Luminal Threonine Concentration Acutely Affects Intestinal Mucosal Protein and Mucin Synthesis in Piglets

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Abstract

Intestinal mucin synthesis is sensitive to dietary threonine supply, which suggests that the gut’s requirement for threonine may comprise a significant proportion of the whole body requirement. We used a continuously perfused gut loop model and intraluminal flooding dose technique in 6 young pigs to study the acute effects of varying luminal availability of threonine on intestinal protein and mucin syntheses. A complete amino acid mixture containing 0, 21, or 56 mg threonine/g total amino acids (whole body requirement ratio is ~28 mg/g) was continuously perfused in isolated loops for 120 min, including a 30-min $^3$H-phenylalanine flooding dose. We measured fractional synthesis rates of total mucosal protein and mucin by analyzing $^3$H-phenylalanine incorporation. Fractional rates of total mucosal protein synthesis were higher in loops perfused with solutions containing threonine at 56 mg/g (66 ± 4%/d) compared with 0 mg/g (42 ± 9%/d) and 21 mg/g (53 ± 6%/d) ($P < 0.05$). For mucin, fractional rates of synthesis differed between 0 mg/g (323 ± 6%/d), 21 mg/g (347 ± 49%/d), and 56 mg/g (414 ± 31%/d) ($P < 0.05$). In addition, total proline and threonine concentrations in the protein hydrolysates increased with luminal threonine concentration ($P < 0.05$), indicating an increase in threonine- and proline-rich proteins. De novo synthesis of mucosal and mucin proteins is acutely sensitive to luminal threonine concentration, which demonstrates the importance of dietary amino acid supply to gut protein metabolism. J. Nutr. 138: 1298–1303, 2008.

Introduction

Although the gastrointestinal tract represents only 4–6% of body mass, it accounts for 25–50% of whole body protein turnover (1–3). Indeed, the extraction of dietary amino acids by the gut has been estimated at 20–70% of dietary indispensable amino acids on first pass (4). Given the considerable demand of the gut for amino acids, gut maintenance and growth must constitute a large proportion of whole body amino acid requirements (5). Bertolo et al. (6) have previously shown that when piglets were fed via total parenteral nutrition (which involves gut bypass and atrophy), the threonine requirement decreased by 55% compared with oral feeding. These findings were supported by Burrin et al. (4,5,7,8), who showed in pigs that 60–90% of the dietary threonine intake was extracted on first pass by the portal-drained viscera, which is dominated by gut metabolism, whereas other indispensable amino acids were extracted at only 14–33%.

One of the primary fates of amino acids extracted by the gut is synthesis of proteins, which are mostly secreted into the lumen (9). These endogenous secretions include large quantities of mucus, which protect the gut from pathogens and antinutritional factors. The main component of mucus is mucins, glycoproteins with a protein core that is particularly rich in threonine, proline, and serine, with threonine alone constituting 28–35% of the total amino acids (10,11). Mucin protein is very resistant to digestion and its amino acids cannot be reutilized by the animal (5,10). Small intestinal mucins, as well as colonic mucins, are readily digested by colonic microbes, but these amino acids are not available to the host because of negligible amino acid absorption in the large intestine. Therefore, intestinal mucin secretion represents a significant net loss of indispensable amino acids to the animal. Indeed, for most indispensable amino acids, gastrointestinal losses at the ileum accounted for 14–33% of the maintenance requirement, but for threonine, the contribution was 61% (12). Together, these data suggest that the gut has a high threonine requirement, which is a significant proportion of the whole body requirement. Indeed, mucin synthesis (13,14) and goblet cell morphology (15) are sensitive to dietary threonine supply. In situations of gut stress that would increase mucus turnover, it follows that threonine utilization would increase in proportion to whole body needs. Given the amino acid costs associated with stress-induced nonspecific protein synthesis, the costs for mucin-associated amino acids, such as threonine, would be much greater due to their specialized role in gut secretions. If threonine that is utilized during the first-pass metabolism for...
mucin synthesis is increased, then the availability of threonine in portal circulation will decrease and in turn decrease threonine that is available for peripheral tissues (16).

Because threonine is an indispensable amino acid and because the threonine requirement of the gut is disproportionately higher than other amino acids, the study of this amino acid in the gut is of particular interest nutritionally. Adegoke et al. (17,18) successfully validated a continuously perfused gut loop model using a luminal flooding dose technique that maintains first-pass nutrient exposure, limits any systemic effects by the perfused nutrients, and allows for multiple loops within an animal, which decreases experimental error. In this study, our objective was to adapt this model to demonstrate the limiting amino acid concept for luminal threonine on protein and mucin synthesis in the gut. We hypothesized that if luminal, first-pass threonine supply is deficient, then protein, and especially mucin, synthesis will be limited acutely, even in animals with adequate threonine status.

