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AGE AND CONTINUOUS LACTOSE CHALLENGE MODIFY LACTASE PROTEIN EXPRESSION AND ENZYME ACTIVITY IN GUT EPITHELIUM IN THE RAT

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The activity of lactase enzyme declines after weaning. This study was to investigate changes in the lactase expression in the whole gastrointestinal tract during the development and the possibility that this and activity can be induced by lactose. Expression of lactase protein in the gut of 1—12-weeks old rats was studied by immunocytochemistry. Possible induction was evaluated by immunohistochemical and biochemical techniques in 8-week-old rats after lactose challenge for seven days. Lactase immunoreactivity was detected only in the small intestine and it decreased 20% during the week after weaning. A steady level of 40% lower than in the sucklings was found in the adult rats. In the lactose-challenged rats the optical density of immunoreactivity increased by about 30% in those that consumed the highest concentration of lactose. In the proximal jejunum, elevation of the enzymatic activity was three-fold. In the rat lactase protein expression decreased rapidly after weaning and expression and activity were induced by lactose-rich diet, most notably in the proximal jejunum.

Key words: enzyme activity, immunocytochemistry, lactase, lactose

INTRODUCTION

Lactose intolerance (lactose maldigestion, lactose malabsorption, lactase nonpersistence, primary acquired lactase deficiency or hypolactasia) is a common clinical diagnosis in Western countries (1). Gastrointestinal symptoms caused by lactose maldigestion have been attempted to attenuate not only by total abstinence of the use of lactose or by lactase substitution, but also by continuous intake of small doses of lactose. It has also been postulated that one possible mechanism of improved tolerance after prolonged lactose exposure could be adaptation of lactose metabolism by colonic microflora,
rather than the induction of the synthesis of new lactase protein by epithelial cells (2, 3).

In animal experiments, greater tolerance to lactose has been shown in suckling animals as a result of high activity of lactase (for review see 1, 4, 5). These high levels of enzyme activity remain until weaning, whereafter a marked and rapid decline to the adult low levels occur. These levels have been described as being approximately one tenth of those found in sucklings. Interestingly, various dietary manipulations with lactose (6–9) or other carbohydrates (10, 11) have been shown to increase the lactase activity in the small intestine by up to 50%. However, other studies could not demonstrate any induction either in rats (12–16), calves (17) or pigs (18). It has also been proposed that lactose feeding cannot prevent the reduction of lactase activity after weaning, but delays the reduction in animals (14, 19, 20) and in man (21, 22). In view of this considerable and controversial literature concerning the potentiality of lactose to cause an induction of the enzyme lactase, it seemed reasonable to re-examine this phenomenon using an animal model in controlled laboratory conditions and sophisticated modern immunohistochemical and biochemical techniques.

MATERIALS AND METHODS

Animals

Female Wistar rats from the breeding colonies of the Laboratory Animal Centre, Helsinki University, were housed at 23°C with a light-dark cycle of 12 h (from 7 am to 7 pm). Tap water or lactose-containing water and commercial rat pellets containing no lactose (R36, Lactamin, Stockholm, Sweden) were fed ad libitum.

Age-related changes study

Twenty seven female rats were divided into seven age groups as follows: 5 days (n = 4), 2 weeks (n = 4), 3 weeks (n = 3), 1 week after weaning i.e. at the age of 4 weeks (n = 4), 2 weeks after weaning i.e. at the age of 5 weeks (n = 4), 8 weeks (n = 4) and 12 weeks (n = 4). Both the dam and the sucklings were housed in the same polypropylene cages until the young rats were weaned at the age of 3 weeks. Sodium pentobarbital (60 mg/kg i.p.) anesthetised animals were decapitated and one-cm-long samples were taken from the proximal part of oesophagus and from the stomach. The small intestine was removed and 6—8 samples (one-cm-long) were taken at equal distances, the first sample from the duodenum and the last from the distal part of ileum. Samples from the colon were taken from the transversal part.

