Expression of intestinal ornithine decarboxylase during postnatal development in neonatal rats

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Abstract

Ornithine decarboxylase (ODC) has been shown to play an essential role in intestinal growth and maturation in rats. However, the regulatory mechanisms have not been fully elucidated. We studied the mechanisms of expression of intestinal ODC during postnatal development. Rat small intestinal mucosa was obtained from postnatal days 10, 15, 17, 19, 21, 24 and 30. Intestinal mucosa was assayed for ODC and sucrase activities. In addition, intestinal ODC mRNA, and ODC protein levels were also measured. The results showed that the intestinal sucrase activity was low before postnatal day 19. The sucrase activity then increased steadily from day 19 up to day 30. Intestinal ODC activities remained low from postnatal day 10 to day 17. A sharp increase in ODC activity was noted on day 19, which peaked on day 24 (a 20-fold increase from its low basal level) and declined on day 30. Intestinal ODC proteins followed the same pattern of postnatal expression as that of ODC activity. In contrast, ODC mRNA did not show significant change throughout the study period. The possible mechanisms by which intestinal ODC mRNA levels remain practically unchanged during postnatal development are discussed. We conclude that the ontogenic increase in sucrase activity, a marker for intestinal maturation, occurs at the same time to that of the induction of ODC activity. We also suggest that the induction of intestinal ODC activity during postnatal development is the result of post-transcriptional events or other cellular mechanisms. A better understanding of the regulation of polyamine biosynthesis during postnatal development of the small intestine will provide insights contributing to the maturation of the small intestine. © 2002 Published by Elsevier Science B.V.

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1. Introduction

Diarrhea is one of the major causes of infant morbidity and mortality worldwide [1]. Young infants are particularly susceptible to the severe sequelae of diarrhea. Intractable diarrhea of infancy is a unique disease entity with a high mortality rate and is quite different from that found in adults [2]. Rossi et al. [3] showed that 70% of infants with intractable diarrhea had persistent mucosal atrophy up to 6 months after presentation. The exact mechanisms of prolonged mucosal injury and impaired mucosal regeneration process in early infancy are not clear. A better understanding of the mechanisms that regulate intestinal development and maturation during the postnatal period is essential for the management of intractable diarrhea of infancy as well as the development of therapeutic strategy. There are similar features in the ontogeny of the small intestine between humans and laboratory rodents [4]. The rat small intestine is not well developed and undergoes major developmental changes during the third postnatal week, which corresponds to weaning [5]. One of the contributing factors of these major developmental changes may be related to polyamine biosynthesis. Although the induction of ornithine decarboxylase (ODC, EC 4.1.1.17) activity and a concomitant accumulation of cellular polyamines have been found to be critical and necessary during the process of adaptation/maturation in the small intestine mucosa [6–8], the cellular mechanisms regulating the expression of ODC activity

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during postnatal development have not been fully elucidated. In the current study, we have investigated genetic mechanisms regulating the expression of intestinal ODC activity during postnatal development at cellular and molecular levels.

2. Materials and methods

2.1. Animals

Timed-pregnant Sprague–Dawley rats (C–D strain) (Charles River, Wilmington, MA) received on the 15th day of gestation, were housed in individual cages and maintained on a 12-h light–dark cycle with lights on at 0600 h. On the expected date of delivery, cages were checked every 4 h for births, and the day of birth was regarded as day 0. On the second postnatal day, all pups were pooled and redistributed, such that each dam had eight to nine pups. Each dam with her group of pups was housed separately. Dams were fed standard rat chow (Rodent Laboratory Chow 5001, Purina Mills, Inc., St. Louis, MO) and water ad libitum. Pups were allowed to suckle freely until the time of death. Daily weights of pups were measured to ensure adequate growth. Pups of both sexes were used in the experiments. To prevent the stress and changes induced by removing animals from a cage, all rat pups in one cage were killed on a single day.

2.2. Tissue collection

Postnatal rats aged 10, 15, 17, 19, 21, 24 and 30 days were used for the experiments. Animals were decapitated between 1100 and 1200 h. Immediately after death, the whole small intestine (from the duodenum to the ileal–cecal junction) was removed, placed immediately on ice, trimmed of excess fat and mesentery and weighed. The intestinal contents were removed by gentle flushing with ice-cold saline. The intestines were split open and mucosa scraped, and frozen immediately in small aliquots at −80 °C. Intestinal homogenates were used for the determination of ODC and sucrase activities as well as the protein content. For total RNA isolation, the intestinal mucosa was processed on the day of tissue procurement. This study protocol was approved by the Animal Investigation Committee of the Wayne State University and complied with the Guide for the Care and Use of Laboratory Animals (1985) of the National Institutes of Health.

