synthesis was decreased in the distal SI compared with COL pigs. The observed reduction in protein synthesis may result from damaged intestinal cells and apoptosis due to the inflammation and NEC found in the FORM piglets. Alternatively, a reduction in protein synthesis by formula feeding may reflect decreased expression and hence synthesis of defensive proteins, such as MUC2, and cause a breach in the intestinal defense system that leads to NEC. Future studies investigating the effect of type of feeding on intestinal protein expression and synthesis before the occurrence of inflammation might offer further insight into this topic.

The importance of threonine availability on protein and mucin synthesis has been shown in neonatal pigs and rats (24-26). In mini-pigs with induced ileal colitis, intestinal threonine utilization for mucin synthesis was increased (44). Interestingly,

FIGURE 2 Fractional synthesis rate of MUC2 in (A) the distal SI and (B) colon in preterm pigs fed COL or FORM for 2 d. Values are mean ± SEM, n = 6. *Different from FORM; P = 0.02.
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