Obesity and Changes of Alkaline Phosphatase Activity in the Small Intestine of 40- and 80-day-old Rats Subjected to Early Postnatal Overfeeding or Monosodium Glutamate

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Summary

To investigate the relationship between development of obesity and the small intestinal functions two experimental models of male Wistar rats were used in the present work: 1) early postnatally overfed rats, nursed from birth to weaning in small litters (SL, 4 pups/nest), and 2) neonatally monosodium glutamate treated rats (MSG 2 mg/g b.w. administered s.c. for 4 days after birth) submitted to the same early nutritional manipulation. After weaning, all animals had free access to a standard pellet diet and at 40 and 80 days of age their body weight, body fat content and food consumption as well as changes of the brush-border-bound duodenal and jejunal alkaline phosphatase (AP) activity were compared with parameters of the offsprings raised under normal feeding conditions (NL, 8 pups/nest). At 40 and 80 days of age the postnatally overfed pups from SL nests became heavier, displayed a significantly increased epididymal plus retroperitoneal fat pad weight (P<0.01) and significantly higher AP activity in both segments of the small intestine (P<0.01) in comparison with rats nursed in NL nests, although their mean daily food intake did not differ from that of non-obese rats during the postweaning periods examined. In contrast, the same treatment of MSG rats had only a small effect on late appearance of obesity, i.e. in early postnatally overfed and normally fed MSG rats a similar pattern of body weight, food intake, adiposity and AP activity was found after weaning. The effect of MSG-treatment was also accompanied by the appearance of normophagia, hypophagia and stunted growth on day 40 and day 80, respectively. Moreover, the size of fat depots and the increase of brush-border-bound AP activity in MSG rats belonging to the SL and NL groups was quantitatively similar to the values size of these parameters observed in SL obese rats subjected to early postnatal overnutrition. These results indicate that postnatal nutritional experience (overnutrition) may represent a predisposing factor in control rats from small litters for the development of obesity in later life. Permanently increased small intestinal AP activity observed after weaning in both models of obesity when hyperphagia is not present suggest that these functional changes and associated alterations in food digestion could be a component of regulatory mechanisms contributing to the maintenance of their elevated body fat weight.

Introduction

Increased food consumption surpassing the energetic requirements of the organism and manifested by excessively increased body fat stores presents a growing human health problem. Qualitative and quantitative nutritional conditions in early life may have persistent effects on body weight and body composition. These late metabolic responses on early nutritional experiences characterized as "metabolic imprinting" has been observed in both human epidemiologic and experimental animal studies (Waterland and Garza 1999, Moura *et al.* 2002). There is no doubt that the gastrointestinal tract and the physiological processes of digestion may also be a significant factor promoting obesity. This is consistent with the more effective absorption of food in the upper part of the intestine of obese subjects (Wright *et al.* 1983, Wisen and Johanson 1992) and with the efficacy of energy intake limitation and decreased intestinal absorption of nutrients in the treatment of overweight and obesity (Muzzarelli 1996, Hollander *et al.* 1998). Besides a genetic predisposition, the mechanisms enabling higher energy utilization and storage of the ingested nutrients in adipose depots and increasing the probability of obesity development also involve the appropriate environmental stimulus, such as nutrition (Bouchard 1991, DeCastro 1993, Hill and Peters 1998).

Despite the great amount of data concerning nutrition in early life, there is no general agreement regarding the way in which nutritional events influence functionality of the small intestine in later life. Application of the experimental model in which overfeeding during the suckling period was induced by reducing the litter size at birth (3-4 pups/mother) demonstrated enhanced lipid synthesis in the liver and increased activity of lipogenetic enzymes in fat cells (Duff and Snell 1982, Dugail *et al.* 1986, Bassett and Craig 1988, Fiorotto *et al.* 1991), as well as increased plasma insulin and triacylglycerol levels (Dugail *et al.* 1986, Plagemann *et al.* 1992, 1999, Schmidt *et al.* 2001), which in turn may stimulate growth of body fat weight (Bassett and Craig 1988, Fiorotto *et al.* 1991, Plagemann *et al.* 1992, Voits *et al.* 1996, Davidowa and Plagemann 2000). Although it is clear that these alterations observed prior to weaning might be attributed to the higher quantity of milk available for the individual suckling pup nursed in smaller litters than normal (Fiorotto *et al.*1991), the mechanisms responsible for persisting hyperphagia and obesity after the weaning period are poorly understood. There is evidence that ontogeny of the small

