Maternal protein homeostasis and milk protein synthesis during feeding and fasting in humans

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Sunehag, Agneta L., and Morey W. Haymond. Maternal protein homeostasis and milk protein synthesis during feeding and fasting in humans. Am J Physiol Endocrinol Metab 285: E420–E426, 2003. First published April 15, 2003; 10.1152/ajpendo.00080.2003.—Little is known about amino acid (AA) and protein metabolism in lactating women. We hypothesized: 1) AA sources other than the plasma amino acid pool provide substrate for milk protein synthesis in humans and 2) if albumin was one such source, then albumin fractional synthesis rate (FSR) is higher in the lactating women. To test these hypotheses, six healthy exclusively breast-feeding women [27 ± 3 yr; body mass index (BMI) 26 ± 2 kg/m²] between 6 wk and 3 mo postpartum and six healthy nonlactating women (28 ± 2 yr; BMI 22 ± 1 kg/m²) were studied two times, in random order, during 22 h fasting or 10 h of continuous feeding with a mixed nutrient drink. Protein metabolism was determined using [1-13C]leucine and [15N₂]urea. In both the fed and fasted states, a significant portion of milk protein (20 ± 5 and 31 ± 6%, respectively) was derived from sources other than the plasma free AA pool. A 70% higher (P < 0.02) FSR of albumin was observed in lactating women during feeding, suggesting that albumin is a likely source of AA for milk protein synthesis. We conclude that plasma free AA contribute only 70–80% of the substrate for milk protein synthesis in humans and that albumin may be a significant source of amino acids for the remainder.

The albumin fractional synthesis rate would be elevated in lactating women when compared with nonlactating women.

MATERIALS AND METHODS

Study Design

These investigations were carried out in conjunction with other studies previously reported (15, 16). The details of the protocol are provided to improve the clarity of the new data presentation.

Subjects

The study was approved by the Baylor College of Medicine Institutional Review Board for Human Research. After written informed consent, six lactating women and their infants were studied. The women were between 18 and 35 yr of age, in good health, and between 6 wk and 3 mo postpartum. The infants of these women were healthy and exclusively breastfed at the time of the study. Six healthy, nonpregnant, age-matched, nonlactating women were recruited as controls. The characteristics of the women are depicted in Table 1. All volunteers had a normal physical examination, normal Hb and screening studies for liver and renal function, and a negative pregnancy test before they were accepted in the study. Each lactating woman was provided Fe supplementation over the course of these studies, if she was not already taking them as part of her postpartum care.

Protocol Design

Each woman and her infant were admitted to the Metabolic Research Unit at the Children’s Nutrition Research Center or the General Clinical Research Center on the evening before study. At 1800 on the evening of admission, two intravenous catheters were introduced in the antecubital fossa or forearm vein under Emla (Astra Pharmaceuticals, Wayne, PA) cream analgesia, one for isotope infusion and the other in the contralateral arm for blood sampling. Subjects were fed a supper meal of 10 kcal/kg at 1800 and a small snack (5 kcal/kg each) at 2000 and were subsequently fasted except for water overnight. At 0600, baseline breast milk and blood samples (5 ml each) were obtained, and the subjects received an oral dose of 2H²O (0.1 mg/kg) to measure the equilibration of body and milk water. Within 3 h of the oral administration of the 2H₂O in both the fasting and fed state, the plasma and milk water enrichments of ²H were essentially identical and remained so throughout the study period.

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Table 1. Demographic data of the subjects studied (protocol 1)

<table>
<thead>
<tr>
<th>Age, yr</th>
<th>Weight, kg</th>
<th>BMI, kg/m²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactating women</td>
<td>27 ± 3</td>
<td>69 ± 5</td>
</tr>
<tr>
<td>Nonlactating women</td>
<td>28 ± 2</td>
<td>62 ± 4</td>
</tr>
</tbody>
</table>

Data are means ± SE. BMI, body mass index.

thus eliminating the possibility of any significant pools of preformed milk (15). In addition, they received primed-constant infusions of [2-13C]glycerol (30 μmol/kg, 2.0 μmol·kg⁻¹·min⁻¹ during feeding and 1.5 μmol·kg⁻¹·min⁻¹ during the fasting study) to measure rates of gluconeogenesis and lipolysis; [U-13C]glucose (20 μmol/kg, 0.33 μmol·kg⁻¹·min⁻¹) to measure total glucose rates of appearance; [1-13C]leucine (6.0 μmol/kg, 0.1 μmol·kg⁻¹·min⁻¹) to measure leucine rate of appearance (Rₐ); and [15N₂]urea (32 μmol/kg, 0.5 μmol·kg⁻¹·min⁻¹) to measure urea Rₑ, an indicator of protein oxidation. Carbon dioxide production and O₂ consumption were measured every 3 h. With the exception of the urea and leucine data, the results of these isotope studies have been published already (15, 16).

