Lactase phlorhizin hydrolase turnover *in vivo* in water-fed and colostrum-fed newborn pigs

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We have estimated the synthesis rates *in vivo* of precursor and brush-border (BB) polypeptides of lactase phlorhizin hydrolase (LPH) in newborn pigs fed with water or colostrum for 24 h *post partum*. At the end of the feeding period, piglets were anaesthetized and infused intravenously for 3 h with L-[4-³H]-phenylalanine. Blood and jejunal samples were collected at timed intervals. The precursor and BB forms of LPH were isolated from jejunal mucosa by immunoprecipitation followed by SDS/PAGE, and their specific radioactivity in Phe determined. The kinetics of precursor and BB LPH labelling were analysed by using a linear compartmental model. Immuno-isolated LPH protein consisted of five polypeptides [high-mannose LPH precursor (proLPH_h), complex glycosylated LPH precursor (proLPH_c), intermediate complex glycosylated LPH precursor (proLPH_i) and two forms of BB LPH]. The fractional

synthesis rate (K_s) of proLPH_h and proLPH_c (approx. 5%/min) were the same in the two groups but the absolute synthesis rate (in arbitrary units, min⁻¹) of proLPH_h in the colostrum-fed animals was twice that of the water-fed animals. The K_s values of proLPH_i polypeptides were significantly different (water-fed, 3.89%/min; colostrum-fed, 1.6%/min), but the absolute synthesis rates did not differ. The K_s of BB LPH was not different between experimental treatment groups (on average 0.037%/min). However, the proportion of newly synthesized proLPH_h processed to BB LPH was 48% lower in colostrum-fed than in water-fed animals. We conclude that in neonatal pigs, the ingestion of colostrum stimulates the synthesis of proLPH_h but, at least temporarily, disrupts the processing of proLPH polypeptides to the BB enzyme.

INTRODUCTION

Lactase phlorhizin hydrolase (LPH) is the predominant disaccharidase in the jejunal brush border (BB) membrane of newborn mammals and is responsible for the hydrolysis of lactose to glucose and galactose [1,2]. Thus the synthesis and abundance of the enzyme are vitally important to the wellbeing of newborn animals.

LPH expression in the BB membrane is the end result of a complex series of transcriptional, translational and post-translational events. Although the initiation of LPH gene expression is regulated by the animal's stage of development, and in most mammals the mRNA is first expressed during the later stages of gestation, the protein is extensively modified after translation and before insertion into the BB membrane [3]. Multiple precursor (proLPH) polypeptides, differing as to type and degree of glycosylation, have been identified [4,5]. In addition, insertion of the enzyme into the BB membrane involves the proteolytic cleavage of the final complex glycosylated precursor protein to a BB enzyme of lower molecular mass [4,5]. There is also evidence for a dimeric form of the BB enzyme [6]. Thus although transcriptional events initiate enzyme expression, the complex post-translational pathway that leads to the final insertion of enzymically active LPH into the BB membrane offers other possible mechanisms for controlling the level of expression of the mature BB protein. These include the potential for dietary modification of post-translational processing.

Valid measurement *in vivo* of BB LPH post-translational processing in the intestinal mucosa is difficult owing to the continuous fluctuation of the free amino acid pools from which the enzyme is formed. These amino acid pools can be derived from arterial circulation, from intracellular proteolysis, and from luminal–serosal transport. Thus systemically administered amino acids entering the mucosal free amino acid pools might be substantially diluted by amino acids from the intestinal lumen and from intracellular proteolysis. In the newborn suckled pig (our best model for human intestinal development), the problems of measuring intestinal LPH synthesis *in vivo* are further compounded because the intestinal enterocyte rapidly endocytoses large quantities of intact protein, particularly IgG, from colostrum, thereby further diluting the tissue free amino acid pool [7,8]. As a result of these problems, the synthesis rates of BB hydrolases have generally been determined from the study of intestinal explants or tissue culture cell lines [4–6,9–11]. In fact, the fractional synthesis rates (K_s) of LPH polypeptides *in vivo* in pigs have never been measured.

Colostrum is a rich source of organic nutrients for the newborn mammal. It contains high concentrations of growth factors and hormones generally presumed to contribute to the growth, and perhaps maturity, of the neonatal small intestine [12,13]. Pig colostrum contains a particularly high concentration of maternal immunoglobulins, especially IgG, and the ingestion and endocytosis of these proteins is vital to the acquisition of passive immunity. We have found that feeding colostrum to newborn

Abbreviations used: BB, brush border; LPH, lactase phlorhizin hydrolase; K_s , fractional synthesis rate; proLPH_c, complex glycosylated LPH precursor; proLPH_h, high-mannose LPH precursor; proLPH_i, intermediate complex glycosylated LPH precursor; SA, specific activity; SR, specific radioactivity.

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pigs rapidly stimulates small-intestinal protein synthesis [14]. However, we have also found that feeding newborn pigs with colostrum, instead of water or milk, lowered the specific activity (SA) of lactase in the jejunal mucosa; the change in SA was associated with an increase in the relative amount of LPH precursor polypeptides [15]. This latter observation led us to hypothesize that ingestion of colostrum alters some aspect of LPH precursor post-translational processing. Here we report the results of a study in which, using isotopic labelling followed by compartmental analysis of the labelling kinetics, we measured for the first time the K_s values of precursor LPH polypeptides *in vivo* as well as that of the mature BB enzyme in the mucosa of 24 h old piglets fed with water or colostrum.