Materials and Methods

Animals and gut loop model. The gut loop model was adapted from that of Adegoke et al. (17,18), who successfully validated a continuously perfused small intestinal loop model using a luminal flooding dose technique to study the regulation of protein synthesis in intestinal mucosa by luminal nutrients. Notably, Adegoke et al. (18) did not observe any differences in protein synthesis when glucose or short-chain fatty acids were included with amino acids. Therefore, we chose to investigate the role of luminal amino acid ratios alone in intestinal protein synthesis.

The present experiment was conducted in accordance with the Canadian Council on Animal Care Guidelines and was authorized by the institutional Animal Care Committee. Six food-deprived, 10-kg Yorkshire pigs (Stuart King Farms) were anesthetized using an initial flow of 4% halothane mixed with oxygen and maintained with 2% halothane and oxygen. Under anesthesia, a midline incision was made to expose the small intestine. The ligament of Treitz was identified and 15 cm distal of the ligament, an inlet polyethylene cannula (i.d., 1.6 mm; Watson Marlow) was inserted into a hole made with an 18-gauge needle through the intestinal wall into the lumen. The cannula was secured with suture tied around the intestine and tubing. A 10-cm section of intestine was isolated and an outlet cannula was inserted similarly at the opposite end of the loop. A total of 4 loops was prepared similarly with 50 cm of intestine between loops. The loops were flushed with PBS (125 mmol/L NaCl, 15.9 mmol/L Na₂HPO₄, 1.2 mmol/L NaH₂PO₄, pH 7.4, 37°C) to remove any chyme that may be present. The intestines were then rinsed with warm 0.9% saline and placed gently back into the abdominal cavity for the duration of the experiment. To prevent adhesion between the handled intestines, adequate moisture levels were maintained by spraying the intestine with warm 0.9% saline and covering the abdomen with plastic wrap. Heart rate, respiration rate, body temperature, and oxygen saturation were monitored throughout the experiment.

Amino acid solution perfusions. The perfusion solutions were continuously warmed in a water bath at 37°C. The inlet and outlet cannulae were connected to a multichannel peristaltic pump (Watson Marlow) that pumped respective perfusion solutions through the loops (~5 mL luminal volume) at 3 mL/min in a closed loop system using a total perfusate volume of 50 mL. This flow rate approximates that estimated for chyme in human jejunum (i.e. ~2–4 mL/min) (19) and ileum (i.e. 2.4 mL/min) (20). The various solutions and loops were randomly allocated. After a 90-min perfusion, each amino acid solution was replaced with an equivalent solution containing 50 μCi ³H-phenylalanine (Amersham Biosciences) in a 2-mmol/L phenylalanine solution. After this flooding dose of phenylalanine was perfused for an additional 30 min, the loop was excised using cautery to prevent bleeding, flushed with cold PBS, slit lengthwise, and scraped with a microscope slide on ice to remove the mucosa. Mucosal tissue was immediately frozen in liquid nitrogen and stored at ~70°C until further analysis. To estimate the amount of

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>mg/g</th>
<th>Total amino acids</th>
<th>mmol/L</th>
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<tr>
<td>Tyrosine</td>
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volume of saturated potassium citrate (Sigma-Aldrich), left on ice for 10 min, and then filtered using a 0.45-µm filter. These samples were frozen and stored until further analysis by HPLC for tissue free amino acids.

The pellet containing the tissue-bound amino acids was disrupted and washed 4 times with 4 mL of ice-cold 2% (wt:v) perchloric acid. The final pellet was resuspended in 1.3 mL of 1 mol/L NaOH by disrupting the pellet with a Teflon pestle; NaOH was added to a total volume of 2.4 mL. The samples were then left to sit in a 37°C water bath for ~1.5 h to solubilize the protein for total protein analysis. To the remaining solution, 1.2 mL of cold 20% (wt:v) perchloric acid was added and the mixture was placed on ice for 20 min to precipitate the protein. The samples were then centrifuged at 2800 × g; 15 min and standards (norleucine and 15N-leucine) were then added to the pellet, which was disrupted with a glass rod in 1.2 mL of 6 mol/L HCl. This acid mixture was then transferred to a 25-mL digestion tube with cap; the tube was rinsed with additional 6 mol/L HCl to a final volume of 5 mL. The samples were digested at 110°C for 24 h for protein hydrolysis. The cooled hydrolysates were transferred to an Erlenmeyer flask and brought to a final volume of 25 mL with deionized water. The contents were filtered with a 0.45-µm filter and stored until further analysis.