intestine and growth of the pups can also be influenced by lipid changes in the milk. Milk lipid concentrations varied considerably in individual rats, although their feeding did not differ (Mozeš *et al.* 1993) and increased in dams nursing small litters (4 pups/nest) as compared to dams nursing large litters (16 pups/nest) (Fiorotto *et al.* 1991). High-fat diet feeding during pregnancy and lactation lead to an increase of milk fat concentrations even in dams nursing normal nests (Del Prado *et al.* 1997, Trotier *et al.* 1998). This was reflected by an increase of body fat, body weight and uptake of glucose and fatty acids in the jejunum of the offspring (Del Prado *et al.* 1997, Trotier *et al.* 1998, Jarocka-Cyrta *et al.* 1998, Guo and Jen 1995). On the other hand, early undernutrition induced by an increased number of rat pups in the nest (16-18 pups) led to changes in the structure of the brush border membrane; it decreased AP and sucrase activity and increased lactase and leucine aminopeptidase activity in the small intestine (Pathak *et al.* 1981). From this point of view, the quantitative and qualitative changes in milk intake may have a pronounced modulational influence on the functional development of the intestinal tract throughout the postnatal period. However, little attention has been paid to the significance of these changes and their consequences on small intestinal function, food intake and body fat during later periods of life.

The model of neonatally monosodium glutamate (MSG)-treated rats is of special interest in the development of obesity which is not a result of overeating. MSG administration during the early postnatal period results in a sequence of events including hypothalamic neuronal injuries (Olney 1969, Sun *et al.* 1991, Dawson *et al.* 1997), increased plasma levels of insulin, corticosterone and triglycerides (Abe *et al.* 1990, Macho *et al.* 1999, 2000, Dolnikoff *et al.* 2001), as well as increased lipogenesis and reduced lipolysis by adipose tissues (Macho *et al.* 2000, Dolnikoff *et al.* 2001) which ultimately supports the development of obesity apparent after 80 days of age. In mature MSG rats, an enhanced capacity to spare body fat stores during fasting, more marked adaptational increase of food intake and more rapid body fat restoration was demonstrated after refeeding (Raček *et al.* 2001). Moreover, permanently increased alkaline phosphatase activity in the brush-border of duodenal enterocytes has also been demonstrated in adult rats postnatally treated with MSG during *ad libitum* feeding (Mozeš *et al.* 2000, Martinková *et al.* 2000) and after dietary manipulations as food restriction, fasting and refeeding (Raček *et al.* 2001). These results indicate that the enzymatic changes

observed in the small intestine of mature MSG rats may be a component of regulatory mechanisms maintaining their obesity at critical values and contributing to the development of the MSG syndrome. Another question is whether MSG obesity and the alterations of intestinal function could also be influenced by early nutritional experiences and if the effect of these changes coincides with the mechanisms that mediate a predisposition to the later development of feeding and body fat perturbation observed in another form of obesity.

We therefore determined the changes on day 40 and day 80 in the brush-border-bound duodenal and jejunal alkaline phosphatase activity, food intake and body growth parameters in postnatally overfed (4 pups/nest) and normally fed MSG rats (8 pups/nest) as well as in their saline-treated control littermates submitted to the same postnatal nutritional manipulations.

Methods

Experimental animals

A total of 22 Wistar rat dams (Velaz Prague, Czech Republic) were used in the present study. The mothers and their offspring were housed from birth to weaning in Plexiglass cages in a temperature-controlled environment of 22±2 °C with a relative humidity of 60±15 % and 12L:12D regime (light on at 06:00-18:00 h) with free access to a standard laboratory diet (Velaz/ Altromin 1520 DOS 2b, Velaz, Prague) and tap water.

To induce early postnatal overnutrition or normal nutrition, the litter size was adjusted on the first day after birth to four pups *per* nest (small litters, SL, n=12) and eight pups per nest (normal litters, NL, n=10). All groups consisted of males and females. Half of the number of pups in each nest received s.c. injection of monosodium salt of L-glutamic acid (2 mg/g b.w., Sigma, St. Louis, MO) (MSG groups) or an equivalent volume of 1.25 % saline solutions (control groups) daily for 4 days after birth. MSG was dissolved in distilled water and the drug concentration was adjusted so that each pup received 50 µl of solution/g/b.w. After weaning (on day 30), the male rats were housed in pairs in Plexiglass cages under the same conditions (diet, water, temperature, light-dark regime) as before weaning.