MATERIALS AND METHODS

Materials

L-[4-³H]Phenylalanine was purchased from Amersham (Arlington Heights, IL, U.S.A.), and endoglycosidase H (Endo H) from DuPont-New England Nuclear (Boston, MA, U.S.A.). Dulbecco's PBS was obtained from Gibco (Grand Island, NY, U.S.A.), and leupeptin, aprotinin and antipain were obtained from Sigma (St. Louis, MO, U.S.A.). Ultrapure hydrochloric acid (12 M) was purchased from J. T. Baker Chemical Co (Phillipsburg, NJ, U.S.A.). HPLC diluent was obtained from Pickering Laboratories (Mountain View, CA, U.S.A.), and poly(vinylidene difluoride)/ProBlott membranes were obtained from Applied Biosystems (Foster City, CA, U.S.A.). Pig colostrum was obtained from Texas A&M University (College Station, TX, U.S.A.). All other chemicals were of the highest analytical grade. All aqueous solutions were prepared with deionized water (Millipore Co., Bedford, MA, U.S.A.).

Infusion and tissue preparation

The protocol for these studies was approved by the Animal Care and Use Committee of Baylor College of Medicine and was performed in accordance with the Guide for the Care and Use of Laboratory Animals [16].

Five pregnant sows were obtained from the colony of standard commercial pigs at Texas A&M University. At delivery two weight-matched piglets were removed from each sow and placed in a warm room (90–95 °C). One animal from each litter was fed with water and the other was fed with pig colostrum *ad libitum* for 24 h. Thus there were five animals per experimental treatment group.

At the start of the infusion study each piglet was anaesthetized with isoflurane and mechanically ventilated via endotracheal tube. Blood gases were monitored at 10 min intervals to ascertain that pH, CO₂ and O₂ remained within the physiological range. Two sterile poly(vinyl chloride) catheters (Argyle, Sherwood Medical, St. Louis, MO, U.S.A.) were then inserted into the abdominal aorta via the umbilical arteries. The catheter used for blood collection was inserted 15–16 cm. The catheter used for isotope infusion was inserted 10 cm and was therefore downstream from the sampling catheter. A laparotomy was performed to expose the jejunum, and the animal was given a primed continuous infusion of 1.3 mCi/kg of L-[4-³H]Phe [specific radioactivity (SR) 1.11 TBq/mmol] during the first minute and 1.3 mCi/h per kg for the next 3 h. At 10, 20, 30, 60, 90, 120 and 180 min after the start of the infusion, blood samples were collected and 8 cm sections of the jejunum were surgically excised beginning 90 cm distal to the peritoneal reflection (analogous to the ligament of Treitz), taking particular care not to disturb the blood supply to the remaining intestinal tissue. A 0.5 cm segment

from each jejunal section was preserved for histological analysis [17]. The remaining jejunal tissue was flushed with 30 ml of cold 154 mM NaCl. The mucosa was then scraped and placed in 10 ml of Dulbecco's PBS containing protease inhibitors, homogenized for 2 min with a Tekmar Tissumizer at a 70 % setting, and frozen at –70 °C [18].

Histological analysis

The jejunal samples were processed and stained with haematoxylin and eosin as previously described [17].

Measurement of the specific activity of lactase

Lactase SA was determined in homogenates of scraped mucosa as previously described [17,19]. Results were calculated and expressed as described below.

Preparation of free amino acid pools from blood and mucosa

Samples of whole blood (200 µl) and the homogenized mucosa (approx. 50 mg/ml) were mixed with an equal volume of 1 M HClO₄. The samples were held on ice for 30 min and centrifuged at 12000 *g* for 20 min to separate precipitated protein. The protein pellet was used to measure the SR of total mucosal protein (see below). The tissue supernatants, after protein removal, were prepared for amino acid analysis as previously described [18].

Hydrolysis of mucosal protein

The precipitated mucosal protein was suspended in water, sonicated with a microprobe (Heat Systems, Farmingdale, NY, U.S.A.) to disperse the pellet and centrifuged at 12000 *g* for 30 min. This procedure was repeated three times and the final pellet was suspended in 12 M HCl. Hydrolysis was performed as described below for LPH polypeptides.

Purification and immobilization of monoclonal antibody

Ascites cells were prepared by Charles River Laboratories (Wilmington, DE, U.S.A.) by using the hybridoma PBB 3/7/3/2 [20]. The IgG in the ascites fluid was purified and bound to divinylsulphone-linked Sepharose-6B as previously described [6].

Immunoisolation of precursor and BB LPH protein

The immunoisolation of LPH polypeptides has been described by us and others [4–6,9]. Briefly, homogenized mucosa was centrifuged at 114000 *g* to separate membrane-bound protein. The protein pellets were homogenized in 25 mM sodium phosphate buffer, pH 8.0, containing 1 % (v/v) Triton X-100 and a mixture of protease inhibitors. LPH was immunoprecipitated from the solubilized membranes by incubating them for 4 h at 22 °C with the immobilized antibody-bead suspension. To demonstrate that the LPH polypeptides seen after SDS/PAGE were not derived from colostrum, triplicate samples of colostrum having the same protein concentration as the mucosal membrane protein solution were likewise reacted with the antibody-bead suspension. Protein was eluted from the beads with 2 % (w/v) SDS sample buffer containing 5 % (v/v) 2-mercaptoethanol used for SDS/PAGE. The bead suspension was heated at 95 °C for 4 min and the beads were removed by centrifugation. For one study the disaccharidases were isolated as usual and the immuno-precipitate-bead complex was used for Endo H digestions [6,21].