Blood samples (6–10 ml) were collected at 3-h intervals, and the infants were breast-fed at 3-h intervals. During one breast-feeding beginning at ~1500, maternal blood samples were obtained at 15-min intervals for 1 h. Infants were fed from both breasts at each nursing. Infants were weighed before and after each breast-feeding to determine the volume of milk consumed (9).

Each woman in both groups was studied on the following two occasions: one time during continuous feedings and one time during a brief fast. For the fed protocol, lactating women drank an equal volume of water at the same time during a brief fast. For the fed protocol, lactating women drank an equal volume of water at the same time during a brief fast. For the fed protocol, lactating women drank an equal volume of water at the same time during a brief fast. For the fed protocol, lactating women drank an equal volume of water at the same time during a brief fast. For the fed protocol, lactating women drank an equal volume of water at the same time during a brief fast. For the fed protocol, lactating women drank an equal volume of water at the same time during a brief fast. For the fed protocol, lactating women drank an equal volume of water at the same time during a brief fast. For the fed protocol, lactating women drank an equal volume of water at the same time during a brief fast. For the fed protocol, lactating women drank an equal volume of water at the same time during a brief fast. For the fed protocol, lactating women drank an equal volume of water at the same time during a brief fast.
chloride, and transferred to an autosampler vial (further dilutions, if required, were made with methylene chloride). The samples were injected on the GC-MS (GC: HP5890; MS: HP5989B) employing the electron-impact mode and using an HP 1701 (25 m × 0.25 mm × 0.25 μm) column with selected monitoring of the cluster of ions at m/z 155–155.

Milk analyses. Defatting milk. A homogeneous sample of milk (1.0 ml) was pipetted in microcentrifuge tubes (1.5 ml conical, screw-capped; CEL Associates, Houston, TX). The tubes were capped and placed upside down in a 20-ml scintillation vial and centrifuged for 10 min at 3,000 rpm at 4°C in a refrigerated centrifuge (model J-6B; Beckman Instruments, Palo Alto, CA). Without disturbing the layer of milk fat, the supernatant was decanted carefully in a new microcentrifuge tube.

DEPROTEINIZING MILK. To a 1.5-ml microcentrifuge tube, 50 μl defatted milk, 450 μl deionized water, 500 μl BaOH, and 500 μl ZnSO₄ were added. The tubes were capped tightly, vortexed, and centrifuged for 10 min at 3,000 rpm at 4°C. Protein precipitate was rinsed with 1.0 ml of deionized water to remove any contamination of free amino acids.

Milk protein hydrolysis. HCl (1.0 ml of 12 N) was added to the protein precipitate in a 4.0-ml screw-top vial and heated to 100°C for 24 h to hydrolyze the proteins. The solution was poured over a 1.0-ml column of Dowex 50W-X8 cation exchange resin; the resin was rinsed with 3.0 ml of water; and the amino acids eluted from the resin with 2.0 ml of NH₄OH. The resultant amino acid solution was dried, derivatized, and analyzed as described above.

Mathematical Models

All calculations of substrate turnover rates and product/precursor relationships were carried out under near-isotopic and substrate steady state, i.e., Ra and the rate of disappearance of labeled and unlabeled substrate are equal. The fraction of milk protein leucine derived from the plasma space was determined using classical product/precursor relations applying the following formula:

fraction milk protein–plasma amino acids = \( \frac{Leu_{\text{milk protein}}}{KIC_{\text{plasma}}} \)

where Leu \(_{\text{milk protein}}\) is the [\(^{13}\text{C}\)]leucine enrichment in the hydrolyzed milk proteins and KIC \(_{\text{plasma}}\) is the [\(^{13}\text{C}\)]KIC enrichment in plasma.

