Nutritional Stimulation of Milk Protein Yield of Cows Is Associated with Changes in Phosphorylation of Mammary Eukaryotic Initiation Factor 2 and Ribosomal S6 Kinase 1

Chanelle A. Toerien, Donald R. Trout, and John P. Cant

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Abstract

Production of protein by the lactating mammary gland is stimulated by intake of dietary energy and protein. Mass-action effects of essential amino acids (EAA) cannot explain all of the nutritional response. Protein synthesis in tissues of growing animals is regulated by nutrients through the mammalian target of rapamycin (mTOR) and integrated stress response (ISR) networks. To explore if nutrients signal through the mTOR and ISR networks in the mammary gland in vivo, lactating cows were feed-deprived for 22 h and then infused i.v. for 9 h with EAA+glucose (Glc), Glc only, L-Met+L-Lys, L-His, or L-Leu. Milk protein yield was increased 33 and 27% by EAA+Glc and Glc infusions, respectively. Infusions of Met+Lys and His generated 35 and 41%, respectively, of the EAA+Glc response. Infusion of EAA+Glc reduced phosphorylation of the ISR target, eukaryotic initiation factor (eIF) 2, in mammary tissue and increased phosphorylation of the mTOR targets, ribosomal S6 kinase 1 (S6K1) and S6. Both responses are stimulatory to protein synthesis. Glucose did not significantly increase mammary S6K1 phosphorylation but reduced eIF2 phosphorylation by 62%, which implicates the ISR network in the stimulation of milk protein yield. In contrast, the EAA infusions increased (P < 0.05) or tended to increase (P < 0.1) mammary mTOR activity and only His, like Glc, decreased eIF2 phosphorylation by 62%. Despite activation of these protein synthesis signals to between 83 and 127% of the EAA+Glc response, EAA infusions produced less than one-half of the milk protein yield response generated by EAA+Glc, indicating that ISR and mTOR networks exert only a portion of the control over protein yield. J. Nutr. doi: 10.3945/jn.109.114033.

Introduction

During lactation, the mammary gland is the site of synthesis and secretion of copious amounts of protein, equivalent to up to 38% of the total amino acid flux through the body of well-fed dairy cows (1). Nutrition of the lactating female influences the rates of protein synthesis in the mammary glands and output in milk. Increases in either energy or metabolizable protein intake can stimulate milk protein yield (2,3). The response is typically attributed to an increase in the supply of essential amino acids (EAA) that limits protein synthesis in the mammary glands. In ruminant animals, the energy effect is often attributed to accelerated protein outflow from the rumen resulting from more rapid pregastric fermentation of dietary carbohydrates. However, dietary energy also stimulates milk protein yield in nonruminants (2) or ruminants given post-ruminal glucose (4,5), which has been explained as being due to a faster mammary blood flow rate that delivers more EAA for mammary consumption (5).
The above explanations are based on a conceptualization of milk component yields as a function of their precursor concentrations according to mass action. However, there are many situations where this action has been shown to be quite low or even negative. A 2-fold increase in plasma EAA concentrations for several hours by close arterial infusion resulted in only a 13% increase in milk protein yield (6). Euglycemic insulin infusion increased milk protein yield whereas EAA concentrations in plasma actually dropped (7,8). Accordingly, alternatives to the hypothesis of simple mass action are that nutrients act either through the endocrine system of the body or directly on the mammary glands to regulate activities of the milk protein-synthesizing machinery.

There are 2 different amino acid-sensing mechanisms that have been identified to operate in mammalian cells. One involves the mammalian target of rapamycin (mTOR) that stimulates mRNA translation by phosphorylation of the ribosomal S6 kinase 1 (S6K1) and eukaryotic initiation factor (eIF) 4E-binding protein (4E-BP1). Via the mTOR network, the postprandial rise in circulating insulin and EAA concentrations, particularly Leu, stimulates protein synthesis in liver and muscle of growing animals (9). A second amino acid detection mechanism is through uncharged tRNA that activate phosphorylation of eIF2 as part of the integrated stress response (ISR), which slows initiation of mRNA translation (10).

