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Differences in Enterocyte Brush Border Enzyme Activities in Ageing Rats Reared in Germ-Free and Conventional Conditions

H. KOZÁKOVÁ, R. ŠTĚPÁNKOVÁ, Z. ŘEHÁKOVÁ, J. KOLÍNSKÁ[1]

Department of Immunology and Gnotobiology, Institute of Microbiology and [1]Institute of Physiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic

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Summary

The aim of this study was to evaluate the levels of disaccharidase and dipeptidyl peptidase IV activities in rat jejunal enterocytes under the influence of long-term germ-free conditions. We found that the brush-border lactase and dipeptidyl peptidase IV activities were two to three times higher in 2-month-old germ-free rats in comparison with their conventional counterparts. The highest effect of germ-free condition was observed on lactase activity in 6-month-old and dipeptidyl peptidase IV in 2-month-old rats. No difference between germ-free and conventional rats in sucrase and glucoamylase activities was found in 2-month-old rats. The difference develops with increasing age, sucrase activity becoming significantly higher in 6- and 12-month-old rats and glucoamylase in 12-month-old germ-free rats.

Key words

Enterocytes – Lactase – Sucrase – Glucoamylase – Dipeptidyl peptidase IV

Introduction

Germ-free animals are ideal models for the study of microbial influences on the development of enterocyte membrane markers. Under conventional conditions, the intestinal microflora is established within a few days after birth; thereafter, continuous stimuli from the intestinal microflora including its metabolites may be essential for normal development and maturation of the host animal and its immune system (Tlaskalová *et al.* 1970, Umesaki *et al.* 1982, Tlaskalová-Hogenová *et al.* 1983, Savage 1984, 1986). The indigenous microflora also influences the turnover of enzymic activities in enterocytes of the small intestine involved in the digestive process. Comparative studies of the possible microbial influences on intestinal morphology and on various disaccharidase activities in small intestinal mucosa homogenates using germ-free (GF) and conventional (CV) mice were presented by Abrams *et al.* (1963) and using rats by Reddy and Wostmann (1966) and Kawai and Morotomi (1978). Abrams *et al.* (1963) showed that the absence of microflora resulted in a marked increase in the life span of ileal epithelial cells. Differences in the activity of several disaccharidases including sucrase, lactase and trehalase between GF and CV rats were not apparent until after weaning when the GF animals exhibited higher activities than the CV rats. When the GF rats were conventionalized by introduction of caecal contents from the CV rats, the disaccharidase activities were reduced to normal conventional levels. Not only the activities of the enzymic glycoproteins of the microvillar membrane, but also the synthesis of sugar chains of membrane-associated glycoproteins

Fax +420 2 293 894
E-mail physres@sun1.biomed.cas.cz
http://www.lf2.cuni.cz/physiolres/physiolres.htm

were affected by the introduction of microorganisms (Umesaki *et al.* 1982, Bry *et al.* 1996). Conflicting results were presented for intestinal alkaline phosphatase of GF animals. While the findings of Kawai and Morotomi (1978) suggested that alkaline phosphatase activity was strongly depressed by the association with the indigenous microorganisms in the epithelial mucosa of the upper small intestine of rats, Whitt and Savage (1988) reported that specific activity of alkaline phosphatase did not differ in cells isolated from GF mice and from those associated with microflora. To our knowledge, no information on peptidases, especially, dipeptidyl peptidase IV activity on the enterocytes of GF rats has yet been presented.

The goal of this study was to compare the activities of lactase, sucrase, glucoamylase and dipeptidyl peptidase IV of isolated brush borders of germ-free and conventional rats at various ages until one year of life. In order to differentiate luminal membrane enzymic markers from α-glucosidases, β-galactosidases or peptidases of other locations in enterocytes, we specifically focused on enzymes of isolated brush-border membrane vesicles. Sucrase (sucrase-isomaltase, EC 3.2.1.48-10) and glucoamylase (maltase-glucoamylase, EC 3.2.1.20) were chosen as markers of changes in digestive functions during weaning and lactase (lactase-phlorizinhydrolase, EC 3.2.1.3-62) for its critical role in nutrition of young rats. These enzymes were often utilized as indicators of the functional state and maturation of the intestinal epithelium (Welsh *et al.* 1989, Kolínská *et al.* 1990). Of special interest was the brush-border associated enzyme dipeptidyl peptidase IV (DPP IV, EC 3.4.14.5). This serine-type protease preferentially cleaves N-terminal dipeptides from polypeptides containing proline as the penultimate amino acid. DPP IV is also known as CD26 lymphocyte membrane marker associated with a number of biological processes such as hormone regulation, immune responses and cellular interactions with the biomatrix (reviewed by Erickson *et al.* 1992, Bristol *et al.* 1995, Ansorge and Kahne 1995).