Ra of urea and leucine were calculated under near-steady-state conditions using standard equations:

\[ Ra = \left( \frac{E_i}{E_p} - 1 \right) \times I \]

where \( E_i \) and \( E_p \) are the enrichments of the isotope in the infused and the plasma, respectively, and \( I \) is the rate of infusion of the isotope of interest.

Leucine values (µmol·kg⁻¹·min⁻¹) were converted to protein (mg·kg⁻¹·min⁻¹) assuming the content of leucine in body proteins is ~8% (17). Thus the rate of proteolysis (mg·kg⁻¹·min⁻¹) in the postabsorptive state was estimated using the following equation:

\[ \text{proteolysis} = Ra_{\text{leucine}} \times 131/0.08 \]

where \( Ra_{\text{leucine}} \) is the rate of appearance of leucine, 131 is its molecular weight, and 0.08 is the fraction of body proteins composed of leucine (17).

During meal ingestion, the total rate of proteolysis (endogenous and exogenous) was calculated using the same formula. The endogenous proteolysis was calculated by subtracting the rate of entry of leucine from the consumed diet from the total leucine \( Ra \). The entry rate of exogenous leucine was calculated by multiplying the content of leucine per milliliter of Sustacal (as provided by the manufacture) times the rate of ingestion of Sustacal (ml·kg⁻¹·min⁻¹). The rate of protein synthesis was measured using the following equation:

\[ \text{protein synthesis} = \text{proteolysis} - \text{protein oxidation} \]

In the postabsorptive state, proteolysis is totally from endogenous sources. In the continuously fed state, proteolysis is endogenous proteolysis plus dietary protein intake.

The fractional rate of albumin synthesis was calculated by dividing the rate of increase of the \([^{13}\text{C}]\)leucine enrichment in the albumin pool by the plasma KIC enrichment (i.e., the slope of the rise of \([^{13}\text{C}]\)leucine enrichment in albumin over the course of the study) as we have described previously (3).

Statistical Analysis

The two overnight fasting (0600) values of substrate and hormone concentrations were averaged for each individual. These values were compared with those obtained during fasting and feeding from 1500 to 1800 (i.e., 19–22 h of fasting or 9–12 h of feeding, respectively). Plasma enrichments were averaged from 1500 to 1800, and \( Ra \) for the fed and fasted conditions were calculated from these values. All data are expressed as means ± SE. Data obtained during steady state, in both the fasting and fed protocols (i.e., study hours 9–12), were analyzed using repeated-measures ANOVA (SPSS for Windows version 11.5; SPSS). No interaction was found between diet (feeding/fasting) and lactation (+/-). Therefore, post hoc analyses were performed using paired Student’s \( t \)-test within each group, unpaired \( t \)-test between the two groups, or Wilcoxon’s signed rank test.

RESULTS

Plasma Substrate and Hormone Concentrations

After a 10-h overnight fast, the plasma concentrations of glucose, lactate, β-hydroxybutyrate, FFA, insulin, and glucagon were similar in the lactating and nonlactating women. After 12 additional hours of fasting (22 h total fast), plasma glucose and insulin decreased similarly in both groups (Table 2), whereas plasma β-hydroxybutyrate and FFA increased (\( P < 0.01 \) in both groups) as anticipated. The increases were similar in both groups of women. Plasma lactate and glucagon did not change in either group with fasting. After 18–22 h of study and 9–12 h of frequent feeding of Sustacal, plasma substrate and hormone concentrations were essentially the same in the lactating and nonlactating women (Table 2; see Ref. 16).

Urea Kinetics

After an overnight fast, the plasma urea concentrations were similar in the lactating and nonlactating women.

In the postabsorptive state, proteolysis is totally from endogenous sources. In the continuously fed state, proteolysis is endogenous proteolysis plus dietary protein intake.

The fractional rate of albumin synthesis was calculated by dividing the rate of increase of the \([^{13}\text{C}]\)leucine enrichment in the albumin pool by the plasma KIC enrichment (i.e., the slope of the rise of \([^{13}\text{C}]\)leucine enrichment in albumin over the course of the study) as we have described previously (3).
women. During fasting, the plasma urea decreased in both groups \( (P < 0.03) \). During the fed study, the plasma urea concentrations increased \( (P < 0.02) \) to a near plateau after 9 h. After both 19–22 h of fasting and 10–12 h of continuous feeding, plasma urea concentrations were higher \( (P < 0.01) \) in the lactating women compared with nonlactating women (fasting 12.0 ± 0.8 vs. 9.0 ± 0.7 mg/dl; fed 20.5 ± 1.3 vs. 15.6 ± 0.9 mg/dl).

