Exogenous leptin controls the development of the small intestine in neonatal piglets

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Abstract

Leptin, a hormone produced and secreted by adipose tissue, muscles and stomach, is involved in the regulation of adipose tissue mass, food intake and body weight in neonatal animals. It is also produced in the mammary glands and secreted into the colostrum and milk. Since leptin receptors are widely distributed in the small intestine mucosa, the aim of the present study was to investigate the effect of exogenous leptin on the development of the small intestine in neonatal piglets. Male neonatal piglets were fed with sow's milk or artificial milk formula. Every 8 h the latter received either vehicle or leptin (2 or 10 µg/kg body weight). The animals were either killed after 6 days of treatment and the small intestine sampled for histology and brush border enzyme activities or were tested for marker molecule (Na-fluorescein and BSA)

absorption *in vivo*. Feeding milk formula slowed the maturation of small intestinal mucosa compared with feeding sow's milk. However, after leptin treatment the length of the small intestine was increased, and intestinal villi length, but not crypt size, was reduced compared with controls. The mitotic index was increased and the percentage of vacuolated enterocytes was reduced in the entire small intestine. Enterocyte brush border protease and lactase activities were reduced in the jejunum. Na-fluorescein marker molecule absorption did not change but that of BSA was reduced 3·8-fold. In conclusion, exogenous leptin administered in physiological doses reversed the maturation of the small intestinal mucosa to the range found in sow-reared piglets.

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Introduction

Leptin is a 167 amino acid protein, first discovered in adipose tissue (Zhang *et al.* 1994) and after that in a wide variety of tissues, including the mammary gland (Smith-Kirwin *et al.* 1998, Aoki *et al.* 1999, Silva & VandeHaar 1999, Bonnet *et al.* 2002). Smith-Kirwin and co-workers (1998) demonstrated the biosynthesis of leptin in human placenta and suggested a plausible role of the hormone in foetal growth. After birth, colostrum and milk take over some of the placenta's functions, especially in regard to the delivery of maternal hormones and growth factors to the neonate. This is true also for leptin, which has been identified in human, mouse, rat and pig colostrum and milk (Casabiell *et al.* 1997, Houseknecht *et al.* 1997, Aoki *et al.* 1999, Estienne *et al.* 2000, Ucar *et al.* 2000). It is noteworthy that in humans, leptin is present in whole breast milk at 30- to 150-fold higher concentrations than in milk formula (Houseknecht *et al.* 1997, Smith-Kirwin *et al.* 1998), and its concentration in breast milk is positively correlated with maternal and/or infant plasma

leptin concentrations. Silva and co-workers (1999) showed that leptin is synthesized in the mammary gland and excreted with milk. In contrast, Casabiell and co-workers (1997) demonstrated that leptin is transferred from the circulation to the mammary gland, then without loss of biological activity, to the milk and after that to the infant's stomach and general blood circulation. The biologically active form of the leptin receptor (OB-Rb) (Cioffi *et al.* 1996, Lee *et al.* 1996) is widespread, and has been localized in the entire length of the small intestine (Morton *et al.* 1998). Using immunostaining, Barrenetxe *et al.* (2002) demonstrated the leptin receptor within the cytoplasm of enterocytes located on both intestinal villi and crypts in the human, rat and mouse small intestine. In human enterocytes, the OB-Rb receptor (long form of the leptin receptor) was also found in the brush border plasma membrane (Barrenetxe *et al.* 2002).

These studies suggest that 'milk-borne' leptin may play a role in the offspring, although its effect on the development of the neonatal gastrointestinal tract has never been experimentally verified. The aim of the present study was

to investigate the role of exogenous leptin in the development of the small intestine of neonatal piglets fed artificial milk formula. Development was evaluated in regard to the microscopic structure of the intestinal mucosa, brush border-specific enzyme activities and *in vivo* intestinal absorption of marker molecules.