Nutritional stimulation of the mTOR and ISR networks has been studied little in lactating mammary tissue. Lactating glands express 3-fold higher levels of the eIF2 and eIF4E proteins compared with nonlactating mammary glands, and the association of eIF4E with 4E-BP1 is reduced and phosphorylation of S6K1 is elevated (11,12). In vitro, epithelial cells of the mouse and cow mammary glands respond to addition of all amino acids or Leu only with phosphorylation of both 4E-BP1 and S6K1 via mTOR activation (13,14). Addition of Lys, His, or Thr to mammary epithelial cell culture media depressed S6K1 phosphorylation, indicative of mTOR inhibition (15). Effects of nutrition on the mammary mTOR and ISR networks in vivo have not been reported previously.

The starvation-refeeding experiment, in which animals are feed deprived for 12–48 h and then administered individual nutrients per os or i.v., has been useful in elucidating which components of a meal elicit the postprandial response (16,17). In lactating rats, the feed deprivation-refeeding model has been used to study the roles of glucose and insulin in stimulation of mammary lipogenesis (18), and in lactating goats, it has been used to characterize responses to glucose and acetate infusions (19,20). Effects on milk protein yield were not reported. It was our objective to apply a feed deprivation-infusion protocol to establish which nutrients elicit an increase in milk protein yield and to test the hypothesis of a mass action effect against the alternative hypothesis of nutrient signaling effects through the mTOR and ISR networks, using S6K1 and eIF2 phosphorylation as markers, respectively.

Materials and Methods

Experimental protocol and sampling. Six multiparous Holstein cows began the experiment at 69 ± 4 d in milk and 630 ± 14 kg body weight. All animal procedures were approved by the Animal Care and Use Committee at the University of Guelph. Cows were randomly assigned to a 6 × 6 Latin square design with 9 h of infusion after 22 h of feed deprivation repeated every 14 d. Between periods, cows were offered a complete mixed ration (Table 1) for ad libitum intake twice daily after milking at 0530 and 1630 h. Cows were weighed 4 d prior to the first period and every 14 d thereafter. Feed samples were collected twice during each of the 6 treatment weeks, stored at –20°C, and then dried at 55°C, pooled, and subsampled for proximate analysis.

Catheters (Angiocath; 14 gauge, 2.1 × 133 mm; Becton Dickinson) were inserted into 1 jugular vein of each cow at the onset of feed deprivation. I.v. infusion treatments were saline (Sal), all 10 EAA+Gluc, Glc, Met+Lys, His, and Leu. Amino acids were infused at the following rates (g/h) equivalent to their secretion in 500 g/d milk protein: i-Arg (0.62), i-His (0.48), i-Ile (1.09), i-Leu (1.87), i-Lys (1.47), i-Met (0.51), i-Phc (0.93), i-Thr (1.00), i-Trp (0.33), and i-Val (1.29). Glucose was infused at 100 g/h, which has been shown to increase milk protein yield after several days in well-fed cows (4,5).

Cows were milked at +1 h (~0800 h) and +7 h (~1400 h) of infusion with 10 IU oxytocin i.v. At the +7-h milking, milk was collected, weighed, and sampled from the nonbiopsied front quarters. Milk samples were refrigerated and analyzed within 24 h.

Blood samples were collected from the jugular catheter at ~22 h, ~1 min, and hourly until +9 h of infusion into heparinized vacutainer tubes (Becton Dickinson) and stored on ice until centrifuged at 1500 g for 20 min. Plasma was transferred into polypropylene tubes and frozen at –20°C.

Mammary tissue was collected by biopsy (21) at +9 h of infusion after sedation with 10 mg xylazine i.v., caudal epidural analgesia with 0.02 mg/kg body weight xylazine, and local subcutaneous xylcocaine. Procaine penicillin G (12 × 106 IU) and ketoprofen (1 mg/kg body weight) were administered i.v. The first hindquarter for biopsy was randomly selected and subsequent samples were collected from the opposite hindquarter. The inner three-quarters of each core sample was rinsed in ice-cold saline, blotted dry, frozen in liquid N2, and stored at –80°C.