Purification and hydrolysis of LPH polypeptides

LPH polypeptides were purified by SDS/PAGE on 5.0 % (w/v) polyacrylamide slab gels 1.5 mm thick [6,18,22]. Electrophoresis was performed at 40 mA with variable voltage for 4 h. The gels were stained overnight with 0.02 % Coomassie Blue R250 and destained over a 24 h period with five changes of 7 % (v/v) acetic acid. Five Coomassie Blue-stained bands representing precursor and BB forms of LPH were seen on the gels. The gels were scanned in an LKB Ultrascan XL Laser densitometer with GelScan XL software (LKB, Broma, Sweden) to estimate the amount of each LPH polypeptide relative to the total amount of LPH immunoprecipitable protein on the gel [6]. Some gels were then dried and used for fluorography; others were used for measurement of the SR of individual LPH proteins [6]. For the latter purpose the five LPH polypeptides (molecular masses 160, 180, 200, 220 and 240 kDa) were cut from the gel. The gel slices corresponding to an individual protein band were dried, hydrolysed in 12 M HCl for 24 h at 110 °C and the hydrolysate dried under N₂ [18]. Water was then added to each tube and residual gel was removed by centrifugation. The amino acid solution was dried by centrifugation under vacuum, dissolved in 1 M acetic acid and applied to a 1.25 ml cation-exchange column (Dowex AG50W-X8). The samples were eluted with 3 M NH₄OH (recovery more than 95 %), dried under N₂, suspended in water and filtered through a 0.45 µm syringe filter.

Amino acid analysis

The Phe in each specimen was separated from other amino acids by HPLC with an anion-exchange column (AminoPac PA1 column; Dionex, Sunnyvale, CA, U.S.A.) as previously described [6,18]. Samples were eluted with 20 mM NaOH and 80 mM sodium acetate at a flow rate of 1 ml/min. The amino acids, as they emerged from the column, were reacted with 0.05 % *o*-phthalaldehyde in Pickering diluent containing 0.06 % Brij 35 and 2 % 2-mercaptoethanol and detected by an on-line fluorimeter. Quantitative measurements were obtained by comparison with a Phe standard of known concentration. Separated amino acids were collected on a Gilson 201 fraction collector (Gilson Instruments, Middleton, WI, U.S.A.), and the appropriate fractions were counted on a Beckman LS3801 scintillation counter (Beckman Instruments, Fullerton, CA, U.S.A.) with external standard channels ratio quench correction. SR is expressed as d.p.m. per nmol of Phe.

N-terminal amino acid analysis of LPH polypeptides

N-terminal amino acid analysis of LPH polypeptides was performed as previously described [6]. Briefly, after electrophoresis, gels were equilibrated in chilled transfer buffer [25 mM Tris/HCl (pH 8.3)/192 mM glycine/0.01 % SDS/5 % (v/v) methanol] for 10 min. At the same time, a poly(vinylidene difluoride)/ProBlott membrane was wetted with methanol and equilibrated in transfer buffer for 10 min. The gels were then electroblotted for 3 h at 4 °C in a Bio-Rad Transblott Cell at 75 V. After transfer, the membranes were washed with water, rinsed with methanol and stained for 2 min in 0.1 % Coomassie Blue R-250 in 40 % methanol/1 % acetic acid. The stained membranes were washed in water, destained in 50 % methanol and air-dried. Individual protein bands were cut from the membrane and analysed by automated Edman degradation sequencing on a pulsed-liquid sequencer (Model 473A; Applied Biosystems) with on-line microgradient phenyl isothiocyanate analysis.

Calculations

SA of lactase

Lactase SA was measured as µmol of glucose/min per mg of protein. To account for the endocytosed protein consistently observed within the intestinal enterocytes of newborn animals fed with colostrum [8,23,24], the SA was converted to nmol of glucose/min per g of mucosa by multiplying by the protein concentration in each mucosal sample. This value was then used in the subsequent calculation of the absolute amount (arbitrary units) of each LPH polypeptide (see below).

Fractional synthesis rate (K_s) of total mucosal protein

The K_s (%/day) of mucosal protein was calculated as follows [18]:

$$K_s = 100 \times (\Delta S_b / S_i) \times (1440 / T) \quad (1)$$

in which ΔS_b is the difference in Phe SR in mucosal protein samples taken after 10 and 20 min of infusion, S_i is the average free Phe SR in the mucosa over this time period, and T is time expressed in days.

In the colostrum-fed animals, the standard method for calculating K_s gives a misleading value because endocytosed protein significantly dilutes the protein-bound isotopic enrichment [8]. On the assumption that the difference in protein concentration between mucosal samples from water- and colostrum-fed piglets represented the contribution of colostrum protein, we corrected the K_s in the colostrum-fed animals by multiplying it by the ratio of the mucosal protein concentration in the colostrum-fed animals to the mucosal protein concentration in the water-fed animals. This in effect calculates the total Phe incorporated per g of mucosa and is equivalent to the parallel correction of lactase SA (see above).

Total amounts of precursor and BB LPH polypeptides

Lactase SA (nmol of glucose/min per g of mucosa) was divided by the relative amount of BB LPH (i.e. the proportional contribution of the Coomassie Blue-stained 160 kDa band relative to the total amount of all Coomassie Blue-stained LPH polypeptides obtained by gel scanning; see Table 1). This gives, in arbitrary units, the total amount of LPH protein (precursors plus BB LPH protein) in 1 g of mucosa. This calculation of total abundance makes the assumption that lactase SA is attributable only to the mature BB form of the enzyme. Naim et al. [10] have reported that in COS-1 cells transfected with the human cDNA for LPH, a precursor form of the enzyme seems to be enzymically active. However, in those cells the mature BB (160 kDa) form of the enzyme seen *in vivo* is not synthesized, and a precursor form of LPH seems to be expressed at the cell surface instead. Thus until it has been conclusively proved that the precursor polypeptides in the pig are enzymically active, it seems reasonable to omit these proteins from all calculations of total abundance. More importantly for this study, the small difference in the relative amount of BB LPH between the two experimental treatment groups (less than 7 %) is not sufficient to alter the calculations of BB LPH absolute synthesis rates significantly (see below).

For each animal the total amount of LPH protein in 1 g of mucosa was then multiplied by the relative amount of each precursor and BB polypeptide to yield the total amount of individual LPH polypeptides in 1 g of mucosa. These values were used in the calculation of absolute synthesis rates (see below).

