A high-protein formula increases colonic peptide transporter 1 activity during neonatal life in low-birth-weightpiglets and disturbs barrier function later in life

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(Submitted 19 December 2013 – Final revision received 12 May 2014 – Accepted 14 May 2014)

Abstract

Dietary peptides are absorbed along the intestine through peptide transporter 1 (PepT-1) which is highly responsive to dietary protein level. PepT-1 is also involved in gut homeostasis, both initiating and resolving inflammation. Low-birth-weight (LBW) neonates are routinely fed a high-protein (HP) formula to enhance growth. However, the influence of this nutritional practice on PepT-1 activity is unknown. Intestinal PepT-1 activity was compared in normal-birth-weight (NBW) and LBW piglets. The effect of HP vs. normal-protein (NP) formula feeding on PepT-1 activity and gut homeostasis in LBW piglets was evaluated, during the neonatal period and in adulthood. Flux of cephalexin (CFX) across the tissue mounted in Ussing chambers was used as an indicator of PepT-1 activity. CFX flux was greater in the ileum, but not jejunum or colon, of LBW than NBW piglets during the neonatal period. When LBW piglets were formula-fed, the HP formula increased colonic CFX during the 1st week of life. Later in life, intestinal CFX fluxes and barrier function were similar whether LBW pigs had been fed NP or HP formula. However, colonic permeability of HP- but not NP-fed pigs increased when luminal pH was brought to 6·0. The formyl peptide N-formyl methionyl-leucyl-phenylalanine conferred colonic barrier protection in HP-fed piglets. Heat shock protein 27 levels in the colonic mucosa of HP-fed LBW pigs correlated with the magnitude of response to the acidic challenge. In conclusion, feeding a HP formula enhanced colonic PepT-1 activity in LBW pig neonates and increased sensitivity of the colon to luminal stress in adulthood.

Key words: Peptide absorption: Low-birth-weight neonates: N-Formyl methionyl-leucyl-phenylalanine: Formulas

The predominant mode of intestinal absorption of protein hydrolysis products in adults and infants is through H⁺-coupled peptide transporter 1 (PepT-1) (1). It allows absorption of a large variety of di- and tripeptides at the apical membrane that are further hydrolysed by peptidases in the cytoplasm of enterocytes. PepT-1 expression (mRNA and/or protein) increases during the fetal period to peak either at birth or between postnatal day (PND) 3 and 5 in rats and piglets. It then declines with age in both species (2–5). An original feature of PepT-1 development is its relatively high expression in the proximal colon of neonatal rats and piglets during the very early life period, i.e. PND2 or PND3 (2–4). PepT-1 expression and activity in adults are responsive to changes in the quantity and composition of the substrate, showing greater activity when the availability of protein or di- and tripeptides is increased (1,5). This regulation by dietary substrate appears to occur by increasing the gene transcription rate and/or mRNA stability (6). Human low-birth-weight (LBW) neonates are usually fed high-protein (HP) formulas to ensure rapid postnatal catch-up growth (7,8). Interestingly, the postnatal development of PepT-1 mRNA in LBW piglets differs from that of normal-birth-weight (NBW) piglets, with significantly greater levels of PepT-1 mRNA in the ileum and proximal colon of LBW compared with NBW piglets at PND2 (4,5). However, it remains to be known whether feeding a HP formula modifies PepT-1 activity in the neonatal period, especially in LBW neonates.

Besides its role as peptide transporter, a body of literature has emerged on the role of PepT-1 in gut homeostasis. Indeed, in addition to dietary peptides, PepT-1 is able to transport bacterial peptides such as N-formyl and muramyl peptides present in gut luminal content, which are potent

Abbreviations: CFX, cephalexin; FD-4, fluorescein isothiocyanate-dextran 4000; f-MLP, N-formyl methionyl-leucyl-phenylalanine; HP, high protein; Hsp, heat shock protein; LBW, low birth weight; MES, 2-(N-morpholino)ethane sulfonic acid; NBW, normal birth weight; NP, normal protein; PepT-1, peptide transporter 1; PND, postnatal day.