Sample analysis. Milk was analyzed by infrared spectroscopy for true protein, fat, and lactose (Foss System 4000, Foss Electric). Proximate composition of the diet was analyzed by near-infrared spectroscopy at a commercial laboratory (Agribrands Purina Canada). Plasma samples were analyzed spectrophotometrically for glucose concentration (22) and inspection of the results (not shown) indicated that glucose reached a plateau by +1 h of infusion. For all other plasma analytes, pooled samples were prepared from those collected +2 to +6 h of infusion and +8 to +9 h, respectively. Pooled plasma samples and those collected at +22 h, ~1 min, and +33 h were analyzed for plasma urea-N (PUN; kit no. B549–150; Teco Diagnostics), α-amino N (AAN; 23), β-hydroxybutyrate (BHB), and acetate (22). Commercial RIA kits were used to measure glucagon and insulin (Linco Research). Plasma insulin-like growth factor (IGF)–I RIA were conducted according to Okere et al. (24). Inter- and intra-assay coefficients of variation, respectively, were 3.9 and 3.2% for glucose, 5.7 and 3.1% for PUN, 6.3 and 3.3% for acetate, 9.4 and 5.7% for BHB, 10.5 and 2.1% for AAN, 2.3 and 1.9% for glucagon, 4.2 and 2.3% for insulin, and 2.0 and 7.1% for IGF-I.

### Table 1 Ingredient and chemical compositions of the total mixed ration offered to all cows between feed deprivation periods

<table>
<thead>
<tr>
<th>Component</th>
<th>Content, % of DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixed hay</td>
<td>3.6</td>
</tr>
<tr>
<td>Mixed haylage</td>
<td>22.5</td>
</tr>
<tr>
<td>Corn silage</td>
<td>33.8</td>
</tr>
<tr>
<td>High-moisture corn</td>
<td>21.4</td>
</tr>
<tr>
<td>Dairy supplement</td>
<td>18.7</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Nutrients</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude protein</td>
<td>17.0</td>
</tr>
<tr>
<td>Neutral detergent fiber</td>
<td>32.3</td>
</tr>
<tr>
<td>Acid detergent fiber</td>
<td>20.3</td>
</tr>
<tr>
<td>Net energy for lactation, MJ/kg</td>
<td>6.65</td>
</tr>
<tr>
<td>Ca</td>
<td>0.90</td>
</tr>
<tr>
<td>P</td>
<td>0.45</td>
</tr>
</tbody>
</table>

1 Obtained from Floradale Feed Mill (ON, Canada) and contains 10.7 MJ/kg metabolizable energy and 33.3% crude protein.
Concentrations of individual amino acids in the +8-/+9-h pooled plasma sample were determined by an isotope dilution method (25,26) on a gas chromatograph-mass spectrometer (Model HP6890, 5973 mass selective detector; Hewlett Packard). The method does not quantify Arg, Asp, Asn, and Pro.