Under near-steady-state conditions, the plasma enrichments (moles %excess enrichment [MPE]) of \(^{15}\text{N}_2\)urea were similar in lactating and nonlactating women in both the fasting (8.23 ± 1.0 vs. 9.6 ± 1.5 MPE, lactating vs. nonlactating women, respectively) and fed (4.5 ± 0.5 vs. 4.5 ± 0.6 MPE, respectively) conditions. As expected, the urea production rates were higher in the fed compared with the fasting conditions \( (P < 0.01) \) for both. There were, however, no differences between the two groups in either the fasting \( (0.298 ± 0.033 \text{ vs. } 0.263 ± 0.032 \text{ mg·kg}^{-1}·\text{min}^{-1}) \) or fed \( (0.536 ± 0.070 \text{ vs. } 0.476 ± 0.049) \) conditions despite the higher rate of protein ingestion in the lactating women.

**Leucine Ra**

The enrichments of plasma KIC were similar in both groups of women in both the fasted \( (5.3 ± 0.3 \text{ vs. } 5.3 ± 0.2 \text{ MPE}) \) and the lactating vs. nonlactating women, respectively, \( P = 0.92 \) and fed \( (3.1 ± 0.3 \text{ vs. } 3.6 ± 0.1 \text{ MPE}) \) \( P = 0.16 \) conditions. \( \text{Ra} \) of leucine was similar in the fasting condition \( (1.88 ± 0.09 \text{ vs. } 1.87 ± 0.07 \text{ µmol·kg}^{-1}·\text{min}^{-1}) \) in the lactating and nonlactating women, respectively, \( P = 0.49 \) and fed condition \( (3.25 ± 0.29 \text{ vs. } 2.78 ± 0.11 \text{ µmol·kg}^{-1}·\text{min}^{-1}) \) \( P = 0.18 \). When the actual rates of leucine ingestion in the fed condition \( (1.91 ± 0.17 \text{ vs. } 1.56 ± 0.09 \text{ µmol·kg}^{-1}·\text{min}^{-1}) \) in the lactating and nonlactating women, respectively) were subtracted from the total leucine \( \text{Ra} \), the endogenous rate of leucine \( \text{Ra} \) during the fed study was \( 1.34 ± 0.15 \text{ vs. } 1.22 ± 0.06 \text{ µmol·kg}^{-1}·\text{min}^{-1} \) in the lactating and nonlactating women, respectively \( P = 0.52 \).

**Whole Body Protein Balance**

During fasting, the rates of proteolysis, protein oxidation, and protein synthesis were similar in the lactating and nonlactating women (Fig. 1). During continuous feeding, as expected, the rate of total protein entry (from the diet and endogenous proteolysis) increased compared with the fasting values \( (P < 0.01 \text{ in both groups}) \). The rate of endogenous proteolysis decreased \( (P < 0.01) \) similarly in both groups. The calculated rates of whole protein synthesis increased \( (P < 0.01) \) in both groups during feeding compared with fasting. However, no differences were observed between the groups (Fig. 1). Net protein balance was negative and similar in the lactating and nonlactating women during the fasting study. During continuous feeding, net balance was positive and again similar in both groups of women (Fig. 1).

**Milk Volumes**

During the fast, the milk volume production was \( 84 ± 10 \text{ ml/feeding} \). During the Sustacal feeding, the milk volume was \( 133 ± 18 \text{ ml/feeding} \), which was higher than that observed during short-term fasting \( (P < 0.05) \).

**Fraction of Milk Protein Derived from the Plasma Amino Acid Pool**

The enrichments of plasma KIC and leucine and the enrichments of leucine in hydrolyzed milk proteins over the course of the study are depicted in Fig. 2. Assuming that the precursor enrichment of leucine in the mammary epithelial cell is similar to that of the plasma KIC pool \( (12) \), the fraction of milk protein derived from the plasma space increased over the course of infusion in both the fed and fasted conditions. In the fasting state \( 69 ± 6\% \) and in the fed state \( 82 ± 7\% \) came from the circulating leucine pool. Were we to use the plasma leucine enrichment, these values would be even less, \( 75 \text{ and } 59\% \) in the fed and fasted conditions, respectively. Conversely, \( 31 ± 6 \text{ and } 20 ± 5\% \) of the milk protein in the fasted and fed conditions, respectively, were derived from substrates other than the plasma free amino acid pool using the conservative estimate of the plasma KIC enrichment.
Fractional Synthesis of Albumin

In the fed condition, the fractional synthesis of albumin was higher ($P < 0.02$) in the lactating women compared with nonlactating women, whereas in the fasted condition the difference did not reach significance (Fig. 3).