Milk composition

The dams were milked on day 8 and 15 of lactation. Two hours prior to milking (at 08:00 h), the dams were separated from their pups which during that time were kept in a constant-temperature chamber at 34±1 ºC. Ten minutes before milking the dams received an intraperitoneal injection of 2 IU of oxytocin. The milk was obtained by a procedure described in detail by Mozeš *et al*. (1993). Milk fat was determined by the crematocrit method of Lucas *et al.* (1978). The fat concentration was expressed in g/100 ml milk by the formula given by Nagasawa *et al*. (1989) milk fat = 0.1 x (crematocrit in % – 0.59)/0.146. Milk protein concentration was analyzed by the method of Lowry *et al*. (1951). The values were expressed in g/100 ml milk.

Enzyme assay

On day 40 or 80 the animals were killed by decapitation after an overnight fast between 08:00 and 09:00 h. Small (0.5 cm) segments of the proximal duodenum and middle part of the jejunum were immediately removed, the lumen rinsed in distilled water, and frozen in hexane (–70 °C). On the day of the experiment a segment of the frozen tissue was cut (8 µm) in a cryostat at –25 °C and the tissue slices were transferred to glass slides and air-dried.

Demonstration of alkaline phosphatase activity was performed by using a modified simultaneous azo-coupling method (Lojda *et al.* 1979). The incubation medium contained 2.0 mM naphthol AS-BI phosphate (Sigma, Deisenhofen, Germany), 0.8 mM Hexazotized New fuchsin (Serva, Heidelberg, Germany), 20 mM N,N-dimethylformamide (solvent of naphtol AS -BI phosphate) and 0.05 M veronal acetate buffer. The sections were incubated at 37 °C for 10 min at pH 8.9 in the presence of 13 % (w/v) polyvinyl alcohol (PVA; 30 000-70 000 m.w. Sigma) (Mozeš *et al.* 1998).

Enzyme activity was cytophotometrically analyzed with a Vickers M85a microdensitometer. The measurements were performed using a x 40 objective, an effective scanning area of 28.3 μm^2 and a scanning spot of 0.5 µm. The integrated absorbance was measured at a wavelength of 520 nm (Frederiks *et al*. 1987). The mask was set over at least 30 brush border areas along the villus length in five sections of the duodenum and the jejunum. The AP activity was calculated as the absorbance values recorded by the instrument/min/μm^3 brush border ± S.E.M. and their mean values were referred to one animal.

From day 34-39 and 74-79 the MSG-treated and control rats were individually housed and food intake was measured at 24-h intervals as the difference between the amount offered and the remains in the cups. Epididymal and retroperitoneal fat was removed after killing the animals and the wet weight of the whole pad obtained.

Statistical analysis

Data were expressed as mean ± S.E.M. Statistical evaluation of the results was carried out by one-way analysis of variance (ANOVA). The post-hoc Bonferroni t-test was used to compare the differences between the groups.

Results

Litter size reduction resulted in a significant increase of milk fat concentration on postpartum day 8 and day 15. In comparison with NL mothers (n=10) the mean values of milk fat concentration in SL nursing dams (n=12) were 19.3±1.0 vs. 16.1±0.8 g/100 ml (P<0.05) on day 8 and 20.5±0.8 vs. 16.5±0.7 g/100 ml (P<0.01) on day 15. As compared to milk fat the concentration of milk protein during these periods did not differ between SL and NL mothers (8.8±0.6 vs. 9.7±0.5 g/100 ml on day 8 and 8.9±0.2 vs. 10.0±0.3 g/100 ml on day 15).

From day 10 until day 30 of age the overfed pups raised in SL became heavier and displayed significantly higher weight gain than those raised in NL. These differences persisted until day 40 (Table 1) in the control rats while in MSG-treated rats they gradually disappeared till weaning. The onset of growth stunting effect of neonatal MSG administration depended to some extent on their preweaning nutritional condition. While in the overfed MSG rats coming from SL body weight appeared to be decreased earlier (on day 40 of age), in NL rats stunted growth was observed as late as on day 80 as compared to their saline-treated littermates (Tables 1 and 2). Tables 1 and 2 showed that overfeeding induced by litter size reduction increased both absolute and relative weight of the fat tissues in control rats (saline-treated as neonates) as compared to the controls raised in normal litters (P<0.01 on day 40 and P<0.05 on day 80). On the other hand, the development of obesity in MSG-treated neonate rats did not solely depend on their previous nutritional experience i.e. the same excess of epididymal plus retroperitoneal fat pad weight was observed in postnatally overfed pups as in postnatally normally fed animals on day 80.