Mammary tissue samples were homogenized at 4°C in Tris purge (Roche Diagnostics) lysis buffer. RNA, DNA, and protein were extracted and recovery and purity of nucleic acid fractions were determined spectrophotometrically at 260 and 280 nm. Western blot analysis of mammary protein abundance was as described previously (11). Briefly, proteins in postmitochondrial supernatants were separated by SDS-PAGE and transferred overnight to polyvinylidene difluoride membranes (Bio-Rad). Membranes were blocked with skim milk (5% by SDS-PAGE and transferred overnight to polyvinylidene difluoride membranes (Bio-Rad). Membranes were blocked with skim milk (5% w/v) for 1 h at room temperature. For detection of eIF2α phosphorylated on Ser51 (peIF2), proteins were separated on 12% gels and membranes were incubated with rabbit anti-human peIF2 (Cell Signaling). After detection, membranes were stripped of peroxidase activity by incubation with 15% H2O2 for 15 min and were reprobed for total eIF2α (27) using goat anti-human eIF2α (Santa Cruz Biotechnology). For ribosomal protein S6 phosphorylated on Ser235/236 (pS6), proteins were separated on 12% gels and membranes were incubated with rabbit anti-human pS6 (Santa Cruz). For S6K1 phosphorylated on Thr389 (pS6K1), proteins were separated on 10% gels and probed with rabbit anti-human pS6K1 (Santa Cruz). Blots were detected by enhanced chemiluminescence on photographic film (Visualizer Western Blot Detection kit; Upstate). Blot density was quantitated using Northern Eclipse software (version 6.0; Empix Imaging). Samples from each period were analyzed on the same gel and the order was randomized to avoid position bias. In addition, samples were analyzed in duplicate and a control sample was included on every gel as a reference to which other blot densities were normalized. These densities were then normalized per unit DNA to avoid differences per unit protein due to the quantity of milk in the sample. Densities of pEIf2 were also normalized for the total eIF2 blotted density of the sample.

Calculations and statistical analyses. Variance in observations (Yijk) for each cow-period during the experiment was analyzed using the MIXED procedure of SAS (28) with cow as a random effect and period and infusion for each cow-period during the experiment was analyzed using the MIXED procedure of SAS (28) with cow as a random effect and period and infusion as fixed effects. Treatment means were separated by Tukey’s test. For plasma constituents, a repeated time factor was included in the ANOVA model and the −22 h value was included as a covariate. Treatment least-squares means for each time point were calculated and separated by the pdiff option of SAS (27) following a Bonferroni adjustment. Differences were considered significant at P = 0.05 and tendencies at 0.05 < P ≤ 0.1.

Results

Animal health and numbers. The repeated feed deprivation-infusion model and mammary biopsy had no long-term impact on milk production. Mean postpeak decrease in daily milk yield was 1.69 ± 0.3%/wk (Supplemental Fig. 1), which is a higher persistency than the mean decline of 1.86%/wk previously reported for well-fed cows (29). Milk production during the 2 d prior to each feed deprivation period deviated <10% from the mean regression line of milk production. Body weight declined to 95.2% of its pretrial value by period 2, yet by period 6, body weight was at 101.5% of that pretrial. One cow was removed from the experiment due to an upset rumen attributed to spoiled silage. As a consequence, there were 5 cows for treatments Glc, Met+Lys, and Leu.

Table 2 Milk and milk component yields from the front mammary glands of feed-deprived cows during 6 h of infusion of glucose and amino acids into the jugular vein1

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Sal</th>
<th>EAA+Glc</th>
<th>Glc</th>
<th>Met+Lys</th>
<th>His</th>
<th>Leu</th>
<th>SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>6</td>
<td>6</td>
<td>5</td>
<td>5</td>
<td>6</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk, kg/6 h</td>
<td>1.83&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.14&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.02&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.17&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.02&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.11</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Protein, %</td>
<td>3.20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.53&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.35&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.08&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.11</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fat, %</td>
<td>7.49&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.60&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.02&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.44&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.43&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.55</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Lactose, %</td>
<td>3.96&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.62&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.48&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.19&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>4.28&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>4.22&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.11</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Protein, g/6 h</td>
<td>58.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>78.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>74.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>65.6&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>66.6&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>61.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.8</td>
<td>0.001</td>
</tr>
<tr>
<td>Fat, g/6 h</td>
<td>137.7</td>
<td>142.3</td>
<td>156.4</td>
<td>146.6</td>
<td>157.1</td>
<td>145.6</td>
<td>12.1</td>
<td>0.613</td>
</tr>
<tr>
<td>Lactose, g/6 h</td>
<td>73.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>141.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>138.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>83.6&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>92.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>85.1&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>4.3</td>
<td>&lt;0.001</td>
</tr>
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</table>

1 Data are least-squares means. Means in a row with superscripts without a common letter differ, P ≤ 0.05.

Milk components. There were no carryover effects on milk production. The negative control caused milk yield to decrease by 54% compared with the 2-d mean pre-feed deprivation (P < 0.05; data not shown). Relative to Sal, infusion of EAA+Glc increased milk and lactose yields by 69 and 95%, respectively (Table 2) and glucose alone resulted in increases in milk and lactose yields of 72 and 91%, respectively (P < 0.05). Infusion of His elevated milk and lactose yields 18 and 27%, respectively (P < 0.05), which was 26 and 28%, respectively, of the EAA+Glc response.