2.3. Biochemical determinations

All reagents were of reagent grade and obtained from Sigma Chemical (St. Louis, MO). ODC activity was measured in the intestine mucosal homogenates by quantitation of 14CO2 liberated from [carboxyl-14C]ornithine according to Seely and Pegg [9] with modifications as described previously [10]. Briefly, intestine mucosal tissues were homogenized 1:10 (wt/vol) in 45 mM phosphate buffer (pH 7.2) containing 0.1 mM EDTA and 3 mM dithiothreitol and then centrifuged at 30,000 × g for 30 min. Samples of supernatant fractions were incubated with a mixture of 0.2 mM pyridoxal phosphate and 0.75 uCi of DL-(1-14C) ornithine hydrochloride (57 mCi/mmol, Amersham Corp., Arlington Heights, IL) for 1 h at 37 °C. ODC activity was expressed as picomoles of CO2 produced per hour per milligram of protein.

Succarase activity was assayed by the method of Dahlqvist [11], which uses sucrase as the substrate. The unit of sucrase activity was expressed as µmole of sucrose hydrolyzed per minute per gram of protein. Protein was determined by the Bradford procedure [12] using the Bio-Rad protein reagent (Bio-Rad Laboratories, Hercules, CA) and bovine γ-globulin as the standard.

2.4. Measurement of ODC mRNA by reverse transcriptase-polymerase chain reaction (RT-PCR)

We applied the RT-PCR assay as described by Wang et al. [13] with modification [14] for the detection of rat-specific ODC mRNA in the intestinal mucosa of developing rats. ODC mRNA was assayed by a PCR amplification of cDNA synthesized by reverse transcriptase using a GeneAmp RNA PCR kit (Perkin Elmer, Norwalk, CT). Intestine mucosal RNA samples (1 µg) were normalized by determining the efficiency of [α-32P]dCTP incorporation into first-strand cDNA before the RT-PCR assay. Reverse transcription for the assay was then performed by using an oligodeoxythymidylylate primer and extending the suggested reaction time to 60 min at 42 °C. The cDNA was then amplified by PCR using the following oligonucleotide primers derived from rat ODC cDNA [15]. The 5’ primer (5’-AGCAGACGGT-CGGACGAT-3’) recognized a sequence at the junction of exons 9 and 10 of rat ODC cDNA. The 3’ primer (5’-AGACATGGCGAGGTCGCCAA-3’) recognized a sequence in exon 12. The design of primers excludes any possibility of genomic DNA from being amplified. These primers produce a 426-bp DNA fragment from rat ODC cDNA. PCR was carried out at 95 °C for 2 min, followed by 35 cycles consisting of the following phases: 1 min at 95 °C (denaturation) and 1 min at 60 °C (primer annealing-extension) in a reaction mixture (100 µl) with final concentrations of: 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 3 mM MgCl2, 2.5 unit Taq polymerase (Perkin-Elmer), 0.15 µM corresponding primers and cDNA from RT reaction. Also included in the reaction was 0.5 attomoles of a mimic DNA fragment generated from a PCR-amplified fragment of Bluescript plasmid DNA with complementary ends to the ODC primers. The mimic DNA was utilized to normalize amplification efficiencies. The PCR products were electrophoresed on a 4% Nu-Sieve GTG agarose gel (FMC BioProducts, Rockland, ME), transferred to Gene Screen membranes and probed with [γ-32P]ATP radiolabeled primers. After film exposure, the radioactive signal in each band was
quantitated by using the AMBIS Radioanalytical Imaging System (AMBIS Systems Inc., San Diego, CA).