Figure 1 Compartmental model used to calculate the kinetics of LPH polypeptide synthesis

LPH synthesis

For fractional synthesis rates of LPH polypeptides, the kinetics of LPH polypeptide synthesis were calculated with a linear compartmental model (Figure 1) originally derived from results obtained in mature rats [6]. The calculations involved the solution of a set of equations of the general form:

$$SR_t/SR_\infty = A - A[e^{-K_s(t-d)}] \quad (2)$$

in which SR_t is the SR (d.p.m./nmol) of Phe in any given LPH protein at time t (min), SR_∞ is the steady-state SR of its immediate precursor, A is the ratio of the steady-state product SR/steady-state precursor SR, K_s is the fractional synthesis rate (%/min), t is the time of infusion (min) and d is a delay time (min) before the first appearance of label in the product. For $proLPH_h$ (high-mannose LPH precursor), A equalled the measured ratio of SR $proLPH_h$ /SR mucosal free amino acid pool at the plateau. For $proLPH_c$ (complex glycosylated LPH precursor), $proLPH_i$ (intermediate complex glycosylated LPH precursor) and BB LPH, A equalled 1. The measured steady-state SR of $proLPH_h$ was therefore used in the calculation of $proLPH_c$, and that of $proLPH_c$ was used to calculate $proLPH_i$ synthesis.

To confirm the validity of all estimates, SR_t was also calculated from the equation:

$$SR_t = SR_{\text{plateau}}[e^{-K_s(t-d)}] \quad (3)$$

and compared with the measured value for SR_t . In this equation SR_{plateau} is the measured steady-state SR of each precursor. This allows the fit to be estimated from the linear regression of measured SR_t against the predicted value. In all cases the square of the regression coefficient (r^2) of the fit was more than 95%. All equations were solved with the Levenberg-Marquardt least-squares nonlinear regression algorithm [25] by using Origin 3.5 (MicroCal Software, Northampton, MA, U.S.A.) with K_s and d as the independent variables.

For each animal, the absolute synthesis rate of each LPH polypeptide (measured in arbitrary units of min^{-1}) was calculated as the product of the K_s and the total amount of each protein (above). If the amount (mass) of each LPH protein could have been measured in g, this would have been equivalent to label flow. However, even though the amount was not measured in g, the absolute synthesis rate allows the calculation of the proportion of a given protein processed to the next member of the pathway (i.e. the efficiency of post-translation processing) from the equation [efficiency of post-translational processing (%)] = $100 \times (\text{absolute synthesis rate of precursor})/(\text{absolute synthesis rate of product})$. It is important to note that there are proteolytic

steps between $proLPH_c$ and $proLPH_i$ and between $proLPH_i$ and BB LPH that result in a loss of peptide mass. Failure to account for this loss would lead to an underestimate of the efficiency of post-translational processing. To account for this loss in the calculation of post-translational processing efficiency, the absolute synthesis rate of $proLPH_i$ was multiplied by 200/180 and that of BB LPH by 200/160 (i.e. by relative molecular masses) before being compared with the absolute synthesis of LPH_h and LPH_c . No adjustment for differences in the mass of $proLPH_h$ and $proLPH_c$ was necessary because the difference in the mass of these polypeptides results from their degree of glycosylation.

Statistics

Unless otherwise specified, results are presented as means $\pm$ S.E.M. Results were subjected to one-way analysis of variance. P values of less than 0.05 were considered statistically significant.

RESULTS

Histology

In both experimental treatment groups the enterocytes were columnar and displayed a characteristic polarized organization. Interstitial cells were limited in number. As previously reported [8,14,15], numerous IgG-filled granules were seen in the cytoplasm of colostrum-fed but not water-fed animals. Within an experimental treatment group, no morphological differences were seen between the jejunal samples obtained after 10 min of infusion and those obtained after 180 min of infusion.

Specific activity of LPH

The values for mucosal protein and lactase SA are shown in Table 1. The mucosal protein concentration/g of mucosa was significantly higher in the colostrum-fed than in water-fed piglets. Lactase SA was lower in the colostrum-fed than in water-fed animals but the differences were not significant.

Purification, abundance and labelling of LPH polypeptides

In both experimental treatment groups, electrophoresis separated immunoprecipitated LPH protein into five bands (molecular masses approx 160, 180, 200, 220 and 240 kDa) as identified by Coomassie Blue staining. Immunolocalization of protein in colostrum samples revealed no bands with a molecular mass over 80 kDa (presumed lactoferrin). The identity of the 160, 200 and 220 kDa bands have been previously described by us and others

Table 1 Protein content, lactase specific activity and the relative amounts of LPH polypeptides in the jejunal mucosa of water-fed or colostrum-fed newborn piglets

* $P < 0.05$, water-fed compared with colostrum-fed.

	Water-fed animals ($n = 5$)	Colostrum-fed animals ($n = 5$)
Protein (mg/g of mucosa)	94.1 $\pm$ 1.71	175.9 $\pm$ 10.3*
SA of lactase (nmol/min per g of mucosa)	12230 $\pm$ 2100	8970 $\pm$ 1800
Relative amounts of polypeptide (% of total amount of LPH polypeptides):		
200 kDa ($proLPH_h$)	1.2 $\pm$ 0.1	3.5 $\pm$ 0.2*
220 kDa ($proLPH_c$)	0.6 $\pm$ 0.1	1.1 $\pm$ 0.1*
180 kDa ($proLPH_i$)	0.8 $\pm$ 0.16	4.8 $\pm$ 1.3*
160 kDa (BB LPH)	97.3 $\pm$ 0.4	90.7 $\pm$ 1.3*

Figure 2 Fluorogram of [^3H]-Phe incorporation into LPH polypeptides in colostrum-fed animals over time

[4,22] and are discussed below. For clarity, we shall refer below to the 160 kDa protein as BB LPH, to the 200 kDa protein as proLPH_h, and to the 220 kDa protein as proLPH_c. For reasons also discussed below, we believe that the 180 kDa band is a precursor of BB LPH and we therefore call it proLPH_i.