Infusion of EAA+Glc and Glc increased milk protein yield 33 and 27%, respectively, relative to Sal (P < 0.05). Infusion of Glc without any amino acids generated a milk protein yield response equal to 80% of that generated by EAA+Glc (P < 0.05). Met+Lys–, His–, and Leu-stimulated protein yields did not differ from Sal (P < 0.10) nor did Met+Lys– and His–stimulated protein yields differ from EAA+Glc (P > 0.10), which indicates an intermediate response to these EAA. While being infused at the same rate as in EAA+Glc, the effect of Met+Lys on protein yield equaled 45% of the effect of EAA+Glc and His generated 41% of the effect.

Fat yield did not differ across treatments (P > 0.10), so with the increased milk volume, fat percentage was lower in EAA+Glc and Glc than Sal (P < 0.05).

Plasma constituents. Levels of plasma constituents did not differ between treatments at the start of the food deprivation (−22 h) or just prior to the start of infusion at −1 min (P > 0.10; Supplemental Table 1). Concentrations of plasma AAN, acetate, PUN, and IGF-I also did not differ between treatments during infusion (P > 0.10; Table 3) except for a tendency for lower AAN after the Glc treatment compared with the Met+Lys treatment (P = 0.06). Glucose and acetate concentrations decreased during the 22-h food deprivation (P < 0.05) whereas PUN concentrations increased (P < 0.05). Acetate continued to decline during infusion of nutrients (P < 0.05).
Whereas plasma glucose concentrations were virtually identical in Sal, Met+Lys, His, and Leu at the different time points (P > 0.10; Supplemental Table 1), infusion of EAA+Glc and Glc doubled concentrations (P < 0.05; Table 3), with a concomitant decrease in BHB (P < 0.05). The change in plasma glucose was accompanied by an increase in insulin and decrease in glucagon concentrations between +2 and +6 h (P < 0.05; Supplemental Table 1). There was also a tendency during this time for insulin to be higher during the Glc treatment than during the EAA+Glc treatment (P = 0.10). By +8.5 h, insulin and glucagon levels after the EAA+Glc treatment returned to values that did not differ (P > 0.10) from Sal (Table 3). With Glc infusion alone, glucagon remained low (P < 0.05) at +8.5 h.

Compared with Sal, infusion of His and Leu elevated their respective concentrations in plasma (P < 0.05) and Met+Lys infusion tended to increase Lys (P = 0.08) and Met (P = 0.06; Table 4). His, Lys, Met, Phe, and Thr concentrations in plasma were all increased (P < 0.05) by EAA+Glc infusion, whereas concentrations of branched-chain amino acids (BCAA) remained unchanged (P > 0.10). In contrast, infusion of Glc by itself increased glucagon, but not glucose (P > 0.05; Table 3).
itself resulted in no change in His, Lys, Met, Phe, or Thr (P > 0.10) and a 67% decrease in concentrations of all 3 BCAA (P < 0.05). Concentrations of Cys and Tyr also decreased during the Glc treatment (P < 0.05) and Gly and Ser concentrations increased (P < 0.05). Otherwise, there were no effects on non-EAA. Summed concentrations of total and EAA were similar for all treatments except Glc, where they were much decreased compared with EAA+Glc (P < 0.05).