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neutrophil chemotactic substances. PepT-1 was therefore believed to promote inflammation. As such, it has been reported that PepT-1 is highly expressed in a chronically inflamed colon in adults. Infusion of the model peptide N-formyl methionyl-leucyl-phenylalanine (f-MLP) elicits neutrophil infiltration and inflammation in the jejunum of adult rats within 4 h. Inhibition of PepT-1 reduced this effect. Infusion of f-MLP also increases ileal permeability in rats through a neutrophil-derived oxidant-dependent mechanism. However, participation of formyl peptide receptors 1 and 2 in gut homeostasis and repair after injury has also been demonstrated. Cell protection mechanisms against oxidative and inflammatory damage by f-MLP through heat shock protein (Hsp) 27 induction seem to be involved in this protective effect.

Recent data indicated that changes in gut homeostasis during early life have a durable impact on gut function. This was first exemplified by early life stress that disturbs the cross-talk between gut microbiota, barrier function and neurons in rat neonates and is followed by greater response to gut inflammation or visceral hypersensitivity in adults. Our group recently demonstrated that HP formula feeding influences distal intestinal homeostasis in pigs both during the neonatal period and in adulthood. Based on these literature data and our published data on the developmental pattern of PepT-1 mRNA in LBW piglets, we hypothesised that LBW piglets have increased PepT-1 activity in the distal intestine that might be accentuated by HP formula feeding. Furthermore, we speculated that changes in PepT-1 activity would have long-term consequences on distal intestine barrier function and homeostasis. The objectives of the present study were therefore (1) to investigate PepT-1 activity in LBW and NBW piglets at different ages, (2) to examine the effect of HP formula feeding on PepT-1 activity in LBW piglets and (3) to evaluate whether distal intestine barrier function is disturbed in adult LBW pigs fed a HP formula during neonatal life.

Materials and methods

Animal procedures

The experimental protocol was designed in compliance with the recommendations of the French and European law (Décret: 2001-464 29/05/01, 86/609/CEE) for the care and use of laboratory animals under the certificate of authorisation to experiment on living animals no. 35-69.

Cross-bred (Piétrain X Large White X Landrace) piglets from the experimental herd of INRA (St-Gilles, France) were used in two separate experiments.


A total of fifteen LBW piglets (average birth weight 1.01 kg, range 0.74–1.21 kg) and fifteen NBW piglets (average birth weight 1.5 kg, range 1.32–1.64 kg) were selected at birth based on birth weight. They originated from eight different litters representative of our herd (number of piglets per litter 15–5 (SEM 0.6) and average birth weight 1.24 (SEM 0.03) kg). LBW piglets were chosen with a birth weight 30% lower than the average birth weight of the litter. Due to this constraint in birth weight, it was not possible to take sex into account. However, the number of same-sex and non-same-sex pairs of piglets was balanced among the three different age groups. Piglets suckled their mother until PND21 and had no access to creep feed. Of the piglets, one-third was killed at PND2, one-third at PND7 and one-third at PND21. Piglets were anaesthetised with isoflurane delivered through a veterinary anaesthesia ventilator. They were then euthanised by intracardiac T-61 injection (Elvetis). After laparotomy, 15 cm segments of jejunum (20 cm distal from the Treitz ligament), ileum (20 cm proximal to the ileo-caecal junction) and colon (15 cm distal from the ileo-caecal junction) were collected, rinsed with cold 0.9% NaCl solution and immediately placed in Ringer’s bicarbonate (composition in mmol/l: Na+ 145, Cl− 128, PO4− 3, 0.32, Ca2+, 2, Mg2+, 1, HCO3− 25, SO42− 1, K+ 6.3, pH 7.4–7.6) for the Ussing chamber experiment (cephalexin (CFX) flux experiment, without electrophysiological measurements).