Materials and Methods

Animal experiments

The experiments and treatments were conducted in compliance with the European Union regulations concerning the protection of experimental animals. The Local Ethics Committee approved the study protocol. A total of 44 male neonatal piglets (Polish Landrace × Pietrain) from nine different litters were purchased from a commercial pig farm. The average birth weight was 1.60 ± 0.15 kg. The piglets were delivered healthy and without complications, and were kept with their sows for 24 h, except six of them that were kept with their sows for 7 days (control group of sow-reared piglets (CSR)). Six animals were killed by pentobarbiturate overdose 24 h after birth, and another six were killed 7 days after birth (CSR), and sampled like those described below. The other 26 animals were installed (initially in groups of four piglets) in the laboratory in a special cage equipped with an 'artificial sow'. The laboratory provided a 12 h light:12 h darkness cycle and an ambient temperature that was decreased from 32 °C to 28 °C over the 6 days of the experiment to ensure a comfortable environment for neonatal piglets. At the beginning of each trial, four piglets were kept together for an approximately 12 h period of adaptation. After that, the piglets were housed individually in a manner providing social contact with each other. Milk-replacer formula for piglets (protein 19.8%; fat 19.7%; ash 8.2%; Milky Farm, Nukamel Olen, Belgium) was distributed to each piglet every 75 min (20 times per 24 h) in equal amounts by means of an artificial sow (Research Centre Foulum-model, Pig's oline; Boss' Produkter a/s, Denmark). The daily amount of milk replacer was calculated on the basis of daily body weight gain and protein intake. Body weight was recorded every morning. Protein intake in neonatal pigs rose gradually from 11.0 to 11.3 g/kg body weight during the first 7 days of life. The concentration of protein in milk formula was 20% of dry matter (V Lesniewska & M Hedemann, unpublished observations). Sow colostrum and milk, artificial milk formula and plasma blood samples collected from pig neonates were measured for leptin concentration using an RIA kit (Leptin Multi-Species; Linco Research Inc., St Louis, MO, USA). The assay was performed in one run.

Two series of experiments were performed in the laboratory. In the first series, 18 animals were randomly divided into three groups that received either vehicle alone (5 ml 0.9% NaCl) – control group ($n=6$), leptin

(Leptin Mouse Recombinant; Sigma, St Louis, MO, USA) at a dose of 2 µg/kg body weight – group L2 ($n=6$), or 10 µg/kg body weight – group L10 ($n=6$) every 8 h via a stomach tube. The pH of the vehicle was 5.8, which is optimal for leptin bioactivity and similar to that of the ingested milk replacer. After 6 days of vehicle or leptin treatment the animals were killed by pentobarbiturate (Vetbutal; Biowet, Poland) overdose, and the gastro-intestinal tract was removed for morphometric analysis and tissue sampling. The stomach and pancreas were weighed and the small intestine's weight and length determined. Whole-thickness samples of the duodenum, proximal (25%), middle (50%) and distal (75%) jejunum and ileum were harvested and fixed in Bouin's solution for histological analysis. The corresponding 15 cm long intestine segments were gently scraped to collect the mucosa for enzyme activity analysis. In the second series, eight piglets were divided into two groups receiving saline (CM, $n=4$) or leptin at a dose of 10 µg/kg body weight (LM, $n=4$) using an identical schedule as in the first series. On day 6 they were implanted with Silastic catheters in the left jugular vein under general halothane anaesthesia, and on the day after, a test of *in vivo* intestinal absorptive capacity was performed. In the morning, the CM and LM groups received a marker molecule cocktail by a stomach tube. The cocktail contained: 500 mg/kg BSA (67 kDa, Cat. No. A-4503; Sigma) and 9.4 mg/kg Na-fluorescein (376 Da; Merck, Darmstadt, Germany). The components were dissolved in 0.9% NaCl immediately before administration and given to the pigs in a volume of 4 ml/kg body weight. For marker molecule analysis, 4 ml venous blood samples were taken just before administration (0 h) and after 0.5, 1, 2, 4, 8 and 24 h. After the last blood sample was taken, the animals were killed by pentobarbiturate overdose. The blood samples were centrifuged at 3000 g (4 °C) for 15 min and the plasma harvested and stored frozen (–20 °C) until analysis.