Mammary translation factors. Treatments did not differ in abundance of total eIF2α in mammary biopsies (data not shown), but the proportion in the phosphorylated form was reduced 49, 62, and 62% by EAA+Glc, Glc, and His, respectively (P < 0.05; Fig. 1). His infusion elicited a similar level of eIF2α dephosphorylation to that of 100 g/h Glc. The mTOR substrate S6K1 was 66 and 71% more phosphorylated (P < 0.05) during the EAA+Glc and Leu treatments, respectively, and tended to be 61% more phosphorylated (P = 0.09; Fig. 2) during Met+Lys and His treatments. Thus, the effect of EAA+Glc on pS6K1 was nearly duplicated by each of the amino acid-containing infusates. The S6K1 substrate S6 was 113, 95, and 101% more phosphorylated (P < 0.05) during the EAA+Glc, Met+Lys, and Leu treatments, but His had no effect (P > 0.10) despite the S6K1 activation. The only treatment causing an increase in S6K1 and S6 phosphorylation that also increased the RNA:DNA ratio above that of Sal was Leu (P < 0.05). Based on S6K1 and S6 phosphorylation states, infusion of 100 g/h Glc did not stimulate a mammary mTOR response (P > 0.10).

Discussion

Milk synthetic response to feed deprivation-infusion model. To study whether the effect of amino acids and glucose on milk protein synthesis is due to a mass-action effect of substrate concentration or to a signaling mechanism, cows were first deprived of feed to decrease yields of milk and milk protein and permit a yield response. The length of the feed deprivation was based on a time previously found to reversibly decrease milk production in high-producing dairy cows (30). This reversibility was demonstrated by an EAA+Glc infusion that increased lactose production from 46 to 76% of its predeprivation level. Plasma glucose concentrations doubled relative to Sal and, because glucose is a precursor for lactose and lactose is the main osmoregulator of milk volume, an increase in milk and lactose yield in response to EAA+Glc and Glc is consistent with a mass-action effect of the milk precursor. In feed-deprived goats or those with a low blood glucose concentration, glucose infusion increased lactose yields whereas well-fed goats and cows did not respond (19,20,22).

It is not as easy to explain the effect of His on lactose yield. His is not a direct substrate of lactose synthesis and is poorly oxidized (31). The glucagon concentration in plasma was numerically the highest during His infusion, which may have stimulated hepatic glucose release, but neither PUN nor glucose concentrations in plasma support this scenario. Thus, neither a substrate nor a hormonal effect readily accounts for the lactose and milk yield responses to His infusion.

The EAA+Glc mixture stimulated milk protein yield. If it can be assumed that the fractions of total milk and protein produced by the fore mammary glands are equal (i.e. 43%; data not shown), then we estimate from the 3.3-g/h increase in protein secretion from the fore mammary glands (Table 2) that 42% of the 9.59 g/h EAA infused after the EAA+Glc treatment was secreted in milk. Apparent recovery of infused mixtures of...
amino acids in milk protein ranges from ~5 to 45% in the fed cow (3). That we did not find a higher efficiency in feed-deprived cows suggests that 45% capture may be an upper limit for the species. In seals that normally fast during lactation, milk protein yield can be as high as 100% of the entry from body protein loss (32).

Infusion of glucose alone, without any additional amino acids, resulted in a milk protein yield response equal to 80% of that of EAA+Glc, although glucose is not used as a direct substrate for protein synthesis. The decrease in plasma EAA concentrations compared with the EAA+Glc infusion, and in BCAA concentrations compared with SAl, indicates that glucose stimulated protein yield independent of amino acid supply. Similar responses of lactating ruminants to glucose or euglycemic insulin infusion have been reported (4,7,8,33).

It is possible that Glc stimulated protein yield by being oxidized and improving the adenylate charge of milk secretory cells. The mechanism by which a low adenylate charge slows protein synthesis is not by mass action, however, but through AMP-activated kinase-mediated inhibition of mTOR signaling (34). The elevated insulin concentration between h 2 and 6 of Glc infusion could also have stimulated milk protein yield. In any case, we conclude that Glc infusion stimulated milk protein yield through a signaling mechanism and not by mass action.