We have demonstrated an increase of intestinal brush-border membrane activities of GF rats as compared to their CV counterparts. For individual enzymes, differences were found not only in the extent but also in the appearance during various stages of ageing.

Material and Methods

Animals

2-, 6- and 12-month old rats of inbred Wistar AVN strain of both sexes were reared germ-free in plastic isolators (inbred F_{89}) and fed steam-sterilized granulated pellets and sterilized water *ad libitum.* Control animals were fed the same diet under conventional conditions (Štěpánková 1979). Rats were fasted for 24 hours before being anaesthetized with ether and decapitated.

Preparation of brush-border membrane vesicles (BBMV)

Brush border membranes were prepared from jejunal scrapings essentially according to the method of Kessler *et al.* (1978). Briefly, the mucosal layer was gently scraped off, weighed, frozen in liquid nitrogen and placed into a deep freeze until BBMV preparation. BBMV were obtained by a calcium precipitation procedure using solid $CaCl_2$ which was added to the homogenate (1:100 in 50 mM mannitol, 2 mM Tris) in a final concentration 10 mM. After standing in the cold (4 °C) for 20 min the homogenate was centrifuged for 15 min at 2000 x *g* to spin down nuclei, mitochondria and most of the basolateral membranes. The supernatant was centrifuged at 20 000 x *g* for 60 min. The pellet containing almost pure vesicles from the brush border membrane was resuspended in 10 mM KCl (AnalaR, BDH Chemicals Ltd., Poole, England) and used for further studies.

Lactase, sucrase and glucoamylase determination

Lactase, sucrase and glucoamylase activities were determined according to Kolínská and Kraml (1972) with 50 mM, 50 mM sucrose and 12 mg/ml starch (all chemicals from Serva, Heidelberg, Germany), respectively. The liberated glucose was measured with the Tris-glucose oxidase-peroxidase reagent (Dahlqvist 1964).

Dipeptidyl peptidase IV activity determination

Dipeptidyl peptidase IV (DPP IV) was determined with 1.4 mM glycyl-L-proline-4-nitroanilide (Sigma-Aldrich, Diesenhofen, Germany) in 66 mM Tris-HCl buffer, pH 8.0 (Křepela *et al.* 1983). The reaction was stopped with 1 M Na-acetate buffer (pH 4.2) (Lachema, Brno, Czech Republic) and the released 4-nitroaniline was measured at 405 nm (Nagatsu *et al.* 1976). Enzyme activities were expressed as nkat/mg protein, one nanokatal being the amount of enzyme that converts 1 nmol of substrate per second under the given conditions.

Protein assay

Protein concentrations in BBMV were determined by the method of Lowry *et al.* (1951) using bovine serum albumin (Serva, Heidelberg, Germany) as standard.

Statistics

The significance of differences was estimated by Student's *t*-test for unpaired values.

Results

Effect of age on sucrase activity in GF and CV rat enterocytes

Significantly higher sucrase activity in jejunal brush border of GF rats in comparison with CV rats was found only in older (6- and 12-month-old) rats. While the sucrase activity in CV animals of all age groups studied did not change significantly, a marked increase of sucrase activity with increasing age (from 2 to 6 months) was observed in GF rats (Fig. 1A).

Effect of age on glucoamylase activity in GF and CV rat enterocytes

The activity of glucoamylase in enterocyte BBMV isolated from the jejunum of GF and CV rats differed only in the oldest group (12-month-old), the activity of glucoamylase in GF being higher than in CV rats. The activity of glucoamylase in both CV and GF rats at the age of 6 months was almost double of that in 2-month-old animals (Fig. 1B).

Effect of age on lactase activity in GF and CV rat enterocytes

The activity of lactase in GF rats of all three age groups studied was significantly higher in comparison with the activity of this enzyme in the jejunal brush borders of the CV counterparts (Fig. 1C). The highest difference in lactase activity between GF and CV rats was seen in the group of 6-month-old animals.