Mucosa histology and laser scanning cytometry (LSC)

Serial histological sections of 5 µm thickness were cut and stained with haematoxylin and eosin for morphometric analysis under a light microscope. After staining, the depth of crypts, length of villi and thickness of the tunica mucosa were measured in the small intestine preparations at a low magnification with a Nikon type 104 (Nikon Corporation, Yokohama, Japan) optical binocular microscope coupled via a camera to a computer. In each slide, 30 well-oriented villi and crypts were measured using Lucia G v4.60 software (Laboratory Imaging Ltd, Prague, Czech Republic). In each slide from the jejunum and ileum, all of the enterocytes in 30 well-oriented villi were analysed for the presence of 'lysosomal vacuoles' (LV) and the percentage of vacuolated enterocytes was calculated (Biernat *et al.* 1999). In each villi, 20 vacuolated enterocytes were measured for the area of LVs (µm²) using a

planimetry feature of the Lucia G v.4.60 software. The mitotic index was estimated in the jejunal crypts by calculating the percentage of mitotic cells in 1000–1200 counted cells (Biernat *et al.* 1999).

The rate of apoptosis in the mid-jejunum was estimated on the basis of chromatin condensation and DNA content in the nuclei following 7-aminoactinomycin D (7-AAD) staining using LSC (CompuCyte Corp., Cambridge, MA, USA). The fluorescence excitation was provided by a 488 nm, 10 mW argon laser beam. The far-red fluorescence of 7-AAD was measured using a combination of dichroic mirrors and filters transmitting at >650 nm (detector offset 2000 and gain 30). For chromatin condensation another parameter was acquired, corresponding to the highest value of DNA-related fluorescence in the cell – the DNA maximal pixel. In the mid-jejunum, the mitotic index to apoptosis ratio was measured.

Brush border enzyme activity

Brush border enzyme activity was measured in the mucosa scrapings from the proximal, middle and distal parts of the jejunum. The mucosa was scraped off with a microscope slide, and deep-frozen in liquid nitrogen (–80 °C). After thawing, the intestinal mucosa was homogenized in cold distilled water (1 g intestinal mucosa/5 ml distilled water) and centrifuged for 5 min at 1000 *g* at 4 °C. The protein content was then determined as described by Hartree (1972), using BSA as the standard. The activities of aminopeptidase A and N were assayed with L-glutamyl-*p*-nitroanilide and L-leucyl-*p*-nitroanilide as substrates respectively (Maroux *et al.* 1973), and that of dipeptidase IV was assayed with glycyl-L-prolyl-*p*-nitroanilide (Nagatsu *et al.* 1976). The resulting enzymatic units (IU) are expressed as $\mu\text{mol } p\text{-nitroanilide released/min}$ at 37 °C. Lactase, maltase and sucrase were determined as described earlier (Dahlquist 1964) with minor modifications.

Marker macromolecule analysis

The concentrations of marker molecules in the plasma samples from the second series of experiments were analysed according to Nejdfor *et al.* (2000). The fluorescence of Na-fluorescein was measured in a 96-microwell plate (Nunc, Roskilde, Denmark) by spectrophotofluorometry (CytoFluor 2300; Millipore, Bedford, MA, USA), using a filter set-up of 485 nm for excitation and 530 nm for emission. The standards for Na-fluorescein were dissolved in PBS. BSA was quantified by electroimmunoassay (Laurell 1966), using purified BSA diluted in swine serum as a standard and rabbit anti-BSA antibodies (Dako, Glostrup, Denmark). Analysis of the area under the curve (Graph Pad Software v.2.0, San Diego, CA, USA) was performed to compare the marker molecule absorption between CM and LM groups.