2.5. Measurement of ODC mRNA by Northern blot analysis

Total intestine mucosal RNA was isolated by the guanidinium thiocyanate method as described by Chomczynski and Sacchi [16]. RNA samples were denatured and subjected to electrophoresis through 1.5% agarose-formaldehyde gel with 20 μg of total RNA per lane. Fractionated RNAs were then transferred to Gene Screen (Dupont NEN, Wilmington, DE) nylon membranes, and hybridized according to the method of Church and Gilbert [17] as previously described [18]. Hybridizations were carried out with pODC10/2H plasmid, which contains a 1.8 kb cDNA for human ODC [19]. The cDNA clone pODC10/2H was a gift from Dr. O. Jänne of the Rockefeller University, New York. CDNAS were labeled with [α-32P]dCTP (New England Nuclear, Boston, MA), by the nick translation method [20] using the kit from Bethesda Research Laboratories (Gaithersburg, MD). The specific activity of the probes was routinely 3–5 × 106 cpm/μg. Filters were prehybridized at 42 °C for 4–6 h in 20 ml solution [5 × SSC, (1 × SSC is 0.15 M NaCl, 0.015 M Na citrate, pH 7.0), 50% formamide, 5 × Denhardt’s reagent, 1% sodium dodecyl sulfate (SDS), 10% Dextran sulfate, 100 μg/ml sonicated salmon sperm DNA]. Hybridization was carried out at 42 °C overnight with constant mixing. After hybridization, filters were washed in 2 × SSC with 0.1% SDS at 60 °C for 1 h, then in 1 × SSC at 60 °C for 1 h, finally in 0.5 × SSC at 60 °C for 1.5 h. Filters were blotted dry and exposed to X-Omat film at –70 °C in the presence of a Cronex intensifying screen. Quantitation of radioactivity was accomplished by scanning the autoradiograms with a computing densitometer (Molecular Dynamics, Sunnyvale, CA) with a capability of separating background exposure from target bands. Filters were rehybridized with a 32p-labeled 7S ribosomal RNA (rRNA) probe as previously described [18], and all samples were normalized to 7S rRNA, which served as an internal control and did not change significantly under our experimental conditions.

2.6. Western blot analysis

To prepare samples for protein analysis, intestinal mucosa was homogenized in homogenizing buffer [25 mM Tris, (pH 7.5), 1 mM dithiothreitol, 0.1 mM PMSF (phenylmethylsulfonyl fluoride), 0.1 mM EDTA, 5 μg/ml of each of the following: Antipain, Pepstatin A, Chymostatin and Leupeptin] at 1:10 dilution (wt/vol). Homogenized samples were centrifuged at 30,000 × g for 30 min and the supernatant fractions were used for the experiment. Intestinal tissues (50 μg protein per lane) were fractionated on a 12% SDS-polyacrylamide gel. Proteins were then transferred to a nitrocellulose membrane using the Multiphor II Nova Blot Unit (Pharmacia, Piscataway, NJ). ODC proteins were detected by using an anti-rat ODC monoclonal antibody (MAb, MP16-2) [21,22] and a goat anti-mouse horseradish peroxidase-linked secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) and visualized with Western Blot Chemiluminescence reagent (Amersham), according to the manufacturer’s instructions. MP16-2 ODC antibody is a mouse monoclonal antibody directed against a synthetic peptide corresponding to amino acids 355–360 of ODC, a sequence that is well preserved in eukaryotic ODC such as mouse, human and rat [21]. Signal intensities were quantitated by using a densitometric system as described in the method of Northern blot analysis.

2.7. Statistics

All results are presented as mean ± S.E. Two-way ANOVA (analysis of variance) was used to evaluate the differences between groups (pre-weaning and weaning groups) with repeated measurements. Differences in mean values between the two groups were evaluated by the Student’s t-test for unpaired data with P < 0.05 considered as statistically significant [23].

3. Results

3.1. Expression of intestinal ODC activity during postnatal development

Intestinal ODC activities were low from postnatal day 10 to day 17 (Fig. 1, upper panel). However, the ODC activities were significantly increased from postnatal day 19 (a 4-fold increase compared to day 10) to day 30 reaching a peak on day 24 (a 20-fold increase compared to day 10) and declined by day 30. There was a 45% decrease in ODC activity at day 30 when compared to the peak increase at day 24. The increase of ODC activity coincided with the weaning, which occurs during the third week of age. By applying a two-way ANOVA, intestinal ODC activity in the weaning phase (postnatal day 19 to day 30) was found to be significantly higher than in the pre-weaning period (postnatal day 10 to day 17) (P < 0.001).