A total of forty-eight LBW piglets were fed from PND2 to PND28 a normal-protein (NP) formula (50 g protein/l) or a HP formula (77 g protein/l; Table 1), and then a pig-dedicated diet until PND160 as described already. Piglets were killed at PND7 (n 6 per group), PND28 (n 6 per group) and PND160 (n 12 per group). They were euthanised by electrocution followed by exsanguination. After laparotomy, the segments of the distal ileum and proximal colon were collected as described above. Pieces of tissue were collected for the Ussing chamber experiment (CFX flux experiment, without electrophysiological measurements at PND7, PND28 and PND160) and barrier function and electrophysiological measurements at PND160). Pieces of tissue (100 mg) were also collected and either placed in RNAlater (Applied Biosystems) for 24 h at 4°C and stored at −80°C until Western blot analysis.

Ussing chamber experiment

Intestinal tissues were stripped of the external muscle layers (except for colonic tissues at PND7 and PND28 where the muscle layers were left intact to avoid deteriorating the mucosa when stripping) and opened along the antimesenteric border and then mounted in the Ussing chamber (World Precision Instrument). The chamber opening exposed 0.67 cm² of tissue surface area to 8 ml of circulating oxygenated buffer (see below for composition) at 39°C.

<table>
<thead>
<tr>
<th>Table 1. Composition of the neonatal formula</th>
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<tr>
<td>Composition</td>
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<tr>
<td>Energy (MJ/l)</td>
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<tr>
<td>NP: 4.75</td>
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<tr>
<td>HP: 5.03</td>
</tr>
<tr>
<td>Sow milk: 4.70</td>
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<tr>
<td>Protein (g/l)</td>
</tr>
<tr>
<td>NP: 51</td>
</tr>
<tr>
<td>HP: 77</td>
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<tr>
<td>Sow milk: 50</td>
</tr>
<tr>
<td>Fat (g/l)</td>
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<tr>
<td>NP: 82</td>
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<tr>
<td>HP: 79</td>
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<tr>
<td>Sow milk: 80</td>
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<tr>
<td>Carbohydrate (g/l)</td>
</tr>
<tr>
<td>NP: 49</td>
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<tr>
<td>HP: 46</td>
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<tr>
<td>Sow milk: 51</td>
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NP, normal protein; HP, high protein.
Peptide transporter 1 in pig neonate colon

PepT-1 activity in different segments of the intestine was evaluated by measuring mucosal-to-serosal fluxes of CFX (CFX, an aminoccephalosporin transported through PepT-1\(^{180}\)) and not metabolised by the intestine\(^{199}\). One piece of proximal jejunal, distal ileal and proximal colonic tissue per piglet (PND2, PND7 and PND21 for Expt 1 and PND7, PND28 and PND160 for Expt 2) was bathed in 10 mM-MgSO\(_4\), H\(_2\)PO\(_4\) 0·3, HEPES Tris 10, glucose 10, pH 7·4) on PND28 and PND160 for Expt 2) was bathed in 10 mM-piglet (PND2, PND7 and PND21 for Expt 1 and PND7, PND7, proximal jejunal, distal ileal and proximal colonic tissue per epithelial barrier sensitivity to inflammatory stimulus (21). On HEPES Tris buffer (in mM: NaCl 137, KCl 54, CaCl\(_2\) 28, MgsO\(_4\) 1, H\(_2\)PO\(_4\) 0·3, MES Tris 10, mannitol 10, pH 6·0) on the mucosal side and a with 10 mM-MES Tris buffer (pH 6·0) on the luminal side and glutation across the intestinal tissues of NBW piglets