Statistical analyses

The data are expressed as means $\pm$ S.E.M. One-way ANOVA followed by the Tukey post-ANOVA test was used to indicate the statistical differences between the groups in the first series, and the unpaired Student's *t*-test or non-parametric Mann–Whitney test was used to indicate the statistical differences between the groups in the second series (Graph Pad Software v.3.03). In all statistical analysis $P < 0.05$ was taken as the level of significance.

Results

After transporting from the farm, four pig neonates were kept together in one cage for 12 h; at that time (usually within 5–6 h) one of them had taught himself how to eat from the artificial sow. The other piglets soon followed the leader; thus, after separating them into individual cages all piglets ate milk formula distributed by the artificial sow. No differences in appetite between the milk formula-fed (control, CM) and leptin-supplemented (L2, L10, LM) piglets were observed, and all piglets ate all of the milk formula dosed by the artificial sow at once. The concentration of leptin in colostrum was 53.7 ± 15.2 ng/ml in the first portions, 71.7 ± 13.0 ng/ml 24 h after that, and in milk collected on day 7 postpartum it was 25.5 ± 2.5 ng/ml. In the milk formula samples the concentration of leptin was 20.3 ± 2.5 ng/ml. In the milk formula-fed piglets the concentration of leptin in the blood plasma was reduced by about 25% with respect to sow-reared piglets. Intragastric administration of leptin had no effect on plasma leptin concentration, which in control and L10 piglets equalled respectively 1.81 ± 0.44 and 1.41 ± 0.13 ng/ml.

Body and organ weights

Neonatal piglets, fed with sow's milk (CSR) between days 1 and 7 of life gained on average 270 ± 81 g daily; however, no data on their daily food intake are available. In milk formula-fed piglets the food intake was 96 ± 13 g dry matter per day. Milk formula-fed piglets gained less, 84 ± 15 g daily, and had a shorter small intestine (jejunum) and lighter pancreas compared with CSR (Table 1). Six days of leptin treatment had no effect on the body weight of the investigated neonatal piglets or on the stomach, liver, or pancreas weights relative to the body weight compared with the control group (Table 1). The length of the jejunum showed a significant increase and that of the ileum a significant reduction in the piglets treated with $10 \mu\text{g/kg}$ body weight of leptin. The length of the entire small intestine in leptin-treated piglets (L10) was not different from that found in piglets fed with sow's milk (Table 1).

Small intestine morphometry

The results of histometry analysis are shown in Table 2. Feeding with milk formula (control) reduced the crypt

Table 1 Body weight, intestine length and organ weight in sow-reared (CSR), control (C) and leptin-treated (L2, L10) piglets on day 7 of life. Values are means $\pm$ S.E.M. ($n=6$)

	CSR	C	L2	L10	P
Body weight (kg)	3.4 $\pm$ 0.5 ^a	2.1 $\pm$ 0.1 ^b	2.1 $\pm$ 0.1 ^b	2.2 $\pm$ 0.1 ^b	0.0003
Small intestine (cm)	499.2 $\pm$ 32.0 ^a	417.5 $\pm$ 15.2 ^b	431.7 $\pm$ 21.5 ^b	459.8 $\pm$ 22.0 ^{ab}	0.0060
Duodenum (cm)	7.7 $\pm$ 0.6	8.50 $\pm$ 1.2	6.7 $\pm$ 1.5	7.0 $\pm$ 0.7	0.0333
Jejunum (cm)	485.0 $\pm$ 32.0 ^a	401.4 $\pm$ 15.4 ^b	418.4 $\pm$ 20.8 ^b	448.8 $\pm$ 21.8 ^{ab}	0.0050
Ileum (cm)	6.2 $\pm$ 0.9 ^a	7.4 $\pm$ 0.8 ^a	6.6 $\pm$ 1.4 ^a	4.1 $\pm$ 0.4 ^b	0.0003
Stomach/body weight (g/kg)	5.3 $\pm$ 0.7	5.4 $\pm$ 0.4	5.1 $\pm$ 0.4	5.2 $\pm$ 0.4	0.4660
Liver/body weight (g/kg)	32.0 $\pm$ 2.7	33.8 $\pm$ 5.1	30.0 $\pm$ 2.3	34.0 $\pm$ 2.2	0.3934
Pancreas/body weight (g/kg)	1.52 $\pm$ 0.2 ^a	1.9 $\pm$ 0.2 ^{ab}	2.0 $\pm$ 0.1 ^b	1.8 $\pm$ 0.2 ^{ab}	0.0129