The relative amounts of proLPH_h, proLPH_c and proLPH_i were approx. 3-fold, 1.8-fold and 6-fold higher respectively in colostrum-fed than in water-fed animals (Table 1). In addition, because these measurements are of relative amounts (percentages of the total amount), the relative amount of BB LPH was, of necessity, lower in the colostrum-fed than in the water-fed animals.

As determined by fluorography, the sequence of appearance of radiolabel in the LPH polypeptides was the same for both experimental treatment groups. A representative fluorogram is seen in Figure 2. After 60 min of infusion, radioactivity was clearly seen in proLPH_h and was just detectable in proLPH_c. After 120 min of infusion the radioactivity of the proLPH_c had intensified and radioactivity had appeared in proLPH_i. After 180 min of infusion, the radioactivity of proLPH_i had increased and radioactivity had appeared in BB LPH. Although the SR of the 240 kDa band paralleled BB LPH (see below), because it was present in small amounts the 240 kDa band was not visible by fluorography. Treatment of immunoprecipitated LPH with endoglycosidase H demonstrated that the molecular mass of proLPH_h was decreased by the enzyme but that the molecular masses of proLPH_c, proLPH_i and BB LPH were not affected (Figure 3).

The results of N-terminal amino acid sequencing are shown in Table 2: 30 N-terminal amino acids were identified for the BB LPH, 13 for proLPH_h, 8 for proLPH_c, 11 for proLPH_i, and 21 for the 240 kDa polypeptide. All signals seemed to be homogeneous, i.e. uncontaminated by minor proteins. The N-terminal amino acids of proLPH_h were homologous with proLPH_c, but

Table 2 N-terminal amino acid sequence of LPH polypeptides

The initial yields were: 8.4 pmol (200 kDa protein), 3 pmol (220 kDa protein), 2.3 pmol (180 kDa protein), 24.6 pmol (160 kDa protein) and 8.6 pmol (240 kDa protein); repetitive yields were: 96.4% (200 kDa protein), 94.1% (220 kDa protein), 93.6% (180 kDa protein), 93.4% (160 kDa protein) and 96.2% (240 kDa protein). As reported by Mantei et al. [26] for the rabbit, the N-terminal sequences for proLPH_h and proLPH_c start at position 1 of the precursor molecule, for proLPH_i at position 610, and for BB LPH at position 1 of the mature, BB enzyme.

Residue no.	ProLPH _h (200 kDa)	ProLPH _c (220 kDa)	ProLPH _i (180 kDa)	BB LPH (160 kDa)	BB LPH (240 kDa)
1	Ser	Ser	Asp/Ser	Ala	Ala
2	Asp	Asp	Pro	Phe	Phe
3			Glu	Ala	Ala
4	Glu	Glu	Thr	Phe	Phe
5			Ala	Pro	Pro
6	Asp	Asp	Val	Ser	
7	Arg	Arg	Asp	Glu	Glu
8	Asn	Asn	Leu	Val	Val
9	Phe	Phe	Pro	Pro	Pro
10	Ile	Ile	Pro	Ser	Ser
11	Leu		Ser	Lys	
12	Ala			Ala	Ala
13	Ala			Lys	Lys
14	Gly			Val	Val
15	Pro			Val	Val
16				Trp	Trp
17				Glu	Glu
18				Lys	Lys
19				Phe	Phe
20				Ser	Ser
21				Asn	Asn
22				Gln	Gln
23				Pro	Pro
24				Lys	
25				Phe	
26				Glu	
27				Arg	
28				Asp	
29				Leu	
30				Phe	

shared no homology and little identity with the N-terminal amino acids of proLPH_i, BB LPH or the 240 kDa polypeptide. The N-terminal amino acids of proLPH_i were not homologous with the N-terminal amino acids of other LPH proteins. The 160 kDa polypeptide (BB LPH) and the 240 kDa polypeptide had identical N-termini.

Free amino acid pools in blood and mucosa

In both experimental treatment groups, the SR of the blood free amino acid pool rose rapidly and reached a plateau between 30 and 60 min of infusion. Between 60 and 180 min the SR of the blood free amino acid pool in both experimental treatment groups was at isotopic steady state (coefficient of variation 5% for water-fed and 6% for colostrum-fed animals; Table 3). However, the values were significantly higher in the water-fed than in the colostrum-fed animals.

In all animals, the SR values of the mucosal free amino acid pools reached isotopic steady state within 60 min and remained at a plateau for the remainder of the infusion (coefficients of variation 12% for water-fed and 5% for colostrum-fed animals; Table 3). Again, the SR values for the mucosal free amino acid pools were significantly higher in the water-fed than in the

Figure 3 Fluorogram of LPH polypeptides before (–) and after (+) treatment with endoglycosidase H

Table 3 Steady-state SR values of Phe in blood and mucosal pools of free amino acids and LPH precursor polypeptides in water-fed or colostrum-fed newborn pigletsValues shown are means $\pm$ S.E.M. * $P < 0.05$, water-fed compared with colostrum-fed.