Table 1. Alkaline phosphatase (AP) activity, body weight, body fat and food intake of rats aged 40 days which were raised in litters of 4 (SL) or 8 (NL) pups treated with monosodium glutamate (MSG) or saline (control) during the early neonatal period.

	Controls		MSG	
	SL (n = 9)	NL (n = 11)	SL (n = 9)	NL (n = 11)
AP activity				
Duodenum	17.4 ± 0.6**	13.3 ± 0.7	19.4 ± 0.9	17.8 ± 0.6[c]
Jejunum	15.7 ± 0.6**	12.3 ± 0.5	17.1 ± 0.8	16.2 ± 0.3[c]
Body weight (g)	184.0 ± 5.7*	160.6 ± 6.3	154.4 ± 6.2[b]	147.7 ± 3.9
Fat (g)	2.28 ± 0.18**	1.46 ± 0.17	1.76 ± 0.11	1.35 ± 0.10
Fat (% b. w.)	1.27 ± 0.07**	0.90 ± 0.08	1.13 ± 0.06	0.91 ± 0.06
Food intake (g/day)	21.5 ± 0.6	19.1 ± 0.9	16.8 ± 0.6[c]	16.6 ± 0.5
Food intake (% b. w.)	11.7 ± 0.2	11.8 ± 0.3	10.9 ± 0.3	11.2 ± 0.2

Data are means ± S.E.M. AP activity is given as the integrated absorbance in min/μm^3 brush border of individual duodenal and jejunal enterocytes at a wavelength of 520 nm. Fat represents epididymal plus retroperitoneal pads. The 24-hour food intake are means of five measurements (day 34-39) for each rat. Significantly different from NL group: *P<0.05, **P<0.01. Significant differences between similarly treated MSG and control groups: [b]P<0.01, [c]P<0.001.

Food intake expressed in g/day or in g/100 g b.w. did not differ between postnatally overfed and normally fed control groups on day 40 and 80 (Tables 1 and 2). On the other hand, animals receiving MSG as neonates, consumed about 20 % and 14 % less food (P<0.001, P<0.05) on day 40, as well as about 14 % and 19 % less (P<0.05, P<0.01) on day 80 compared to their saline-treated littermates. However, this tendency was not confirmed (except in MSG rats arising from NL), when food intake was expressed in % of body weight (Tables 1 and 2).

Table 2. Alkaline phosphatase (AP) activity, body weight, body fat and food intake of rats aged 80 days which were raised in litters of 4 (SL) or 8 (NL) pups treated with monosodium glutamate (MSG) or saline (control) during the early neonatal period.

	Controls		MSG	
	SL (n = 8)	NL (n = 9)	SL (n = 8)	NL (n = 9)
AP activity				
Duodenum	22.5 ± 0.8***	17.7 ± 0.7	23.5 ± 0.5	21.6 ± 0.4[c]
Jejunum	20.3 ± 0.6*	17.1 ± 0.7	20.9 ± 0.9	19.8 ± 0.7[a]
Body weight (g)	423.3 ± 15.9	411.1 ± 9.5	356.4 ± 11.1[b]	358.0 ± 8.9[b]
Fat (g)	12.7 ± 0.8*	9.0 ± 0.9	11.2 ± 1.0	10.9 ± 0.8
Fat (% b.w.)	3.0 ± 0.11*	2.18 ± 0.18	3.14 ± 0.28	3.05 ± 0.23[a]
Food intake (g/day)	28.7± 1.2	29.5 ± 1.0	24.5 ± 1.2 [a]	23.8 ± 1.0[b]
Food intake (% b.w.)	6.8 ± 0.08	7.2 ± 0.08	6.9 ± 0.15	6.6 ± 0.21[a]