Different letters in a row indicate statistical significance (one-way ANOVA followed by Tukey post-ANOVA test).

depth in the duodenum, reduced the length of villi and thickness of mucosa in the proximal jejunum and increased the length of villi and thickness of mucosa in the ileum compared with natural milk feeding (CSR). Exogenous leptin caused a significant reduction of villi length and correspondingly a reduction in mucosa thickness in the middle and distal jejunum and ileum compared with milk formula-fed control piglets. Although the size of intestinal crypts did not change, the mitotic index was the lowest in the jejunum and ileum in the milk formula-fed group, and the highest in the leptin-supplemented group (Table 2). The apoptotic index analysed using LSC in the mid-jejunum in the sow-reared group (CSR) was $21.1 \pm 5.0\%$. In the milk formula-fed (control) and

leptin (L10) groups it was respectively 23.2 ± 3.0 and $19.7 \pm 1.2\%$ ($P=0.069$). The mitotic index to apoptosis ratio calculated for this segment of the jejunum in the leptin-supplemented group was, however, 1.9-fold higher compared with the control group, and only 1.16-fold higher compared with the CSR group.

In sow-reared piglets (CSR) large LVs were present in the enterocytes only in the distal jejunum and ileum, whereas in the milk formula-fed and leptin-supplemented piglets they were also in the mid-jejunum. However, in the leptin-supplemented group (L10) there were only a few LVs in the jejunum and ileum, and of smaller size compared with the CSR and control groups (Table 2 and Fig. 1).

Table 2 Histological parameters (μm), mitotic index (%), cross-section area of LV (μm^2) and percentage of vacuolated enterocytes (%) in sow-reared (CSR), control (C) and leptin-treated (L10) piglets on day 7 of life. Values are means $\pm$ S.E.M. ($n=6$)

	Crypt depth	Villi length	Tunica mucosa	Mitotic index	LV area	% LV
Duodenum						
CSR	154 $\pm$ 24 ^a	655 $\pm$ 67	848 $\pm$ 94	3.8 $\pm$ 0.2 ^a	—	0
C	116 $\pm$ 10 ^b	534 $\pm$ 105	712 $\pm$ 107	4.2 $\pm$ 0.1 ^b	—	0
L10	133 $\pm$ 7 ^{ab}	528 $\pm$ 73	673 $\pm$ 141	5.7 $\pm$ 0.2 ^c	—	0
Jejunum (25%)						
CSR	139 $\pm$ 35	831 $\pm$ 72 ^a	1010 $\pm$ 79 ^a	4.2 $\pm$ 0.2 ^a	—	0
C	109 $\pm$ 9	509 $\pm$ 65 ^b	638 $\pm$ 29 ^b	3.7 $\pm$ 0.1 ^b	—	0
L10	116 $\pm$ 6	468 $\pm$ 22 ^b	624 $\pm$ 24 ^b	5.6 $\pm$ 0.1 ^c	—	0
Jejunum (50%)						
CSR	124 $\pm$ 21	967 $\pm$ 88 ^a	1108 $\pm$ 116 ^a	5.2 $\pm$ 0.3 ^a	—	0
C	115 $\pm$ 8	865 $\pm$ 235 ^a	972 $\pm$ 224 ^a	3.5 $\pm$ 0.2 ^b	80 $\pm$ 11	43 $\pm$ 2
L10	118 $\pm$ 7	443 $\pm$ 75 ^b	603 $\pm$ 64 ^b	5.8 $\pm$ 0.2 ^c	63 $\pm$ 6*	2 $\pm$ 1***
Jejunum (75%)						
CSR	114 $\pm$ 14	842 $\pm$ 92 ^a	992 $\pm$ 103 ^a	3.6 $\pm$ 0.2 ^a	85 $\pm$ 30	72 $\pm$ 3 ^a
C	103 $\pm$ 8	886 $\pm$ 107 ^a	939 $\pm$ 157 ^a	3.0 $\pm$ 0.1 ^b	88 $\pm$ 13	72 $\pm$ 3 ^a
L10	114 $\pm$ 11	477 $\pm$ 100 ^b	658 $\pm$ 128 ^b	6.0 $\pm$ 0.1 ^c	70 $\pm$ 6	5 $\pm$ 1 ^b
Ileum						
CSR	118 $\pm$ 20	564 $\pm$ 89 ^a	713 $\pm$ 94 ^a	3.3 $\pm$ 0.1 ^a	79 $\pm$ 11 ^{ab}	96 $\pm$ 1 ^a
C	113 $\pm$ 10	798 $\pm$ 106 ^b	926 $\pm$ 113 ^b	3.0 $\pm$ 0.1 ^b	114 $\pm$ 33 ^a	98 $\pm$ 4 ^a
L10	118 $\pm$ 8	371 $\pm$ 47 ^a	519 $\pm$ 45 ^a	5.7 $\pm$ 0.2 ^c	54 $\pm$ 8 ^b	21 $\pm$ 2 ^b