**Mucin purification and hydrolysis.** Our method to purify mucin glycoprotein was a modified version of that developed by Faure et al. (25) in rats that exploits mucin’s resistance to protease action. Approximately 300 mg of mucosal scrapings was homogenized for 45 s in 2 mL of ice-cold 50 mmol/L Tris (pH 7.5) using a Polytron homogenizer at 30%. A solution containing 20 mg of Flavourzyme (a fungal complex of exopeptidases and endoproteases; Novozymes) was added to the homogenate and left to incubate under agitation in a 37°C water bath overnight. After 18 h, the enzymatic activity of Flavourzyme was stopped by placing the homogenate on ice for 10 min. Guanidium hydrochloride and dithiothreitol were added to 4 mol/L and 10 mmol/L concentrations, respectively, and the mixture was left at room temperature for 2 h under agitation. Finally, 25 mmol/L iodoacetamide was added to the homogenate and left overnight at room temperature under agitation. The mucins in this homogenate were then purified using gravity gel filtration chromatography using 10 mL PD-10 columns (Amersham Biosciences) filled with Sephacryl S-300 High Resolution resin (Sigma-Aldrich). The resin was equilibrated with 20 mL of 50 mmol/L Tris (pH 7.5) containing 2 mol/L guanidium HCl. The column was loaded with 1 mL of the homogenate followed by 12 mL of the equilibration buffer. Twelve fractions of 1 mL were collected, placed in dialysis tubing (Spectra/Por, MWCO 12–14,000; VWR International), and dialyzed against deionized water for 48 h. Following dialysis, the fractions were freeze-dried and resuspended in 0.1 mL of deionized water. The mucin-containing fractions were then identified by SDS-PAGE (4% stacking/7.5% migrating, at 20 mA) with periodic acid–Schiff’s base staining for glycoproteins and Coomassie Blue staining for contaminating proteins. The mucin-containing fractions were identified by SDS-PAGE results and then combined in a digestion tube with 5 mL of 6 mol/L HCl and internal standards (norleucine and 15N-leucine) and hydrolyzed at 110°C for 24 h and stored for HPLC analysis.

**Amino acid analysis.** Samples were analyzed for amino acids using the Waters Pico-tag method, which uses phenylisothiocyanate derivatization and separation on a C18 reverse-phase column (Waters) (26). Leucine and phenylalanine fractions were collected and fraction radioactivity was determined using a liquid scintillation counter and Biodegradable Counting Scintillant (Amersham Biosciences).

**Calculations and statistical analysis.** Protein and mucin fractional synthesis rates were calculated using the formula developed by Garlick et al. (27), using the following equation:

$$K_s = \frac{SRA_f}{SRA_b} \times 100$$

where $K_s$ is the fractional rate of protein or mucin synthesis in percentage of the respective protein pool synthesized per day; $SRA_b$ is the specific radioactivity of protein-bound phenylalanine; $SRA_f$ is the specific radioactivity of phenylalanine in the precursor pool (tissue free amino acids); and $t$ is the time allowed for incorporation of the radioactive phenylalanine into protein.

Data were analyzed using a 1-way ANOVA with repeated measures (to account for blocking within pigs) and Student-Newman-Keuls multiple comparisons using Graph Pad Prism 4. Means ± SEM were considered significantly different at $P < 0.05$.

**Results**

**Animals.** All pigs were stable throughout anesthesia with no significant changes in heart rate, oxygen saturation level, respiratory rate, or body temperature during the perfusion periods.

**Mucin purification.** We found the mucin purification method of Faure et al. (25) to be just as effective as the classical method using CsCl density gradient centrifugation (28) but with better efficiency in time and labor (data not shown). Our initial results following the procedures of Faure et al. (25) indicated the presence of contaminating protein bands in the glycoprotein-containing fractions when stained with Coomassie Blue. These results suggested inadequate proteolysis by Flavourzyme, so we increased the amount of Flavourzyme from 10 mg to 20 mg and increased the incubation period from 2 h to overnight (18 h). These modifications did not change periodic acid-Schiff’s-staining patterns for glycoproteins but did eliminate Coomassie Blue-staining bands in the mucin-containing fractions (Supplemental Fig. 1).