Effect of age on dipeptidyl peptidase IV activity in GF and CV rat enterocytes

An important increase of DPP IV activity was found in 2-month-old GF rats in comparison with CV animals of the same age. The brush-border DPP IV activity in the two older groups of GF animals was much lower, almost half of that found in 2-month-old rats, and did not differ significantly from the DPP IV activity of their CV counterparts (Fig. 1D).

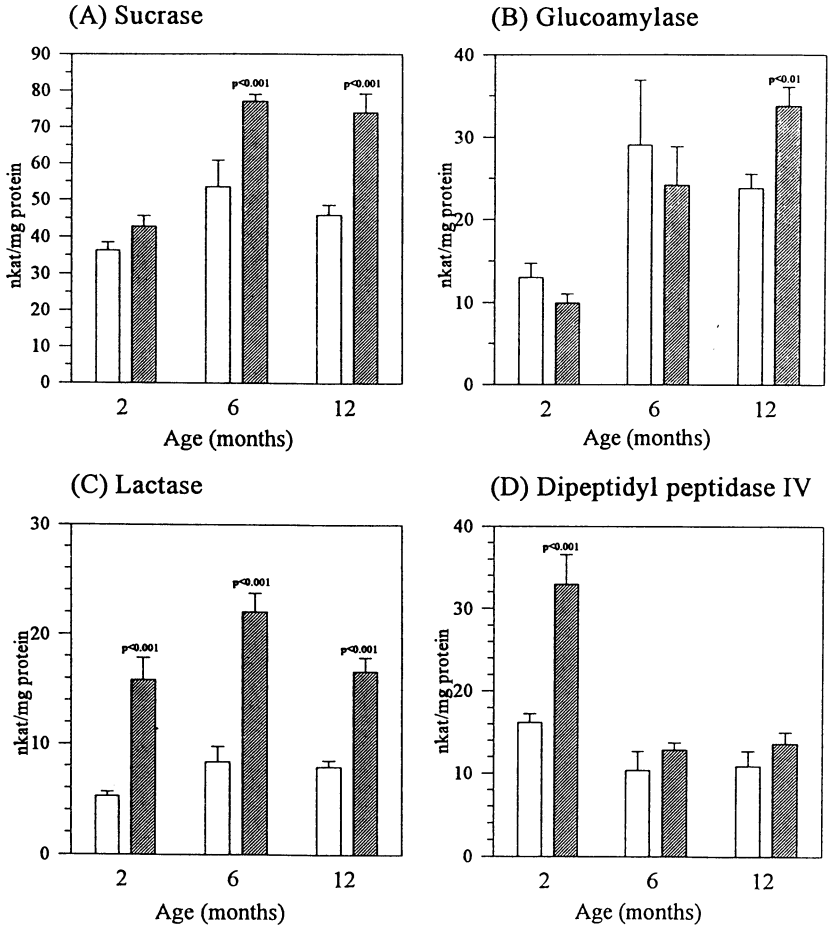

Fig. 1. *Effects of the environmental conditions and age on jejunal enterocyte brush border enzymes in conventional rats (open columns) and germ-free rats (full columns). (A) sucrase, (B) glucoamylase, (C) lactase, and (D) dipeptidyl peptidase IV. Results are means ± S.E.M. of 10–15 animals.*

Discussion

The present data reveal a common difference between CV and GF rats, with a higher expression of all enzymes studied in the latter. There are two major events through which intestinal microflora could mediate the amount of digestive brush-border hydrolyses: the increased rate of cellular renewal along the crypt-villus axis of the intestinal epithelium and changes in the level of cytokines and growth factors