Thereafter, two adjacent segments of tissues were mounted with 10 mM-HEPES Tris buffer (pH 7·4) on the serosal side. After a 20 min equilibration period, 100 mM-f-MLP was added to the mucosal side of one of these later chambers. Since f-MLP absorption through PepT-1 requires acidic conditions on the mucosal side, we omitted the pH 7·4 + f-MLP condition. After 10 min, 10 mM-horseradish peroxidase and fluorescein isothiocyanate-dextran 4000 (FD-4) were added to the luminal side in all chambers, and serosal samples were collected every 30 min for 120 min. Concentrations of horseradish peroxidase and FD-4 were determined as described already\(^{180}\). PepT-1 activity in different segments of the intestine was evaluated by measuring mucosal-to-serosal fluxes of CFX (CFX, an aminoccephalosporin transported through PepT-1\(^{180}\)) and not metabolised by the intestine\(^{199}\). One piece of proximal jejunal, distal ileal and proximal colonic tissue per piglet (PND2, PND7 and PND21 for Expt 1 and PND7, PND28 and PND160 for Expt 2) was bathed in 10 mM-MgSO\(_4\), H\(_2\)PO\(_4\) 0·3, HEPES Tris 10, glucose 10, pH 7·4) on PND28 and PND160 for Expt 2) was bathed in 10 mM-piglet (PND2, PND7 and PND21 for Expt 1 and PND7, PND7, proximal jejunal, distal ileal and proximal colonic tissue per epithelial barrier sensitivity to inflammatory stimulus (21). On HEPES Tris buffer (in mM: NaCl 137, KCl 54, CaCl\(_2\) 28, MgsO\(_4\) 1, H\(_2\)PO\(_4\) 0·3, MES Tris 10, mannitol 10, pH 6·0) on the mucosal side and a with 10 mM-MES Tris buffer (pH 6·0) on the luminal side and glutation across the intestinal tissues of NBW piglets

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The concentration of CFX in samples was determined by HPLC using a C18 column (AccQ-Tag, 15 cm length, 3·9 mm internal diameter; Waters) and a mobile phase of methanol–100 mM-acetate

buffer, pH 6·0 (30:70, v/v), 1 ml/min flow rate, 262 nm wavelength and column oven temperature 40°C.

Quantitative PCR

Quantitative PCR was performed to determine PepT-1 mRNA levels in the ileum and colon of NP- and HP-fed piglets at PND7, PND28 and PND160. Analyses were performed as described previously\(^{4,16}\) using specific primers for PepT-1 (5'-TCTTACAACTGCATGACCT-3'; 5'-AGGGCACGTGCACAGA-3'). Transcript concentration was normalised to the transcript concentration of glyceraldehyde 3-phosphate dehydrogenase (Gapdh, reference gene, 5'-CATCCATGCAACCTCGGCA-3'; 5'-GCATGGACTGTGGTTCATGAGTC-3') in the same sample. Gapdh expression was not affected by the tested factors.

Western blot analysis

Colonic mucosa protein was extracted in a TEX 1X(10 mM-Tris-HCl, 0·1 mM-EDTA, 0·01% Triton X-100, 60 mM-Tris-base, pH 6·8, 10% glycerol, 3% SDS, 5% β-mercaptoethanol and a protease inhibitor cocktail (104 mM-4-(2-aminoethyl)-benzenesulfonyl fluoride, 80 µM-aprotinin, 4 mM-bestatin, 1·4 mM-E-64, 2 mM-leupeptin, 1·5 mM-pepsatin A, Sigma-Aldrich) buffer. Levels of Hsp27 and Hsp70 were then determined by Western blot as described previously\(^{22}\). Densitometry of blots was measured and expressed relatively to β-actin density of the corresponding sample.

Statistical analysis

Data were analysed using the program GraphPad Prism (GraphPad Software, Inc.). In Expt 1, a two-way ANOVA was performed, testing piglet age, piglet birth weight status and the interaction between these two factors. Data from Expt 1 and Expt 2 (PND7 and PND28) were combined and analysed by one-way ANOVA. The t-test with Bonferroni correction was used as a subsequent multiple comparison test. Data from piglets at PND160 in Expt 2 were analysed using a t-test for basal parameters. Colonic barrier function responses to luminal stress were analysed using a two-way ANOVA, testing the neonatal diet, luminal treatment and the interaction between these two factor effects. The t-test with Bonferroni correction was used as a subsequent multiple comparison test. Data are presented as means with their standard errors. A P value ≤0·05 was considered significant.