DISCUSSION

The present studies, to our knowledge, are the first exploring the impact of lactation per se on maternal amino acid and protein metabolism and measuring the sources of amino acids for milk protein synthesis using stable isotope dilution methodologies. Despite some small differences in the plasma concentration of urea, lactation per se had little impact on maternal amino acid and protein metabolism. We have reported that plasma glucose was the primary but not only source of milk lactose (15). Similarly, we found that, over the course of 8–12 h of isotope infusion, the leucine enrichment in milk proteins was always less than that of the plasma leucine and KIC enrichments. These data...
strongly suggest that a portion of the amino acids for milk protein synthesis is derived from some source (presumably a protein source) other than the plasma free amino acid pool. In addition, we demonstrated a striking difference in the fractional rate of albumin synthesis in the fed study condition between the two groups of women, suggesting that the plasma albumin pool may, in fact, be a significant contributor to the unlabeled amino acid pool contributing to milk protein synthesis.

In the present studies, we applied a new model to the determination of protein metabolism in vivo. Traditionally, extrapolations of the rate of leucine entering body protein are made by subtracting the measured rate of leucine oxidation from the Ra of leucine (12). Because a primary end product of amino acid oxidation is the production of urea, we used the Ra of urea as a measure of amino acid oxidation (4, 5, 7), and the rate of proteolysis was extrapolated from the leucine Ra based on the content of leucine in human protein (17). This methodology has the advantage of using multiple 13C-labeled compounds in the same study or using either [1-13C]- or [5,5,5-2H3]leucine to measure its Ra (12) together with a urea tracer to measure urea production (an indicator of protein oxidation; see Ref. 7).

Because of the relatively small contribution (8–12 g/day) of maternal protein (or amino acid) metabolism (100–130 g/day) to milk protein production, it is not surprising that, with the tools utilized, we were unable to demonstrate significant differences between the lactating and nonlactating women with regard to proteolysis, protein oxidation, and protein synthesis.

To our knowledge, there are no known large intracellular pools of leucine or KIC in mammary tissue and no large pool of protein as that existing in muscle. Thus it would be logical to assume that the entire source of amino acids for milk protein synthesis would be derived from the plasma free amino acid pool. Lower leucine enrichments in milk protein, compared with plasma leucine or KIC enrichments, may be a result of a delay in the incorporation of labeled amino acids into milk proteins and their subsequent appearance in milk. However, we think this is unlikely because 1) we previously demonstrated that only a 3-h delay was required to achieve full isotopic equilibration of orally administered 2H2O in milk water, suggesting no real “hidden pools” of milk within the lacteals (15); 2) we utilized a 9-h isotope infusion during which the breast was emptied at least four times; and 3) because of the emptying of the milk contents from the breast with each feeding, the labeled proteins do not have to intermix with a large slowly turning over pool of previously synthesized proteins.

Were there another source of leucine for milk protein synthesis other than the plasma free amino acid pool, we believe that it would most likely be that of albumin. Albumin is a unique body protein. It is one of the largest single protein pools in the body; it is made exclusively in the liver and is used by nearly every tissue in the body (18). Because of the very low enrichment achieved in the plasma albumin pool compared with that of the leucine and KIC pools in this study, it would be impossible to demonstrate a direct transfer of labeled leucine from the plasma albumin to milk proteins. Were albumin to make a significant contribution to milk protein synthesis, we hypothesized that it would be used at a more rapid rate in lactating than nonlactating women. Thus, to maintain normal albumin concentrations, the fractional synthesis rate of albumin would have to be higher in the lactating women. This was, in fact, the case. Although the FSR was higher in the lactating women under short-term fasting conditions, the difference did not reach significance with the relatively small number of women studied. However, during feeding, the FSR of albumin in the lactating women was nearly 70% greater than that of the nonlactating women. Further study will be required to determine the precise role of albumin in the synthesis of milk proteins.

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DISCLOSURES

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