Lactose challenge study

Twenty four female rats (8 weeks; 230—270 g) were divided into four groups and were housed in groups of six in polypropylene cages. For seven days each group received either 3%, 10% or 20% lactose-containing water or tap water (controls) ad libitum. Lactose was purchased from Valio
Ltd (Helsinki, Finland). The fluid intake was measured daily by weighing the drinking bottles. Animals were weighed the day before the lactose challenge and the day before termination. At the end of the experiment the animals were fasted overnight before they were killed by decapitation under CO₂ anaesthesia.

Samples were taken from the proximal part of the oesophagus and the stomach. The first samples from the small intestine were cut from the middle of the duodenum, and the remaining small intestine was divided into four equal sections (the first, middle and latter parts of the jejunum, and the ileum). Samples from the colon were taken from the transversal part. Two samples, one for immunocytochemistry and one for the enzyme activity assay, were always taken from the beginning of each part: the first was fixed and the latter was placed into ice-cold 0.9% saline for subsequent determination of lactase activity as shown below.

**Preparation of intestinal samples**

**Fixing samples**

One-cm-long samples were washed in 0.01 M phosphate buffered 0.15 M saline (PBS), pH 7.1, and fixed immediately with 1% paraformaldehyde (Fluka Chemie AG, Buchs, Switzerland) in PBS. After fixation for 6—8 hours at room temperature, all samples were immersed in 15% sucrose (BDH, Poole, England) in PBS buffer with 0.01% sodium azide (Riedel-deHaen AG, Seelze, Germany) at +4°C. The buffer was changed after 1—2 days.

**Mucosal homogenates**

Two-cm-long segments of the small intestine were opened longitudinally and cleaned carefully in ice-cold 0.9% saline. The mucosa was scraped off, weighed and frozen immediately at −20°C for lactase measurement. Before the assays, the samples were diluted in 6 parts of ice-cold 0.9% saline, homogenised by Ultra-Turrax (T8, Ika Labortechnik, Staufen, Germany) for 15-20 seconds in ice and centrifuged at +4°C 1400 × g for 10 minutes. All measurements were made from supernatant fraction stored frozen at −20°C.

**Immunoperoxidase labelling**

Cryostat sections (6 μm for the age related changes study, 8 μm for the lactose challenge study) were cut, transferred onto 3-aminopropyltriethoxysilane (Sigma Chemical Co., St Louis, MO, USA) coated slides and allowed to dry for 1 hour. Endogenous peroxidase was inhibited with 0.3% (v/v) hydrogen peroxidase in methanol for 30 minutes. Non-specific binding sites were blocked by incubation with normal horse serum and normal rat serum (1:30, Tissue Culture Services, Buckingham, UK) for 30 minutes at room temperature. Sections were then incubated overnight at +4°C with monoclonal antibodies raised against either human (23) (1:30 mlac1, mlac3, mlac4, mlac5, mlac6, mlac8, mlac9, mlac10) or rat lactase (24, 25) (1: 200, FBB3/4, YBB2/61). The sections were then incubated with biotinylated horse anti-mouse IgG (Vector Laboratories, Peterborough, UK) for 30 minutes, and then with avidin-biotin-peroxidase complex (Vector) for 60 minutes at room temperature according to the manufacturer's instructions. Finally, the specimens were incubated for 5 minutes in a chromogen solution containing glucose oxidase-3,3'-diaminobenzidine-nickel ammonium sulphate (Sigma) to amplify the reaction product. Omission of the primary antisera was included as a method control, and conventional hematoxylin and eosin staining of sequential sections was used for comparative histopathological analysis.
**Optical density measurements**

Optical density measurement was used in order to obtain a semi-quantitative measurements of lactase protein expression. Tissue sections were imaged at a magnification of $\times 20$ on an Olympus BH2 microscope (Tokyo, Japan) using bright field optics. Sony CCD monochrome video camera model XC-77CE (Tokyo, Japan) connected to a Seescan Symphony image analysis system (Seescan, Cambridge, UK) was coupled to the microscope. The captured image was automatically digitised to a $512 \times 512$ pixel array with each pixel having been assigned one of the 256 grey levels. Image segmentation was achieved by interactive thresholding to separate immunoreactive epithelium from non-immunoreactive structures. Optical densities were determined from the amount of the incident light absorbed by the reaction product present within epithelium. Illumination was adjusted optimally to prevent saturation of the camera, and transmitted light outside the section was subtracted from the final optical density.