Results

Peptide transporter 1 activity in suckling low-birth-weight and normal-birth-weight piglets during the neonatal period

CFX fluxes across the proximal jejunum mucosa were similar in LBW and NBW piglets, irrespective of their postnatal age (Fig. 1(a)). In the ileum, no difference in CFX flux was observed between the NBW and LBW piglets at PND2. However, LBW piglets exhibited a 5·3-fold greater flux of CFX (P=0·035; Fig. 1(b)) at PND7. Flux of CFX across the ileum
tended to stay greater in LBW than NBW piglets ($P = 0.09$; Fig. 1(b)) at PND21. In the proximal colon, although the CFX flux was increased 3-fold in LBW compared with NBW piglets at PND2, the difference did not reach significance ($P = 0.17$; Fig. 1(c)). No difference was observed thereafter.

**Effect of a high protein formula on peptide transporter 1 activity and barrier function in low birth weight piglet ileum and colon**

**During the neonatal period.** Formula feeding did not change CFX flux across the ileal tissue of LBW piglets at PND7, irrespective of the level of protein in the formula (Fig. 2(a)). At PND28, no difference in ileal CFX flux was observed between the NP- and HP-fed piglets (Fig. 2(a)). In the colon, NP formula feeding did not alter CFX flux across the mucosa compared with suckled LBW piglets. However, feeding a HP formula resulted in greater CFX flux across the colonic mucosa, especially at PND7 where CFX flux was enhanced 3.6-fold in HP- compared with NP-fed piglets (Fig. 2(b)). *PepT-1* mRNA relative expression was unchanged by the type of formula in both segments and at both ages (Table 2).

**Later in life.** At PND160, neither ileal barrier function nor CFX flux across the ileum was different between the NP- and HP-fed pigs (Table 3). Stress conditions (pH 6.0 buffer or pH 6.0 buffer + f-MLP) did not modify ileal permeability (data not shown). Basal colonic barrier function parameters as well as CFX flux across the colonic mucosa of NP or HP formula-fed piglets were also similar between the NP- and HP-fed pigs (Table 3). When luminal pH was brought to 6.0, colonic flux of FD-4 dramatically increased compared with pH 7.4 in the colonic tissue of HP- but not NP-fed pigs (Fig. 3). The addition of f-MLP to the mucosal side of the colonic tissue restored FD-4 flux in HP-fed pigs ($P < 0.001$, Fig. 3). Horseradish peroxidase flux across the colonic mucosa was not significantly altered by acidic pH or by the addition of f-MLP, irrespective of the pig neonatal diet (data not shown).
Table 2. Ileal and colonic peptide transporter 1 (PepT-1) mRNA levels at postnatal day (PND) 7 and PND28* (Mean values with their standard errors)

<table>
<thead>
<tr>
<th></th>
<th>PND7</th>
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<th>PND28</th>
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<tbody>
<tr>
<td></td>
<td>NP</td>
<td>HP</td>
<td>NP</td>
<td>HP</td>
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<tr>
<td></td>
<td>Mean SEM</td>
<td>Mean SEM</td>
<td>Mean SEM</td>
<td>Mean SEM</td>
</tr>
<tr>
<td>ileum</td>
<td>1.0 0.2</td>
<td>0.8 0.1</td>
<td>0.7 0.1</td>
<td>0.5 0.03</td>
</tr>
<tr>
<td>colon</td>
<td>1.1 0.6</td>
<td>1.0 0.2</td>
<td>1.6 0.4</td>
<td>2.2 0.4</td>
</tr>
</tbody>
</table>

NP, normal protein; HP, high protein.* Levels of PepT-1 mRNA (normalised to that of glyceraldehyde 3-phosphate dehydrogenase (Gapdh)) were measured at PND7 and PND28 in the ileum and colon of low-birth-weight piglets that were fed the NP or HP formula from PND2 to PND28.