	SR (d.p.m./nmol)	
	Water-fed animals	Colostrum-fed animals
Arterial blood (after 60 min)	18685 $\pm$ 974	8370 $\pm$ 261*
Gut mucosa (after 60 min)	9067 $\pm$ 679	3621 $\pm$ 153*
LPH _h (200 kDa) (after 60 min)	4474 $\pm$ 305	3093 $\pm$ 107*
LPH _c (220 kDa) (after 90 min)	3964 $\pm$ 374	2791 $\pm$ 115*
LPH _i (180 kDa) (terminal sample)	3615 $\pm$ 361	2645 $\pm$ 229*
Mucosa/blood (%)	50 $\pm$ 4	42 $\pm$ 3
LPH _h /mucosa (%)	48 $\pm$ 3	87 $\pm$ 3*
LPH _c /LPH _h (%)	85 $\pm$ 18	88 $\pm$ 17
LPH _i /LPH _c (%)	103 $\pm$ 5	95 $\pm$ 6

Figure 4 Time course of labelling of proLPH_h (●), proLPH_c (○), proLPH_i (▲) and BB LPH (values multiplied by 10 before plotting) (Δ) in water-fed animalsSymbols represent measured experimental values (means $\pm$ S.D.). Lines represent values calculated from the compartmental model.

colostrum-fed animals. However, the ratios of the isotopic enrichment of the mucosal to the blood amino acid free pools were not significantly different between experimental treatment groups.

Fractional synthesis rates of mucosal protein

The K_s for mucosal protein of the water-fed animals was 64 ± 21 %/day. After correction for exogenous protein from colostrum, the K_s in the colostrum-fed animals was significantly higher (121 ± 21 %/day).

Synthesis of LPH polypeptides

Figures 4 and 5 show the time course of labelling of proLPH_h, proLPH_c, proLPH_i and BB LPH for both experimental treatment groups (symbols in Figures 4 and 5 represent actual experimental values). Regardless of experimental treatment, the SR of proLPH_h rose rapidly during the first 30 min of infusion and reached an isotopic steady state by 60 min. At steady state, the

Figure 5 Time course of labelling of proLPH_h (●), proLPH_c (○), proLPH_i (▲) and BB LPH (values multiplied by 10 before plotting) (Δ) in colostrum-fed animalsSymbols represent measured experimental values (means $\pm$ S.D.). Lines represent values calculated from the compartmental model.**Table 4** Kinetics of LPH polypeptide synthesis in the jejunal mucosa of water-fed or colostrum-fed newborn piglets K_s is the fractional synthesis rate. * $P < 0.05$, water-fed compared with colostrum-fed.

	Water-fed ($n = 5$)		Colostrum-fed ($n = 5$)	
	Delay (min)	K_s (%/min)	Delay (min)	K_s (%/min)
ProLPH _h (200 kDa)	5.1 $\pm$ 0.9	5.0 $\pm$ 0.7	7.4 $\pm$ 0.6	4.9 $\pm$ 0.9
ProLPH _c (220 kDa)	19.2 $\pm$ 2.2	5.9 $\pm$ 0.8	24.9 $\pm$ 2.2	4.8 $\pm$ 0.8
ProLPH _i (180 kDa)	42.9 $\pm$ 4.8	4.0 $\pm$ 0.8	38.8 $\pm$ 2.8	1.6 $\pm$ 0.2*
BB LPH (160 kDa)	56.8 $\pm$ 8.5	0.031 $\pm$ 0.003	60.6 $\pm$ 4.6	0.042 $\pm$ 0.005

SR of proLPH_h was significantly higher in water-fed than in colostrum-fed animals (Table 3). The steady-state SR of proLPH_h in water-fed piglets was on average 24 ± 1.5 % (coefficient of variation 13 %) of blood free amino acid pools and 48 ± 3 % (coefficient of variation 13 %) of mucosal free amino acid pools. In the colostrum-fed piglets, the SR of proLPH_h was on average 37 ± 1.8 % (coefficient of variation 10 %) and 87 ± 3 % (coefficient of variation 8 %) of blood and mucosal free amino acid pools respectively.

The SR of proLPH_c reached isotopic steady state after 90 min of infusion in both treatment groups and again the steady-state values in the water-fed animals were significantly higher than in the colostrum-fed animals. In both treatment groups the steady-state SR values of proLPH_c were lower than, but not significantly different from, the steady-state SR of proLPH_h. The SR of proLPH_i reached a steady state after 120 min of infusion in the water-fed animals, and by 180 min in the colostrum-fed animals. Within an experimental treatment group, the steady-state SR values of proLPH_i and proLPH_c were the same, but the values in the water-fed animals were significantly higher than in the colostrum-fed animals (Table 3).

In both experimental treatment groups, the SR of BB LPH rose very slowly during the first 120 min of infusion and rapidly thereafter, but did not reach isotopic equilibrium. By 180 min the SR of BB LPH was 202 ± 22 d.p.m./nmol in the water-fed animals and 138 ± 13 d.p.m./nmol in the colostrum-fed animals.

Table 5 Total amount, absolute synthesis rates and post-translational processing efficiencies of LPH polypeptides in water-fed or colostrum-fed newborn piglets

Absolute synthesis rate is the product of total amount and K_s (min^{-1} ; Table 4). Post-translational processing efficiency is the ratio of absolute synthesis rates of product to precursor, expressed as a percentage. * $P < 0.05$, water-fed compared with colostrum-fed.

	Total amount (arbitrary units)		Absolute synthesis rates (arbitrary units)		Post-translational processing efficiency	
	Water-fed	Colostrum-fed	Water-fed	Colostrum-fed	Water-fed	Colostrum-fed
ProLPH _n (200 kDa)	149 ± 23	278 ± 54*	6.9 ± 1.6	12.8 ± 2.3*	72 ± 6	39 ± 12*
ProLPH _c (220 kDa)	82 ± 9	85 ± 13	4.8 ± 0.7	4.3 ± 1.1	90 ± 28	125 ± 19
ProLPH _i (180 kDa)	101 ± 20	386 ± 38*	3.7 ± 0.8	4.9 ± 1.0	102 ± 8	75 ± 12*
BB LPH (160 kDa)	12230 ± 2100	8870 ± 1800	3.8 ± 0.6	3.7 ± 0.4	62 ± 11	32 ± 6*

The SR of the 240 kDa polypeptide was the same as that of BB LPH in all animals (results not shown).