**Lactase activity assay**

Lactase enzyme (EC 3.2.1.23) activity was assayed spectrophotometrically by the method of Dahlqvist (26) after 60 minutes incubation at pH 6.0 at $+37^\circ C$ in a medium containing 25 mM lactose as substrate. Lactase activity was expressed as units (one μmol of lactose hydrolysed per minute at $+37^\circ C$) per gram of total protein present in homogenates. All the chemicals were obtained from Sigma. The protein content of the homogenates was assayed by the method of Lowry et al. (27), using human serum albumin (Sigma) as a standard.

**Ethics**

The study protocols were approved by the Animal Experimentation Committee of the University of Helsinki.

**Statistics**

All data are expressed as the mean±SE. Statistical significance was determined by analysis of variance (ANOVA), and multiple comparisons were performed by the Dunnet's post test.

**RESULTS**

**Age-related expression of lactase**

In order to study possible changes in the lactase protein expression several antibodies were tested. Of the antibodies raised against human lactase only mlac6 demonstrated immunoreactivity in the rat, but unsatisfactory background staining also appeared. Antibodies FBB/3/4 and YBB2/61 (raised against rat lactase) both exhibited strong characteristics of lactase immunoreactivity in the brush border of the small intestine in the young age groups studied. FBB3/4 was selected to immunostain the upper and lower parts of the small intestine of all ages groups, and the entire gastrointestinal
tract, i.e. the oesophagus, the stomach and the colon, in age groups of 1 week, 3 weeks and 5 weeks old (i.e. age of 2 weeks after weaning).

Lactase immunoreactivity was detected in no other part of the gastrointestinal tract except in the small intestine, and no significant differences were seen between the upper and lower part of the small intestine. In the youngest animals (one and two weeks), lactase immunoreactivity was strongly expressed in all small intestinal epithelial cells (Fig. 1A and 1B). In the suckling three-week-old rats, immunoreactivity was reduced with patchy appearance. Immunoreactivity was also markedly reduced in rats after one week of weaning (Fig. 1C and 1D). In the three older age groups, lactase immunoreactivity was even lower and mostly confined to the tips of the intestinal villi.

In order to get a semi-quantitative measurement of the lactase protein expression, optical density measurement was used for the first jejunal samples taken two cm from the end of the duodenum. It was found that lactase
immunoreactivity was significantly lower in rats three weeks after weaning compared with that of the one-week-old animals used as a reference group (Table 1).

**Effects of lactose challenge on lactase protein expression and its enzymatic activity**

Weight gain in 3%, 10% and 20% lactose-challenged groups was less compared with the controls, 21 g/week and 44 g/week, respectively (Table 2). In all the lactose receiving groups the average fluid intake was 19 ml/rat/day compared with the controls 32 ml/rat/day. Therefore, the actual amounts of lactose consumed were 0.7 g, 2.1 g and 2.6 g/rat/day, respectively.

Immunocytochemistry showed that in the control group, lactase immunoreactivity was weak and at the same level as seen in adult animals in the first part of the study (Table 1 and 3). There was a clear induction of lactase protein in epithelial cells in those groups which received lactose-rich diet, this being most evident in those that received 20% lactose (Table 3). Increase in lactase immunoreactivity was 26% in those who received 3% or 10% and 42% in those who received 20% lactose solution.

**Table 1.** Optical densities (arbitrary units) of lactase immunoreactivity at the beginning of the rat jejunum in relation to age.

<table>
<thead>
<tr>
<th>Age</th>
<th>Suckling</th>
<th>Weaned</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weeks</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>0.32 ± 0.01</td>
<td>0.31 ± 0.02</td>
</tr>
</tbody>
</table>

Analysis of one-way variance (ANOVA) with Dunnett’s multiple comparison test (control 1 wk vs. test group). n = 3—4.

* p < 0.05  
** p < 0.01.