To evaluate whether Hsp was involved in the protective effect of f-MLP on barrier function, we measured the levels of Hsp27 and Hsp70 in the colonic mucosa by Western blot at PND160. No difference between the dietary groups was observed (Hsp27: NP 0.42 (SEM 0.08) v. HP 0.41 (SEM 0.07), P=0.92 and Hsp70: NP 0.95 (SEM 0.09) v. HP 0.93 (SEM 0.10), P=0.88). Interestingly, Hsp27 levels in colonic mucosa correlated negatively with FD-4 fluxes across the colonic mucosa in luminal acidic conditions in HP-fed pigs (Fig. 4(a)). The flux increase from neutral to acidic conditions also correlated negatively with Hsp27 levels, whereas the decrease induced by the addition of f-MLP correlated positively with Hsp27 levels in HP-fed pigs (Fig. 4(b) and (c), respectively). No such correlation was observed in NP-fed animals or with Hsp70 levels (data not shown).

Discussion

PepT-1 is a singular intestinal transporter exhibiting multifaceted function, from peptide and amino-acid supply to bacterial–host communication controlling gut homeostasis and inflammation. It has also unique developmental features with transient high expression in the colon of neonatal animals during very early life and many regulatory pathways in health and disease. The present study extends this pattern of peculiar regulatory pathways to LBW neonates whose nutrient requirements, intestinal development and gut homeostasis characteristics are overlooked but yet of great importance for neonatal care givers. We demonstrated that LBW neonatal piglets exhibited greater PepT-1 activity in the ileum during the neonatal period compared with NBW piglets. Furthermore, increasing dietary protein supply with HP formula feeding increased PepT-1 activity in the colon. Later in life, colonic barrier function of HP-fed pigs was more sensitive to luminal stress (modelled here by a mild change in luminal pH) than that of NP-fed pigs. Furthermore, f-MLP conferred protection to HP colonocyte barrier function. This protective effect of the bacterial peptide was unlikely accounted for a higher entry into colonocytes since PepT-1 activity was similar in the colon of NP- and HP-fed pigs at that age. Conversely, a role for Hsp27 is conceivable as demonstrated by the negative correlations between Hsp27 levels in the colon and the colonic response to stress and the positive correlation between Hsp27 levels and barrier function restoration by f-MLP.

Peptide transporter 1 activity in low-birth-weight piglets

LBW piglets are characterised by delayed maturation of intestinal functions during the 1st days of life compared with NBW piglets18,23 followed by catch-up for some but not all intestinal functions, resulting in no apparent gross anatomical difference between LBW and NBW piglets but showing transcriptomic, proteomic and functional differences at the end of the neonatal period or immediately after weaning24–26. Our data demonstrate increased PepT-1 activity, illustrated with CFX flux, in the ileum of LBW compared with NBW piglets with a significant difference at PND7. One could argue that CFX crosses intestinal mucosa not only through PepT-1 but also through tight junctions in the paracellular space. However, paracellular transport of CFX is unlikely for two reasons. First, we (G Boudry and I Le Huerou-Luron, unpublished results) and others18 demonstrated that CFX transport is a saturating process, whereas paracellular leak would be non-saturating. Second, we previously observed lower paracellular

Table 3. Ileal and colonic barrier function parameters and cephalaxin (CFX) fluxes at postnatal day (PND) 160* (Mean values with their standard errors)