Fractional synthesis rates of LPH polypeptides

The system parameters for LPH synthesis for the two experimental treatment groups are shown in Table 4. Figures 4 and 5 show the lines generated from the equations for the delay times and fractional synthesis rates as determined with the compartmental model (symbols are the measured values). For any one animal, the fit had an r^2 value of more than 95%. Figures 4 and 5 also show qualitatively that the rate at which label equilibrated into proLPH_n and proLPH_c differed little between the two groups, and the formal calculations of the K_s values (Table 4) confirm this observation. The rate of label equilibration into proLPH_i was, however, significantly slower in the mucosa of colostrum-fed than water-fed piglets.

Absolute synthesis rates of LPH polypeptides

Table 5 shows the calculations of the absolute rate of synthesis and the efficiency of post-translational processing of each LPH polypeptide. The absolute synthesis rate of proLPH_n in the colostrum-fed piglets was double that in the water-fed piglets, whereas the absolute synthesis rates of other LPH polypeptides, including BB LPH, were not significantly different. The proportion of newly synthesized proLPH_n that was processed to proLPH_c was significantly lower in colostrum-fed than in water-fed animals. Although in both experimental treatment groups the proportion of proLPH_c processed to proLPH_i was not significantly different, the proportion of proLPH_i processed to BB LPH was again significantly lower in the colostrum-fed than in the water-fed animals. Overall, in the colostrum-fed piglets only 32% of the newly synthesized proLPH_n was processed to BB LPH.

DISCUSSION

The results from the present study represent the first reported estimates of the synthesis kinetics of both the precursor and BB forms of LPH *in vivo* in the pig. In addition, the results demonstrate that LPH synthesis can be modified by diet, in this case by feeding with colostrum, a rich source of organic nutrients generally thought to contribute to the newborn's growth and development.

We conclude from this study that proLPH_n synthesis is increased by feeding with colostrum, and at the same time the efficiency with which this LPH precursor is processed to the BB protein decreases. These conclusions are, however, dependent on

the relation between the protein bands seen in neonatal animals after SDS/PAGE of LPH immunisolated with highly specific monoclonal antibodies. The relation between the 200, 220 and 160 kDa bands has been established by pulse-chase studies *in vitro* [4,22]. However, the identities of the 180 and 240 kDa bands seen by SDS/PAGE have not been defined in this species by similar techniques.

In the present work, fluorography and direct SR measurements of samples obtained at accurately timed intervals during the isotope infusion confirmed the precursor-product relation between the immunisolated LPH polypeptides seen *in vitro*. Isotope appeared sequentially in the 200, 220, 180 and 160 kDa bands. Further, within a treatment group the SR values of the 200, 220 and 180 kDa proteins reached isotopic steady states that were not different from one another. Because the rate of rise of label incorporation into the 180 kDa band lagged approx. 20 min behind isotope incorporation into the 220 kDa polypeptide but was more rapid than isotope incorporation into 160 kDa protein (BB LPH), we believe it to be an intermediate in the pathway leading to BB LPH. The SR of the 240 kDa band paralleled, and was equal to, the SR of the 160 kDa band, and like the SR of the 160 kDa band did not reach a steady state during the infusion. The kinetics and magnitude of the isotopic labelling of this polypeptide led us to conclude that it is a dimer of BB LPH (160 kDa).

N-terminal amino acid sequencing further clarified the identity of each protein. The N-terminal amino acid sequence of the 200 and 220 kDa proteins, presumed to be proLPH_n and proLPH_c, were homogeneous and were 100% similar to one another. They were not similar to the N-terminal amino acid sequences of the 240, 180 or 160 kDa polypeptides. The N-terminal amino acid sequence of the 200 kDa protein was approx. 85% similar to the N-terminal amino acid sequence of proLPH_n in humans and rabbits, and 100% similar to proLPH_n in the rat [6,26]. The N-terminal amino acid sequences of the 160 and 240 kDa proteins were also homogeneous, were 100% similar to one another, and were 93%, 89% and 72% similar to the N-terminal amino acid sequences of BB LPH from humans, rabbits and rats [6,26]. Beginning with residue 610 of proLPH_n, the 11 N-terminal amino acids of proLPH_i were 55% similar to the amino acid sequence of the corresponding region of human proLPH [26]. As would be expected if the protein were an intermediate precursor synthesized from proLPH_c, the N-terminus of the 180 kDa protein had no similarity to the N-terminus of the other LPH polypeptides.

Digestion with endoglycosidase H demonstrated that only the 200 kDa polypeptide contained sufficient quantities of mannose to alter its migration by SDS/PAGE. Thus it seemed that this

protein was the first detectable LPH translation product and that the other polypeptides had undergone post-translational processing [6]. On the basis of SDS/PAGE migration, the steady-state SR, N-terminal amino acid sequencing and endoglycosidase H data, we concluded that the 200 kDa band is indeed pro-LPH_n, that the 220 kDa band is proLPH_c and that the 180 kDa band is a proteolytically cleaved intermediate between proLPH_c and the BB enzyme, which we term proLPH_i. We also concluded that, as in the rat [6], the 240 kDa band is a dimer of the BB polypeptide.

The infusion strategy for this study was specifically designed to bring the enrichment of the blood and tissue pools of free amino acids, but more importantly of proLPH_n, to isotopic steady state and to do so rapidly without perturbing the normal metabolic pools. At steady state the SR of the mucosal free amino acid pool was on average 46% of the blood pool of free amino acid regardless of experimental treatment, but the SR of proLPH_n at steady state was 48% and 87% (water-fed compared with colostrum-fed animals) of the SR of the mucosal free amino acid pool and was not what we should have predicted on the basis of the well-documented dilution effects of intestinal luminal amino acids in fed animals [18].