Data are means ± S.E.M. AP activity is given as the integrated absorbance in min/μm^3 brush border of individual duodenal and jejunal enterocytes at a wavelength of 520 nm. Fat represents epididymal plus retroperitoneal pads. The 24-hour food intake are means of five measurements (day 74-79) for each rat. Significantly different from NL group: * $P<0.05$, *** $P<0.001$. Significant differences between similarly treated MSG and control groups: [a]$P<0.05$, [b]$P<0.01$, [c]$P<0.001$.
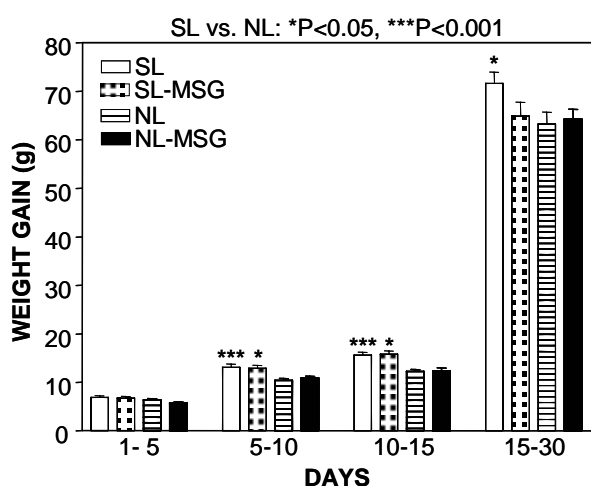

Fig. 1. Mean weight gain of MSG-treated (n=17) and saline-treated (n=18) rat pups raised four per litter (SL) or eight per litter (NL) (MSG n=18 and control n=18, respectively) from birth until weaning.

Alkaline phosphatase (AP) activity in the duodenum and jejunum of 40- and 80-day-old rats is shown in Tables 1 and 2. Compared with preweaning overnutrition, the obese rats (neonatally saline-treated) from SL nests displayed a significantly increased enzyme activity when compared to NL reared lean controls. The differences of AP activity between SL and NL rats were about 28 % and 23 % in the duodenum and 25 % and 19 % in the jejunum on day 40 and 80, respectively. Despite a similar tendency, no significant differences between their AP activity was found at these periods of

life in neonatally MSG-treated SL and NL rats. AP activity in the small intestine also corresponded with the higher values observed in obese SL controls. Moreover, an increase of brush-border-bound AP activity in the NL MSG rats on day 40 preceded the development of excessive body fat accumulation.

Discussion

Significantly increased body fat observed in overfed intact males as compared with normally fed rats is in agreement with earlier studies (Bassett and Craig 1988, Fiorotto *et al.* 1991, Plagemann *et al.* 1992, Voits *et al.* 1996, Davidowa and Plagemann 2000) in which obesity was induced by early litter size reduction. It was also demonstrated in some experiments that these obese rats showed an enhanced food intake which persisted from weaning until day 90 of age (Plagemann *et al.* 1992, Voits *et al.* 1996). This is at variance with our results suggesting nearly similar food consumption of obese and lean rats on day 40 and 80. This discrepancy might be due to the higher range in the number of pups between reduced and normal nests (3-4 vs. 10-12 pups/nest) or to the fact that the observed higher mean food intake/rat in small litter rats might also be attributed to their higher body weight. Experimental studies concerning long-term consequences of early overnutrition in various hyperphagic obesity models also showed conflicting results. Evidence has been provided that food restriction after weaning in obese male rats raised from small litters

inhibited their weight gain and decreased epididymal fat weight (Bassett and Craig 1988). On the other hand, ob/ob mice and Zucker rats with a genetic predisposition to overeating that emerges during the suckling period and persists after weaning (Wilson *et al.* 1989, Kowalski *et al.* 1998) demonstrated an ability to develop full obesity throughout life despite nutritional deficiency induced at birth by increasing the litter size (Johnson *et al* 1973, Cleary *et al.* 1980). Consistently with the concept that MSG-induced obesity occurs without an elevated food intake, no differences were observed in our experiments between both absolute and relative food consumption after weaning in neonatally overfed or normally fed MSG rats. From this point of view, our results suggest that the development of excess body fat in MSG-treated rats and their control littermates may be a critical consequence of early overfeeding despite the different pathogenesis of obesity, whereas from the aspect of long-term modulation the maintenance of their adiposity at higher levels does not solely depend on postweaning overnutrition.