**Mammary signaling to translation apparatus.** Extracellular insulin and amino acid concentrations and intracellular AMP concentrations are communicated to the protein synthetic apparatus in muscle and liver via mTOR (9,34). Active mTOR phosphorylates S6K1, which, in turn, phosphorylates ribosomal protein S6 and several other proteins to accelerate mRNA translation. Amino acid deficiencies and other cellular stresses inhibit mRNA translation through the ISR (10). The ISR involves 4 different cellular kinases that can phosphorylate eIF2α on Ser51, converting it from a substrate for GDP/GTP exchange to an inhibitor of the exchange (35). Accordingly, p-eIF2 inhibits mRNA translation. In an attempt to differentiate which of the 2 translation regulation networks may be responsible for the effects of glucose and amino acids on milk protein synthesis in the bovine mammary gland in vivo, we measured phosphorylation states of the downstream targets of the ISR and mTOR networks, eIF2α and S6K1, respectively.

Activation of S6 is achieved through sequential phosphorylation of Ser235, 236, 240, 244, and 247 by S6K1 (36). Ser235 and 236 can also be phosphorylated by p90RSK, a target of mitogen-activated protein kinase signaling. Although treatment effects on pS6 reflected effects on pS6K1, the possibility exists that the increases in phosphorylation of S6 on Ser235/236 we observed were due to p90RSK activation and not via mTOR.

The positive control of EAA+Glc, which stimulated milk protein yield the most, influenced both signaling networks, as evidenced by dephosphorylation of eIF2α and phosphorylation of S6K1. The pS6K1 effect confirms findings in vitro, where addition of a complete mix of amino acids to the media in which bovine mammary epithelial cells are cultured increases mTOR-dependent S6K1 phosphorylation on Thr389 several-fold (13,14). The 0.7-fold increase in mammary pS6K1 abundance in vivo is consistent with 0.3- to 1.4-fold increases observed in liver and skeletal muscle of young pigs fed or infused i.v. with insulin and amino acids after feed deprivation (26,37,38).

The effect of EAA+Glc on mammary p-eIF2 has little precedent. Phosphorylation of eIF2α has not been found to change with refeeding after feed deprivation in either liver or muscle (26,39). Glucose reduces p-eIF2 in yeast (40) and in some mammalian cells in vitro (41,42). Here, we show for the first time, to our knowledge, a prominent in vivo effect of EAA+Glc on mammary pS6K1 and p-eIF2.

Like EAA+Glc, infusion of His alone tended to influence both networks, whereas only mTOR targets were, or tended to be, stimulated by Leu and Met+Lys infusions, respectively. The ISR response to amino acid deficiency is mediated through the eIF2α kinase GCN2, which is activated by uncharged tRNA (10). The lack of effect of Met, Lys, and Leu infusions on p-eIF2 suggests that the charging of their respective tRNA was not affected by their infusion. On the other hand, tRNA charging was apparently increased by His infusion, suggesting that, of the EAA, His was uniquely in deficient supply for milk protein synthesis during the feed deprivation. Despite a greater p-eIF2 response than with EAA+Glc and 93% of the pS6K1 response, His infusion stimulated milk protein yield to only 41% of that with EAA+Glc. This indicates that additional factors beyond the ISR and mTOR networks were responsible for the milk protein stimulation of the EAA+Glc treatment.

The results of Leu and Met+Lys infusion lead to a similar conclusion. The mTOR-activating effect of Leu in vivo is well established in many splanchnic and peripheral tissues of the growing animal (16,43). In vitro, mammary epithelial cells respond to Leu with increased S6K1 phosphorylation and milk protein synthesis (13,15). Our findings in vivo confirm the effect of Leu on mammary S6K1, which was 7% higher than the EAA+Glc treatment, but milk protein yield was lower during the Leu infusion than during the EAA+Glc infusion. Similarly, protein synthesis rates did not increase in liver and cardiac tissue of feed-deprived rats given Leu per os, despite increases in S6K1 and 4EBP1 phosphorylation (16). Leu infusion into pigs stimulated muscle protein synthesis for 2 h until EAA concentrations in plasma fell to deficient levels (44). We observed no effect of Leu infusion on plasma amino acid concentrations and Leu has not previously stimulated milk protein yields (45,46).