Different letters in columns indicate statistical significance (one-way ANOVA followed by Tukey post-ANOVA test). * $P<0.05$, *** $P<0.001$ (Student's *t*-test followed by Mann-Whitney test).

Figure 1 Representative photographs of the villi in the ileum of milk formula-fed (A) and leptin-supplemented (B) piglets. Note that the villi in the milk formula-fed piglets are much longer than those in the leptin group, and almost all enterocytes in the milk formula-fed piglets contain large LVs in contrast to the leptin-treated group. Haematoxylin and eosin staining. Scale bars=100 μ m.

Brush border enzyme activities

In sow-reared piglets, the activity of the examined brush border enzymes (aminopeptidase A and N, dipeptidase IV, lactase, maltase and sucrase), showed normal dynamic changes during the first week of life (data not shown) related to development. Compared with sow's milk, feeding milk formula resulted in elevation in aminopeptidase A and N, dipeptidase IV and lactase activity and reduction in lactase, maltase and sucrase in the respective segments of the jejunum (Table 3). Notably, in leptin-treated piglets there was a general trend towards recovery of aminopeptidase A and N and dipeptidase IV activity to levels observed in sow-reared piglets, the activity of lactase in the mid- and distal jejunum was further decreased below the activity present in sow-reared piglets (Table 3). The activity of maltase and lactase in the proximal and distal jejunum of leptin-supplemented piglets was not different from that in piglets receiving sow's milk (CSR) or milk formula (controls) whereas in the mid-jejunum it was as low as in the milk formula-fed group (Table 3). The effects of leptin were dose-dependent (data not shown). The activity of maltase was reduced only in the distal

jejunum in leptin-treated piglets compared with the controls. Sucrase activity did not show any differences between the leptin-treated animals and controls.

Marker molecule absorption

After administration of marker molecules, the blood levels of the markers increased in both groups ($P<0.01$). The peak blood plasma concentration differed between the marker molecules in a size-dependent manner, and the peaks were seen after 30 min for Na-fluorescein and after 8 h for BSA (Fig. 2). Area under the curve analysis revealed that plasma levels for Na-fluorescein did not differ significantly between the two groups whilst that of BSA was 3.8-fold reduced ($P=0.016$) in the leptin group compared with the control. This indicates less intestinal absorption of large-size molecules in the leptin-treated piglets.