<table>
<thead>
<tr>
<th></th>
<th>Ileum</th>
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<th>Colon</th>
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<tr>
<td></td>
<td>NP</td>
<td>HP</td>
<td>NP</td>
<td>HP</td>
</tr>
<tr>
<td></td>
<td>Mean SEM</td>
<td>Mean SEM</td>
<td>Mean SEM</td>
<td>Mean SEM</td>
</tr>
<tr>
<td>Isc (μA/cm²)</td>
<td>50 10</td>
<td>85 19</td>
<td>60 21</td>
<td>66 19</td>
</tr>
<tr>
<td>R (mS/cm²)</td>
<td>21 2</td>
<td>18 3</td>
<td>22 7</td>
<td>12 4</td>
</tr>
<tr>
<td>FD4 flux (ng/cm² per h)</td>
<td>597 64</td>
<td>518 69</td>
<td>711 96</td>
<td>852 114</td>
</tr>
<tr>
<td>HRP flux (ng/cm² per h)</td>
<td>40 9</td>
<td>64 17</td>
<td>120 16</td>
<td>95 23</td>
</tr>
<tr>
<td>CFX flux (μmol/cm² per h)</td>
<td>205 26</td>
<td>197 30</td>
<td>671 138</td>
<td>652 106</td>
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</table>

NP, normal protein; HP, high protein; Isc, short-circuit current; R, transepithelial resistance; FD4, fluorescein isothiocyanate-dextran 4000; HRP, horseradish peroxidase.* Intestinal barrier function parameters (Isc, R, flux of FD4 (4kDa) and HRP (40kDa)) as well as flux of CFX across the ileum and colon mounted in Ussing chambers were measured in PND160 pigs that had been fed the NP or HP formula from PND2 to PND28.
permeability in the ileum of LBW compared with NBW piglets at PND28 (27), which would not fit with enhanced CFX flux if it was a paracellular passage.

Our previous data showed that the delay in PepT-1 mRNA maturation was transient in early life and that by PND5, PepT-1 mRNA levels were similar in NBW and LBW piglets in the ileum and colon (4). Functionally, this did not translate into enhanced PepT-1 activity at PND2 but a few days later (PND7) in the ileum with no significant difference in PepT-1 activity in the colon. Discrepancy between mRNA levels, protein expression and activity of PepT-1, especially in the colon, has already been observed either in healthy or diseased states (28). A very recent report has demonstrated increased PepT-1 mRNA but not protein expression in LBW rat pups born to dams fed a low-protein diet compared with NBW pups (29), corroborating differences between LBW and NBW neonates at the gene expression level that do not translate into difference in function.

**Effect of high-protein formula during the neonatal period**

Several studies have demonstrated that HP or high peptide intake increases PepT-1 expression in vitro in intestinal epithelial cell lines (30, 31) or in vitro in the small intestine of adult rodents (32, 33). PepT-1 regulation by its own substrate during the neonatal period has not been reported yet. In our case, the HP formula, which provided 40% more protein than the NP one, failed to induce any change in small-intestinal PepT-1 activity but increased the activity in the colon, especially at PND7. Formula feeding per se did not appear to alter PepT-1 activity when protein content was matching that of sow milk as demonstrated by similar ileal and colonic CFX fluxes between NP formula-fed and suckled piglets. We previously observed that protein level in the distal ileum was increased (25%) at PND7 but not at PND28 in HP- compared with NP-fed piglets (17), suggesting that protein concentration in the luminal content was greater in our HP-fed piglets at least during the 1st week of HP formula feeding. The reason why PepT-1 activity was not enhanced in the small intestine by the increased substrate availability is unclear. It can be speculated that PepT-1 expression and activity in the small intestine are maximal during the neonatal period as illustrated by the spectacular fall in PepT-1 mRNA level between 3-week-old unweaned and 6-week-old fully weaned Yucatan piglets (5) and cannot be further enhanced by dietary protein. In the colon, PepT-1 regulation by protein might be more flexible and the 25% increase of protein...
flowing through the ileo-caecal junction might be sufficient to enhance PepT-1 activity. Another possible pathway for specific up-regulation of PepT-1 activity in the colon could be pro-inflammatory signals since PepT-1 expression and activity have been shown to be induced by TNF-α and interferon-γ (34). However, our previous data did not show any sign of inflammation in the colon of piglets fed a HP formula (17), weakening this hypothesis.