These findings can be explained by observations from protein synthesis studies *in vitro*. Barnes et al. [27] have shown that at isotopic equilibrium the SR of tRNA in cultured macrophages is closer to that of cellular protein than to that of either the intracellular or the extracellular free amino acid pools. These results suggest that amino acids from intracellular proteolysis are a primary source for charging tRNA. Because of the lack of luminal amino acid absorption in the water-fed piglet, intracellular proteolysis might be the predominant source of the amino acids for protein synthesis because the SR of proLPH_n was significantly lower than that of the mucosal free pools.

The situation seems to be different in the colostrum-fed neonatal pig. In these animals, large quantities of intact colostrum protein, such as IgG, are absorbed into lysosomal vacuoles [8]. These vacuoles might come to occupy as much as 50% of the cytoplasmic space adjacent to the apical membrane [8,23,24]. If degraded, these proteins would not only dilute the free amino acid pool (as measured in acid extracts) but could also represent a separate compartment in the intact enterocyte. High concentrations of unlabelled amino acids could accumulate at the circumference of endocytotic vacuoles in the vicinity of the BB membrane, while radiolabelled amino acids coming from the blood could accumulate in the perinuclear cytoplasm where the Golgi apparatus is typically seen [8]. Therefore in the colostrum-fed animals, LPH precursors, translated and glycosylated in the ER and Golgi apparatus [3], might be exposed to a free amino acid pool considerably more enriched than the free amino acid pool at the apical membrane. Under these conditions, the SR of proLPH_n would be high relative to the SR of the 'bulk' (i.e. acid-extracted) free amino acid pool. Although these observations are of biological importance, it is important to recognize that, by design, the calculated synthesis rates for this study are independent of the absolute isotopic enrichment of the mucosal free amino acid pool.

The results of this study demonstrate that feeding with colostrum stimulated mucosal protein synthesis *in vivo*. The results also show that *in vivo* the K_s values of proLPH_n, proLPH_c and proLPH_i were 19–60-fold higher than the K_s values of total mucosal protein. The K_s of BB LPH was not affected by dietary treatment and was on average significantly lower in these 1-day-old animals (0.037%/min) than our previous observations in 4-week-old pigs (0.069 ± 0.013%/min) [22]. These findings are consistent with the increase in lactase enzyme activity observed in piglets during the first week of life [2,28] and imply that during

the immediate postnatal period, LPH synthesis is not fully 'activated'. Unfortunately, because of the infusion design of our earlier study we were unable to estimate the K_s values of proLPH polypeptides and therefore do not know whether the age-related change in BB LPH synthesis rates is the result of a change in the rate of precursor post-translational processing.

In contrast with K_s , the absolute synthesis rate (a reflection of the amount of an individual protein synthesized/g of mucosa) of proLPH_n in the colostrum-fed animals was approx. double that in the water-fed animals. No significant differences in the absolute synthesis rates of proLPH_c, proLPH_i or BB LPH were observed. Thus although feeding colostrum seemed to stimulate proLPH_n synthesis, the LPH precursor accumulates within the enterocyte. Only 32% of the pool is processed to the BB enzyme in the colostrum-fed animals compared with 62% in the water-fed animals, causing the amount of BB LPH per unit weight of jejunum to be higher in water-fed animals than in the colostrum-fed animals. It should be noted, however, that these conclusions make two assumptions: first, as discussed above, that 'mature' BB LPH is the only LPH polypeptide with enzyme activity, and secondly that the increased amounts of protein in the intestine of newborns, colostrum-fed animals are exogenous (i.e. from colostrum) and therefore not derived from increased mucosal protein synthesis. Although for this study we could not formally quantify endogenous as opposed to exogenous intracellular protein, microscopy demonstrated numerous intracellular vacuoles, which have been shown by others to occupy as much as 50% of the intracellular space and to contain large amounts of colostrum IgG [8,23,24]. Thus, although we cannot deny the possibility that some protein is derived from mucosal protein synthesis, it seems unlikely that newly synthesized mucosal protein causes serious errors in the calculations.

Although we cannot explain the decreased post-translational processing efficiency of proLPH_n observed in the colostrum-fed animals, it is interesting to speculate about the underlying mechanism. Studies of BB hydrolase synthesis in organ culture have shown repeatedly that glycosylation inhibitors block expression of the mature enzyme in the BB membrane by preventing the synthesis of conformationally mature precursor polypeptides [29]. Further, it has been shown that patients with congenital BB hydrolase deficiency, particularly sucrase-isomaltase deficiency, might have impaired intracellular transport, which again seems to result from the conformational immaturity of the precursor polypeptides [29]. Thus in these colostrum-fed animals the translocation of LPH precursors to the BB membrane might be impaired because of an unidentified colostrum component that prevents conformational maturing of proLPH polypeptides.

An alternative explanation relates to the sorting of the intracellular vesicles containing LPH polypeptides. Using immunogold electron microscopy, Heath et al. [30] have observed a mixing of the secretory pathway for the BB hydrolases and the transcytotic pathway for colostrum protein absorption in colostrum-fed neonatal piglets. The accumulation of large IgG-containing vacuoles beneath the BB membrane in colostrum-fed animals could impair the ability of the exocytotic vesicles containing proLPH polypeptides to translocate from the Golgi apparatus to the apical surface of the cell. Whatever the underlying mechanism, the present results show the potential for differential nutrient effects on LPH translation (i.e. synthesis of proLPH_n) and post-translational processing, and underscore the necessity for measuring the entire process to obtain a full understanding of this complex protein synthesis system.

We thank Leslie Loddeke for editorial assistance and Adam Gillum for help with the illustrations. This project of the USDA/Agricultural Research Service Children's

Nutrition Research Center, Department of Pediatrics, Baylor College of Medicine and Texas Children's Hospital, has been funded in part with federal funds from the USDA/ARS under co-operative agreement number 58-625001-003. The contents of this publication do not necessarily reflect the views or policies of the U.S. Department of Agriculture, nor does mention of trade names, commercial products or organizations imply endorsement by the U.S. government.

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