Discussion

Our studies demonstrate for the first time that leptin present in colostrum and milk may be important for

Table 3 Brush border enzyme activity (μmol/min per g mucosa) in sow-reared (CSR), control (C) and leptin-treated (L10) piglets on day 7 of life. Values are means ± S.E.M. (n=6)

	Aminopeptidase A	Aminopeptidase N	Dipeptidase IV	Lactase	Maltase	Sucrase
Jejunum (25%)						
CSR	10.0 ± 1.9 ^{ab}	2.1 ± 0.4	0.6 ± 0.1 ^a	10.8 ± 1.6	7.9 ± 2.1	4.0 ± 1.1
C	11.0 ± 2.2 ^a	3.6 ± 1.1	2.0 ± 0.6 ^b	10.6 ± 4.3	9.4 ± 3.0	2.6 ± 1.1
L10	6.6 ± 1.4 ^b	3.8 ± 1.0	1.0 ± 0.3 ^a	8.0 ± 0.8	7.4 ± 1.4	2.5 ± 1.1
Jejunum (50%)						
CSR	10.4 ± 1.4 ^a	1.7 ± 0.4 ^a	1.9 ± 0.4 ^a	10.6 ± 1.8 ^a	7.4 ± 2.2 ^a	2.6 ± 0.8 ^a
C	23.9 ± 5.5 ^b	11.6 ± 2.5 ^b	5.9 ± 0.9 ^b	16.7 ± 3.6 ^b	3.2 ± 0.6 ^b	0.6 ± 0.3 ^b
L10	6.7 ± 0.8 ^a	3.9 ± 1.3 ^a	1.1 ± 0.3 ^a	5.0 ± 0.3 ^c	3.8 ± 0.8 ^b	0.3 ± 0.1 ^b
Jejunum (75%)						
CSR	13.8 ± 2.6 ^a	10.4 ± 2.3 ^{ab}	4.6 ± 1.3	12.1 ± 1.0 ^a	1.9 ± 0.3	1.8 ± 0.3 ^a
C	27.6 ± 5.3 ^b	15.4 ± 3.4 ^a	6.3 ± 0.7	6.2 ± 1.6 ^b	3.3 ± 0.8	0.4 ± 0.3 ^b
L10	15.4 ± 3.5 ^a	6.5 ± 1.2 ^b	4.1 ± 0.9	3.6 ± 1.1 ^c	1.9 ± 0.5	0.7 ± 0.2 ^{ab}

Different letters in columns indicate statistical significance (one-way ANOVA followed by Tukey post-ANOVA test).

controlling the development of small intestine structure and function in neonatal piglets. Importantly, the clear-cut effects of leptin supplementation on intestinal mucosa morphometry, presence of LVs in the enterocytes, proliferation of mucosa epithelial cells, enterocyte brush border enzyme activities and marker molecule absorption were observed following doses of leptin that can be considered physiological.

The concentrations of leptin in sow's milk, and in the blood plasma in piglets, correspond to those previously reported (Estienne *et al.* 2000), although no data on leptin in sow colostrum and milk at the very beginning of lactation are available. In our study, the concentration of leptin in milk formula, being roughly one-third of that in the colostrum and four-fifths of that in milk, was much higher than previously reported for human milk formula (Houseknecht *et al.* 1997, Resto *et al.* 2001). The difference is most probably related to the applied technologies of producing milk formula (Resto *et al.* 2001). Thus, the higher dose of leptin in our study was sufficient to supplement the neonates in the colostrum period, and the lower dose was sufficient thereafter. Unfortunately, we did not have a chance to access the sows for additional

sampling to get more precise kinetics of the leptin concentration in colostrum and milk. In milk formula-fed piglets, plasma leptin did not increase in response to enteral administration of leptin, which may suggest that: (i) leptin is not transferred from the gut into the circulation, which is in contrast to the findings in suckling rats by Casabiell *et al.* (1997), and (ii) the effects observed in the present study depend on leptin receptors localized in the intestinal mucosa (Morton *et al.* 1998, Barrenetxe *et al.* 2002).