**Table 2.** Effect of seven day lactose challenge on weight gain and daily water and lactose consumption in rats Lactose concentration in the drinking water.

<table>
<thead>
<tr>
<th></th>
<th>0%</th>
<th>3%</th>
<th>10%</th>
<th>20%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight gain (g)</td>
<td>44.3 ± 5.4</td>
<td>20.2 ± 5.4*</td>
<td>18.2 ± 7.3*</td>
<td>23.8 ± 7.9*</td>
</tr>
<tr>
<td>Water intake (ml/rat)</td>
<td>32.4 ± 0.7</td>
<td>23.3 ± 2.1*</td>
<td>20.6 ± 2.6*</td>
<td>12.7 ± 1.5**</td>
</tr>
<tr>
<td>Lactose intake (g/rat)</td>
<td>0.0 ± 0.0</td>
<td>0.7 ± 0.1</td>
<td>2.1 ± 0.3</td>
<td>2.6 ± 0.3</td>
</tr>
</tbody>
</table>

Analysis of one-way variance (ANOVA) with Dunnett’s multiple comparison test (control 0% vs. test group). Mean ± SE, n = 5—6. * p < 0.05.
Table 3. The effect of seven days lactose challenge on optical densities† of lactase immunoreactivity at the beginning of the jejunum and on lactase enzyme activities‡ in rats

<table>
<thead>
<tr>
<th></th>
<th>0%</th>
<th>3%</th>
<th>10%</th>
<th>20%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Optical density</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jejunum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First part</td>
<td>0.19±0.01</td>
<td>0.24±0.01*</td>
<td>0.24±0.01*</td>
<td>0.27±0.02**</td>
</tr>
<tr>
<td><strong>Lactase activity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duodenum</td>
<td>2.2±0.2</td>
<td>2.0±0.2</td>
<td>2.8±0.5</td>
<td>4.5±0.7*</td>
</tr>
<tr>
<td>Jejunum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First part</td>
<td>5.2±0.8</td>
<td>6.6±1.1</td>
<td>8.4±1.7</td>
<td>17.4±3.7*</td>
</tr>
<tr>
<td>Middle part</td>
<td>16.8±2.6</td>
<td>18.8±1.4</td>
<td>26.4±1.8*</td>
<td>26.7±1.8*</td>
</tr>
<tr>
<td>Last part</td>
<td>23.3±3.0</td>
<td>18.9±2.1</td>
<td>28.6±1.3</td>
<td>26.4±6.0</td>
</tr>
<tr>
<td>Ileum</td>
<td>6.8±1.5</td>
<td>7.2±1.7</td>
<td>9.2±1.2</td>
<td>5.8±1.2</td>
</tr>
<tr>
<td>Colon</td>
<td>3.5±0.4</td>
<td>2.5±0.2</td>
<td>5.1±0.5</td>
<td>4.7±0.8</td>
</tr>
</tbody>
</table>

Analysis of one-way variance with Dunnett's multiple comparison test (control 0% vs. test group). Mean±SE, n = 5—6.
† arbitrary units
‡ unit = μmol lactose hydrolysed at +37°C/min/g protein
* p < 0.05
** p < 0.001

Lactase activity in the control group as measured by spectrophotometry, was higher in the middle and last part of the jejunum in comparison to the duodenum and the ileum (Table 3). However, measurable enzyme activities were also found in the large intestine, but not in any other parts of the gastrointestinal tract (Table 3). Lactase activity increased approximately 60% in the epithelial cells homogenates from the middle part of the jejunum on 10% lactose group and, more markedly, 200% in the duodenum and 330% in the proximal and 60% in the middle part of the jejunum in those who received 20% lactose.

DISCUSSION

The present study shows that lactase enzyme immunoreactivity is distributed in a uniform fashion in the suckling rat small intestine epithelium and that there is an age related decrease in immunoreactivity after weaning. In addition, the results show that both lactase immunoreactivity and enzyme activity can be induced in young adult rat epithelial cells by lactose challenge.