produced by cells of the intestine immune system or by epithelial cells. It is known that enterocytes of GF rats migrate along the villus at a slower rate than they do in animals with normal microflora. The villi of the proximal small intestine of GF rats have significantly more cells than those of CV animals. Accordingly, the villus height in GF rats is significantly higher than in CV rats and the cell cycle in GF rats is longer than that observed in their CV counterparts (Uribe *et al.* 1990). Thus, Olsen and Korsmo (1982) suggest that elevated disaccharidase levels in GF animals are mainly the consequence of an increased number of mature enterocytes at the villus tips. However, the extent of this stimulation depends on the type of digestive enzyme and its developmental profile. In the rat, a typical activity gradient of each digestive enzyme from villus tip to crypt base develops from birth on through to the adult age (reviewed by Koldovský 1981). It is established shortly after weaning and the distribution patterns of the enzymes along the crypt-villus axis in 2-month-old rats can be considered as already fully developed. Sucrase and glucoamylase of suckling rats appear first in the crypt zone and remain there at a maximum until day 20 (Raul *et al.* 1977). In the adult age, high-activity sucrase is evenly distributed along the villus and the crypt cells continue to synthetize this enzyme (Beaulieu *et al.* 1989). The activity of two α-glucosidases, sucrase and glucoamylase, which develop to a mature status during the weaning period are, according to our results, still lower in young (2-month-old) rats in the brush-border membrane than in older (6-month-old) rats. As these two enzymes are evenly distributed along the villi at the adult age and are synthetized in the crypt zone, the increased height of the intestinal villus in GF rats does not markedly participate in the changes of specific activity of these enzymes. It should be noted, however, that the specific sucrase activity is significantly elevated in 6- and 12-month-old and glucoamylase activity in 12-month-old GF rats. Lactase activity, the highest at birth, is no longer detectable in the crypts by day 20 and it is restricted along the villi (Simon *et al.* 1979). Based on these findings, we could speculate that the increased villus height and increased amount of mature enterocytes in GF rats profoundly affect their specific lactase activity. Indeed, our results show a 2–3 fold increase of the specific activity of brush-border lactase in all the groups studied. They support the findings of Reddy and Wostmann (1966) that the lactase activity in mucosal homogenates from 2-month-old GF rats is enhanced.

DPP IV, already detected at birth, is confined to both the crypt and the villus zone during the weaning period of rats with a maximum activity in the villus tip of adults (Kolínská *et al.* 1986). The pattern of differentiation and development of DPP IV seems to be intermediary between lactase and sucrase. Similar to lactase, the DPP IV is high at birth and like sucrase it is also confined to the crypts in adults. According to our results, a two-fold increase of DPP IV activity is apparent only in younger 2-month-old GF rats in comparison with CV rats. In view of the fact that some brush-border hydrolases (e.g. lactase) are more dependent on cell maturity than others (glucoamylase, sucrase), the former enzymes would be more affected by events occurring after microbial interactions.

Intraluminal microflora influences the release of biologically active peptides and participates in the regulation of gastrointestinal endocrine cells and the epithelial structure (Uribe *et al.* 1994). The epithelium is in direct contact with the intestinal lumen and represents an ideal communication between the intestinal environment and immune cells of the mucosa. Immune response to bacterial antigens – production of cytokines – influences the structure and function of epithelial cells. Cytokines are released from the cells of the immune system and also by epithelial cells. Thus, the intestinal epithelium was reported to produce constitutively, or after stimulation, cytokines IL-1, IL-6, IL-8, IL-10 and transforming growth factor-β (TGF-β) (reviewed by Stadnyk 1994, Tlaskalová-Hogenová *et al.* 1995) and IL-7 which stimulates proliferation of $\gamma\delta$ intraepithelial lymphocytes (Kagnoff 1996). Recent observations of Ziambaras *et al.* (1996) show that inflammatory cytokines IL-6 and interferon-γ (INF-γ) mediate down-regulation and tumour necrosis factor-α (TNF-α) up-regulation in the expression of the sucrase-isomaltase gene in the intestinal epithelial cells of acutely inflamed Crohn's ileum. Our results are in accord with the enhancing effect of TNF-α on stimulation of intestinal sucrase activity in GF piglets by intragastrically applied *Nocardia* delipidated cell mitogen (Kozáková *et al.* 1994), which was found to be a potent stimulator of TNF-α secretion (Mege *et al.* 1993, Trebichavský *et al.* 1993, 1995). These observations raise the possibility for a local release of IL-6, INF-γ and TNF-α in the inflamed intestine; the results may provide evidence that immune signalling molecules affect not only the expression of immunologically active receptors but also some enterocyte-specific differentiation-dependent integral membrane protein(s). Even with the limited data available we could expect that the amount of digestive brush-border hydrolases in the intestine of CV rats would be regulated by certain levels of cytokines and would differ from the enzyme activities of GF animals in which the immune signalling molecules are not stimulated by the microflora. The specific effect of cytokines and growth factors at the level of transcription points to additional mechanisms for brush-border enzyme regulation, especially during inflammation and injury.

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Reprint requests

H. Kozáková, Department of Immunology and Gnotobiology, Institute of Microbiology, Academy of Sciences of the Czech Republic, CZ 549 22 Nový Hrádek, Czech Republic.