We observed a reduction in the body weight of artificially reared vs sow-reared piglets; however, in contrast to previous studies in neonatal rats (Yuan *et al.* 2000), we did not observe body weight reduction following leptin treatment. This can be related to either the much higher doses of leptin applied and/or different route of leptin administration (i.p.) in the rat study. Possibly, the lack of differences in the body weight between milk formula-fed and leptin-supplemented piglets in our study could also be related to similar intake of milk formula in both groups. Nevertheless, we postulate that body weight regulation is rather not a direct target for milk leptin. Earlier studies suggested that leptin can restrict body energy

Figure 2 Mean blood plasma levels of marker molecules, Na-fluorescein and BSA during 24 h after marker gavage feeding to milk formula-fed (○; n=4) and leptin-treated (●; n=4) neonatal piglets.

accumulation indirectly through a local mechanism in the small intestine controlling the absorption of nutrients (Lostao *et al.* 1998, Morton *et al.* 1998) or intestinal motility (Woliński *et al.* 2001). Lostao *et al.* (1998) demonstrated that leptin has a rapid inhibitory effect on D-galactose uptake in the rat intestinal mucosa *in vitro*. In the same year, Morton *et al.* (1998) showed that leptin can reduce the transcription of genes responsible for apolipoprotein biosynthesis (APO-AIV) in the enterocytes in mouse jejunum in response to olive oil administration. Correspondingly, in the present *in vivo* study we observed marked activity reduction of all examined brush border enzymes (except sucrase) in leptin-supplemented piglets. It should be borne in mind, however, that in contrast to natural feeding, milk formula enhances the activity of aminopeptidases and dipeptidase IV, and leptin supplementation depressed their activity to the level seen in the sow-reared piglets. Recently, we have shown that repetitive intragastric administration of leptin influences *in vitro* small intestine contractile activity (Woliński *et al.* 2001) thus revealing a possible effect of leptin on digesta passage. Leptin increased the spontaneous and acetylcholine-stimulated activity of the duodenum and reduced the activity of the jejunum (Woliński *et al.* 2001).

Replacement of sow's milk with milk formula inhibits the growth of the small intestine as well as slows the maturation of the intestinal mucosa as evidenced by low mitotic index, abundance of vacuolated enterocytes in the mid-jejunum and high lactase and peptidase activities. Enteral supplementation with leptin in part reverses these effects. Namely, leptin selectively and dose-dependently increases the length of the small intestine, reduces the thickness of intestinal mucosa by decreasing the height of villi, and significantly reduces the activity of brush border lactase and proteases, regarded as enzymatic markers of gut maturation (Henning 1987, Klein 1989). Stem cell proliferation in the intestinal crypts is enhanced and simultaneously cell apoptosis slightly reduced, thereby the mitotic index to apoptosis ratio becomes similar to that in sow-reared piglets. These effects are consistent with previous immunocytochemical studies (Barrenetxe *et al.* 2002) showing the presence of leptin receptors in the mucosa on the entire length of the small intestine. Small numbers of LVs in the jejunum and ileum following leptin-supplementation together with an increased mitotic index imply that the irreversible process of replacing foetal-type enterocytes with adult-type ones (Baintner 1994) has been accelerated (Biernat *et al.* 1999, Radberg *et al.* 2001). The decrease in the endocytosis rate due to the presence of adult-type enterocytes may explain the sharp reduction in BSA absorption in leptin-supplemented piglets in the present study (Smith & Peacock 1980). No difference in the absorption of the inert and passively transported small-size molecule (Na-fluorescein) suggests that functionally the intestinal absorptive area is not restricted following leptin supplementation.

In conclusion, feeding neonatal piglets with milk formula slows the maturation of the small intestinal mucosa, and exogenous leptin given into the stomach (in a range that is present in colostrum and milk) may reverse the process as evidenced by morphological and functional studies.

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