**Effect of the high-protein formula on colonic barrier function later in life**

The present study provides evidence that feeding a HP formula during the neonatal life has long-term consequences on colonic homeostasis. We show that a mild stress (slightly acidic pH) alters barrier function in the colonic mucosa of HP- but not NP-fed pigs. In Caco-2b cells, a decrease in pH from 7.4 down to 6.0 does not modify epithelial permeability, whereas a pH of 5.5 dramatically increases monolayer permeability, suggesting that intestinal epithelial cells are able to maintain epithelial barrier integrity up to a certain level of metabolic stress (20). Our data suggest that neonatal HP feeding alters the sensitivity of intestinal epithelial cells to metabolic stress in adulthood. The effect of pH change was rapid since a change in permeability was observed within the 2 h of the Ussing chamber experiment. Colonic barrier function in basal conditions was not different between the NP- and HP-fed pigs, suggesting that expression of tight junction proteins was similar between the two groups. The change in colonic permeability with the acidic pH was therefore either linked to the change in tight junction localisation or phosphorylation and cytoskeleton reorganisation as already established with other metabolic stresses (35), or to altered metabolic rescue pathways required under metabolic stress. These data are in line with our previous data showing that female colonic mucosa of pigs fed a HP formula during early life was more sensitive to oxidative stress or inflammatory mediators than the colonic mucosa of NP-fed pigs (37).

Interestingly, addition of f-MLP, a pro-inflammatory peptide transported by PepT-1, reduced the colonic hyper-permeability observed with acidic conditions in HP-fed animals. Previous reports showed that pretreatment with f-MLP protected intestinal epithelial cells from oxidant- or inflammatory mediator-induced depolymerisation of actin and a decrease in transepithelial resistance. This effect was probably mediated by the induction of Hsp27 in epithelial cells since silencing Hsp27 expression inhibited the protective effect of f-MLP (14). A similar Hsp27-driven protective effect by f-MLP is plausible in the present study since Hsp27 levels correlated negatively with the magnitude of response to the acidic challenge and positively with the magnitude of response to f-MLP. However, we must stress out that Hsp27 measured here is constitutive Hsp27 and not Hsp27 induced by the acidic challenge or f-MLP incubation. Moreover, these correlations were observed only in HP- not NP-fed animals that displayed no reaction to the acidic challenge. Finally, the mean level of Hsp27 in colonic mucosa was not different between the NP- and HP-fed pigs. A complex relationship between priming of epithelial cells during the neonatal period induced by a greater invasion of bacterial peptides into colonocytes through PepT-1 and response to f-MLP and induction of Hsp27 later in life in HP-fed pigs can therefore be hypothesised.

Taken together, these experiments demonstrate that LBW neonates have unique intestinal peptide transport capacity that can be greatly influenced by the diet, especially in the colon. The nutritional management of LBW infants that are routinely fed a HP formula may result in enhanced activity of the transporter in the colon during early life. This practice seems also to have consequences on colonic homeostasis later in life. Considering the high level of potentially harmful bacterial peptides present in that part of the gastrointestinal tract at both periods of life, immunological consequences and mechanisms warrant further investigations.

**Acknowledgements**

The authors would like to thank all the individuals involved in animal care. They also acknowledge the help of Soraya Benghabrit and Nadine Mezière.

The present study was supported by the Agence Nationale de la Recherche, PROTNEONAT- ANR-05-PNRA-09.

The authors’ contributions are as follows: G. B. and I. L. H.-L. formulated the research question; G. B., A. J., G. S. and I. L. H.-L. designed the study; G. B., V. R., C. P., A. J., G. S. and I. L. H.-L. carried out the study; G. B., V. R., C. P. and G. S. analysed the data; G. B. and I. L. H.-L. wrote the paper.

The authors declare that they have no conflict of interest.

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