Lactase immunoreactivity was studied throughout the gastrointestinal tract using monoclonal antibodies raised against human lactase. Those antibodies failed to detect the protein, apart from an antibody coded mlac6 which has
been previously used in rabbit tissue. However, because we were unable to
detect lactase in a consistent manner with this antibody, two different specific
monoclonal antibodies raised against rat lactase were tested. Both antibodies
gave similar results. An antibody coded FBB3/4 was selected for further
immunocytochemical studies and the findings were found to be consistent with
those attained by lactase enzyme activity assay.

Lactase immunoreactivity was not detected in any other part of the
gastrointestinal tract, neither in the suckling or weaned rats, except in the small
intestine. This agrees with earlier reports in which only lactase activity assay
were used to study distribution of the enzyme (for review 1).

We found that lactase immunoreactivity decreased already one week after
weaning. During the second week a further reduction occurred, and the
expression remained at this steady level for the whole follow-up period up to 12
weeks. This is in agreement with earlier reports on rapid decrease of lactase
activity after weaning (28, 29). After weaning, lactase immunoreactivity was
present only in patches of epithelial cells, located principally in the upper part
of the villi. In the suckling rat, the lactase protein was present in the villus
epithelial cells along the entire small intestine. This is in agreement with
a previous study (28). However, in adult animals the expression of lactase
protein was very low and there were no apparent differences between the
proximal and distal parts of the small intestine, contrary to earlier observations
(28) where different expression was found in proximal and distal regions of the
small intestine compared to the middle parts of the small intestine.

For the lactose challenge studies we selected 8-week-old rats, in which the
jejunal epithelial cells normally expressed any measurable lactase
immunoreactivity. This was also confirmed in the second part of the present
study, in which this age group revealed very similar, low optical density values
as was seen in the first part. By using semi-quantitative immunocytochemistry
and specific enzyme activity assay it was found that both lactase protein and its
activity can be induced by feeding the animals with lactose-rich diet. The
reliability of the findings was strengthened by the dose depending of lactose
feeding. After a one-week lactose challenge there was a clear induction of
lactase even in the group which received 3% lactose. However, 10% or 20%
lactose challenge was needed to show a significant increase in the enzyme
activity. This may indicate that a longer lactose feeding period, e.g. more than
five weeks is needed to augment the enzyme activity (7, 8, 9), although clear
up-regulation of protein expression (up to 42%) was already seen even in this
short-term treatment study.

Lactose challenge was found to reduce weight gain and water intake. The
possibility of mixing lactose in the feed rather in the drinking fluid may be
advantageous to avoid different patterns in weight gain. However, all animals
were healthy throughout the study and daily observations showed no visible
differences in fecal consistency e.g. diarrhoea between the four groups. However, the frequency of defecation and the total amount of feces may have varied according to the amount of received lactose. This requires additional study.

Interestingly, there was some activity in the large intestine in rats which received lactose feeding. Whether this activity was due to the induction of endogenous lactase in the epithelial cells or originated somehow from the large intestine bacteria adapted to the lactose-containing diet, as described earlier (30), remains to be clarified.

It was found that lactose challenge increased the enzymatic activity of lactase proportionally more than its protein expression, as determined by galactose-oxidase assay for enzymatic activity and immunocytochemistry and image analysis quantification for protein expression. Reduced lactase activity after weaning have been explained to be due of decreased synthesis and increased movement of cells along the villus. Although these mechanisms may also vary in different animal species (for review see 4, 5, 6). Multiple complex mechanisms during transcription, translation and conversion of the inactive form of lactase to active may be influenced by the lack of lactose in the feed, probably not often investigated in detail in previous studies. These facts can be responsible for the loss of activity with maturation and possibly also for the increase of lactase with continuous lactose challenge.

In conclusion, the data presented here shows clearly that in the rat lactase protein and its enzymatic activity can be up-regulated by even a short term lactose challenge suggesting epithelial cell adaptation to lactose rich diet. Whether the findings bear correlation to lactose intolerance in man is not clear, but this model provides a useful tool for investigating mechanisms of induction of the lactase enzyme, and the actions of other substances possibly involved in the regulation of the enzyme’s translation and activity.

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