**Protein and mucin fractional synthesis rates.** The fractional synthesis rates of mucosal protein and mucin are presented in Figures 1 and 2, respectively. Blocking within each pig (by repeated measures analysis) was significant ($P < 0.05$) and so data are presented as means for each threonine level (Figs. 1A,2A) and as connected data within individual pigs (Figs. 1B,2B) to demonstrate the effects of blocking. With respect to total mucosal protein, fractional rates of synthesis were higher in loops perfused with solutions containing threonine at 56 mg/g compared with 0 and 21 mg/g ($n = 6$; $P < 0.05$) (Fig. 1). For mucin glycoprotein, fractional rates of synthesis differed between 0, 21, and 56 mg/g ($n = 5$; $P < 0.05$) (Fig. 2); we did not have enough mucosa for mucin purification in one of the pigs. The control loops that were perfused with nutrient-free PBS had protein synthesis rates of 113 ± 11%/d and mucin synthesis rates of 461 ± 65%/d. Untreated adjacent intestinal tissue not directly exposed to an amino acid mixture was also extracted and analyzed to determine the extent of arterial extraction of the luminal flooding dose and to correct raw data for protein synthesis rate calculations. The protein-bound and free phenylalanine specific radioactivity in this untreated tissue was 3–11% and 6–8%, respectively, of that found in the luminaly flooded loops; these data were similar to those found by Adegoke et al. (17).
Mucosal amino acid composition. Because of their particular abundance in mucins, we analyzed the threonine, proline, and serine concentrations of mucosal protein hydrolysates. Expressed as a percentage of total amino acids, threonine and proline in mucosal protein increased with luminal threonine concentrations in the loops (Table 3) \((P, 0.05)\). Proportions of these amino acids in mucosal hydrolysates from PBS and untreated loops were similar to each other; for threonine and proline, proportions were lower than the respective proportions in loops perfused with 56 mg threonine/g amino acids. Free threonine and serine concentrations were also measured in mucosal homogenates and did not differ across treatments (Table 3).

**Discussion**

We used the concept of the rate-limiting amino acid to assess whether intestinal tissue is dependent on the luminal, first-pass amino acid supply to synthesize proteins. When the limiting amino acid (i.e. threonine) was not present in the lumen, we hypothesized that intestinal protein synthesis would be minimized, even in the presence of adequate arterial threonine supply in a well-nourished pig. As the luminal threonine level was increased, we expected the rate of protein synthesis to acutely increase in response. With this in mind, it was expected that the perfusion solution with no threonine would result in the lowest rate of protein synthesis for both total mucosal protein and for mucin alone, which was observed in our study. As the threonine level was increased, the protein synthesis rates increased between 0 and 21 mg/g threonine levels (for mucin) and from 21 to 56 mg/g (for mucin and total protein). We previously observed, with the use of the indicator amino acid oxidation technique, that the mean threonine requirement in orally fed piglets was 28 mg/g of amino acids (6). This requirement estimate reflects the proportion of total amino acids that must be threonine to maximize whole body protein synthesis. Because the small intestine preferentially utilizes threonine (4–8), we expected the gut’s threonine requirement ratio to be much greater than the 28 mg/g amino acids needed for the whole body. Indeed, our study suggests that the gut acutely responds to luminal, first-pass threonine supply up to 56 mg/g amino acids. Although others have demonstrated that chronic whole body threonine restriction can reduce intestinal protein and mucin syntheses (13–15), our results are profound in that protein synthesis was acutely dependent on the luminal, first-pass supply of amino acids, even in the presence of adequate arterial supply to the loops.

It is also possible that threonine is directly stimulating protein synthesis independent of its role as a limiting precursor. Indispensable amino acids, particularly leucine, are now known to act as nutrient signals to stimulate translation initiation and induce protein synthesis via a mammalian target of rapamycin (mTOR)-regulated pathway (29). The absence of individual amino acids inhibits p70 S6 kinase activity, a key enzyme facilitating the translation of capped mRNA (30,31). Although more focus has been placed on the potency of branched-chain amino acids (30) and arginine (32), deletion of threonine alone has been shown to moderately diminish p70 S6 kinase activity (31). In our

**Figure 1** Fractional synthesis rates of mucosal protein (percent per day) at varying threonine levels expressed as mg threonine/g total amino acids. Data are means \(\pm\) SEM (bars) \((A)\) and individual data for loops within the same pig that are connected by lines \((B)\). Data for \(n = 6\) were analyzed by ANOVA with repeated measures (i.e. within pig) and Student-Newman-Keuls multiple comparisons. In A, means without a common letter differ, \(P < 0.05\).

**Figure 2** Fractional synthesis rates of mucin (percent per day) at varying threonine levels expressed as mg threonine/g total amino acids. Data are means \(\pm\) SEM (bars) \((A)\) and individual data for loops within the same pig that are connected by lines \((B)\). Data for \(n = 6\) were analyzed by ANOVA with repeated measures (i.e. within pig) and Student-Newman-Keuls multiple comparisons. In A, means without a common letter differ, \(P < 0.05\).
In conclusion, this study demonstrated that during acute restriction of luminal threonine supply, the small intestinal mucosal and mucin protein synthesis rates are limited in young, well-nourished pigs. This finding is particularly profound considering that the arterial supply of threonine was adequate, suggesting that in the fed state, intestinal protein synthesis is primarily dependent on luminal, first-pass amino acids. These results have key nutritional implications and may help explain why a significant supply of enteral nutrients is necessary to maintain intestinal mass and integrity, even when parenteral nutrients are adequate (34).
Literature Cited