Another difference between in vitro and in vivo responses of the mammary mTOR network is that His, Lys, and Thr were all inhibitory to S6K1 phosphorylation in mammary epithelial cells in vitro (15), whereas we found higher mammary S6K1 phosphorylation with His and Met+Lys. The in vitro effects were noted within 10 min of amino acid addition and in vivo effects were detected after 9 h of infusion. Moreover, Nicklin et al. (47) showed that glutamine could activate mTOR in HeLa cells by stimulating Leu uptake via a Gln exchange antipporter. Thus, differential effects of EAA administration on Leu transport into mammary cells in vitro and in vivo may explain the opposing effects on pS6K1.

The infusion of Glc alone did not significantly increase S6K1 or S6 phosphorylation but did reduce eIF2α phosphorylation and increase milk protein yield. This implicates regulation via the ISR network and not through mTOR, which is a novel finding for the control of milk protein synthesis. Insulin was elevated transiently between h 2 and 6 of infusion, which in muscle may be sufficient to activate mTOR signaling and protein synthesis (9,17,37), but it is well established that insulin does not acutely stimulate milk protein yield in ruminants (48,49). The absence of an mTOR response to Glc may have been due to the decline in plasma concentration of Leu, which we found to be an activator of mammary mTOR. However, the fact remains that milk protein yield was stimulated by glucose, independently of mTOR. Similarly, the stimulation of protein synthesis in glycolytic skeletal muscle of neonatal pigs by euaminoacidemic, eunisulnemic glucose infusion was not associated with phosphorylation of the mTOR targets S6K1 or 4EBP1 (17). Although
the signaling pathway responsible for the glucose effect in muscle was not identified, we show here that mammary peIF2 decreased 62% by Glc infusion alone.

The stimulatory effect of glucose on protein synthesis in pancreatic β-cells has also been attributed to a dephosphorylation of eIF2α (42,50). The PKR-like endoplasmic reticulum kinase (PERK) is activated by glucose deprivation to phosphorylate eIF2α (40,42). Inhibition of protein synthesis by PERK is required as part of the unfolded protein response in cells that secrete high volumes of protein, such as the pancreatic β-cell, to mitigate the severity of endoplasmic reticulum stress (51). Nonsecretory cells have little need of the PERK pathway. The mammary epithelial cell, in contrast, is highly active in protein secretion and may be expected to routinely use PERK-mediated phosphorylation of eIF2α as a translational control mechanism. Indeed, expression and activity of PERK in the mouse mammary gland is highest during lactation and perk deletion significantly reduced peIF2 abundance (12).

In conclusion, glucose and His infusions in feed-deprived cows generated responses in yields of milk components for which they are not substrates. The stimulation of milk protein yield by Glc did not involve an increase in circulating EAA concentrations and was accompanied by a decrease in BCAA in plasma. Insulin concentrations also rose transiently during Glc infusion. Glucose infusion did not significantly increase mammary S6K1 or S6 phosphorylation but did reduce eIF2α phosphorylation by 62%. The findings implicate stimulation of milk protein yield via the ISR network and not through mTOR. In contrast, the EAA infusions increased (Leu) or tended to increase (Met+Lys and His) mammary mTOR activity and only His, like Glc, decreased peIF2 abundance by 62%. The effect of EAA+Glc on mammary translation signaling was a combination of EAA-induced mTOR stimulation and Glc-induced peIF2 dephosphorylation. Despite stimulation of these signaling pathways to between 83 and 127% of the EAA+Glc response, EAA infusions produced less than one-half of the milk protein yield response generated by EAA+Glc, indicating that ISR and mTOR networks do not exert 100% of the control over protein yield. Part of the control may lie in the mass-action effect of higher EAA concentrations in plasma during EAA+Glc infusion.

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