The exact mechanism by which postnatal overnutrition may lead to permanent functional changes of the small intestine has not been fully elucidated. Until day 15, the rat intestine is exclusively exposed to a liquid diet (milk) which is high in protein and fat but low in carbohydrates (Godbole *et al.* 1981, Mozeš *et al.* 1993). The high growth rate observed during this time period is in agreement with the increase of length, thickness and weight of the small intestine (Sakata and Setoyama 1997) and with the presence of transporter systems and their uptake capacity for glucose, aminoacids and fats (Flores *et al.* 1989, Toloza and Diamond 1992). The milk intake increased from birth up to day 15 and its participation in the overall food intake after this time decreased and was gradually changed by solid food rich in carbohydrate but poor in lipids and proteins. The development of the gastrointestinal tract, besides the nutritional factors, is substantially influenced by hormones. Among the hormones that may have influenced the intestinal enzymes and thus explained the increased AP activity in the present experiment, insulin and corticosteroides should be considered as eligible candidates. Exogenous administration of these hormones to suckling rodents around day 12-15 resulted in a precocious increase of lactase, sucrase, aminopeptidase and AP activity (Arsenault and Menard 1984, Buts *et al.* 1990, 1998, McDonald and Henning 1992, Yeh *et al.* 1994). In addition, significantly increased basal plasma insulin levels were observed on day 14 in rats from small litters

as compared with rats from normal litters (Plagemann *et al.* 1992).

Alkaline phosphatase (AP) is a representative brush-border enzyme functionally involved in nutrient (fat) absorption and the transport of long-chain fatty acids in the intestinal mucosa (Takase and Goda 1990, Bernard *et al.* 1992). In mature rats, AP activity increased in the duodenal enterocytes after eating fat (Alpers *et al.* 1995, Zhang *et al.* 1996); it displayed circadian fluctuations in the duodenum in a close relation to the rhythm of food intake (Martinková *et al.* 2000) and markedly decreased after food deprivation (Raček *et al.* 2001). In suckling rats the activity and the levels of AP mRNA in the small intestine gradually increased from day 12 to day 24 (Yeh *et al.* 1994) and changes in nutrition (i.e. fasting) on day 14 led to a decrease of membranous AP levels in the entire small bowel as compared to normally fed pups (Young *et al.* 1981). Our present results concerning the increased AP activity in 40-day-old obese control as well as MSG rats, when both groups displayed normophagia, clearly indicate that these changes might be a consequence of their early postnatal overnutrition. It could be speculated whether abundance of food in the gastrointestinal tract during lactation or hormonal/ metabolic changes, or both, may be of importance for precocious maturation and adjustment of enzyme activity at the higher level. Although similar AP variations after weaning were observed in both groups of overfed rats raised in reduced litters, some differences between MSG and control rats do exist. As compared to the controls, MSG-treated rats exhibited a significantly higher AP activity even when they were postnatally normally fed which suggest that MSG administration may *per se* lead to the development of lasting functional changes of the small intestine to nutritional changes. Such a possibility did not exclude the similar AP values observed on day 40 in MSG as well as in neonatally overfed control rats. Considering the finding that enzymatic changes were proportional to the size of previous overfeeding in these different models, it is possible that the excess of overnutrition was sufficient in MSG rats to induce maximal elevation of enzyme values but this was not the case in their saline-treated littermates. Such a possibility suggests the absence of an additional increase of AP activity and development of more marked obesity in control rats.

One of potentially interesting aspects of our present results is that the increased size of body fat tissue observed in postweaning rats is closely related to the

enhanced AP activity in the small intestine. It was also found that in comparison to their lean littermates genetically obese rats and mice exhibited a significantly higher intestinal enzyme activity (Flores *et al*. 1990, Adachi *et al* 1999) that surprisingly precedes the development of excessive body weight. From this point of view similar mechanisms of an increased enzyme "set point" may be involved in the modulation of body fat in several forms of obesity in which body weight and body fat changes cannot be explained by hyperphagia only. Our results have also revealed that unspecific early postnatal overnutrition could represent a predisposing factor for the permanent increase of brush-border-bound duodenal and jejunal AP activity. These results extend our knowledge about the effect of early life nutrition, (its variation in quantity or macronutrient composition), upon the functional maturation of the small intestine in obese rats and allow better understanding of the mechanisms that may limit or prevent the obesity risk in later life. However, with regard to the presented data, further detailed studies will be needed to clarify whether the effectiveness of nutrient absorption, the sustained increase of AP activity or both may be of importance for the maintenance of elevated body fat weight once obesity had been established.

Acknowledgements

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