3.2. Expression of intestinal sucrase activity during postnatal development

Although intestinal sucrase activities were low or not detectable from postnatal day 10 to day 17 (Fig. 1, lower panel), it showed a steady increase from postnatal day 19 and reached the highest level on day 30. The increase of sucrase activity from postnatal day 19 coincided with weaning, which occurred during the third week of age, and change of diet from high-fat maternal milk to high-carbohydrate laboratory chow [5]. Intestinal sucrase activity in the weaning period (postnatal day 19 to day 30) was significantly higher than the pre-weaning period (postnatal day 10 to day 17) (P < 0.001).
3.3. Intestinal ODC mRNA abundance by RT-PCR

To determine whether the marked induction of ODC activity during the third postnatal week could partly be due to the transcriptional activation on ODC gene, the expression of intestinal ODC mRNA was studied, using the RT-PCR analysis, on intestinal homogenates from different postnatal days (10-, 15-, 17-, 19-, 21-, 24-, and 30-day-old rats). Fig. 2 shows the steady-state levels of intestinal ODC mRNA during postnatal development. The ethidium bromide staining of ODC (426 bp) and mimic (209 bp) PCR products showed no significant changes among the different age groups. The intensity of each band of RT-PCR products as shown in Fig. 2, lower panel, were quantitated by Southern blot analysis and hybridization with 32P-end-labeled ODC primers. The results are depicted in Fig. 2, upper and middle panels. As opposed to the sharp induction of ODC activity during the third postnatal week, the increase of ODC mRNA was quite small. The ODC mRNA at postnatal day 24 showed a 40% increase (compared to postnatal day 10), whereas the ODC activity at day 24 showed a 20-fold increase when compared to postnatal day 10. There was a slight decrease (18%) of ODC mRNA at day 30 when compared to day 24, while ODC activity showed a 45% reduction during the same period.

3.4. Intestinal ODC mRNA abundance by Northern blot analysis

To confirm our findings of intestinal ODC mRNA by RT-PCR, we performed the measurement of intestinal ODC mRNA by Northern blot analysis. As depicted in Fig. 3, the...
ODC mRNA showed no significant changes during postnatal development. There was a maximal increase of 60% of ODC mRNA at day 24 (compared to postnatal day 10), whereas ODC activity showed a 20-fold increase when compared to postnatal day 10. The pattern of ODC mRNA expression measured by Northern blot analysis is similar to that obtained through RT-PCR analysis of ODC mRNA.

3.5. Expression of intestinal ODC protein during postnatal development

Intestinal ODC protein levels during postnatal development were shown in Fig. 4. The ODC monoclonal antibody (MAb, MP16-2) reacted with a 52-kDa protein. Other weaker bands with higher molecular weight were observed in Western blot, which might represent complex forms of ODC [21]. As depicted in Fig. 4, the intestinal ODC protein levels were low on postnatal day 10 and day 15. The ODC protein increased sharply from postnatal day 19 (an 8-fold increase), peaked at day 24 (a 10-fold increase) and declined at day 30 (a 4-fold increase). There was a 58% decrease in ODC protein level at day 30 when compared to the peak increase at day 24. Intestinal ODC proteins in the weaning period (postnatal day 10 to day 30) were significantly higher than in the pre-weaning period (postnatal day 10 and day 15) (P<0.001). The expression pattern of ODC protein is similar to that of ODC activity (i.e., a sharp increase at day 19, peak at day 24, and a decline at day 30).

4. Discussion

Our current study demonstrated sharp increases of ODC activity from postnatal day 19 to day 30 (peak at day 24). This is in agreement with reports by Luk et al. [7] and Buts et al. [6]. The ontogenic increase in sucrase activity, a marker for intestinal maturation [4], occurs at the same time to that of the induction of intestinal ODC activity. Both intestinal ODC activity and sucrase activity showed a significant increase from postnatal day 19. However, the induction of ODC activity was only transient, with peak at day 24 and decline at day 30. In contrast, the sucrase activity showed a steady increase from day 19 and reached the maximal level at day 30. The transient induction of intestinal ODC activity during postnatal development is consistent with the characteristics of ODC, i.e., a highly regulated enzyme with the shortest half-life among cellular proteins, ranging from several minutes to more than one hour [24].
Intestinal ODC mRNA study using RT-PCR and Northern blot analysis demonstrated no significant increase of ODC mRNA during the weaning period (third postnatal week). The ODC mRNA as measured by RT-PCR at postnatal day 24 showed a 40% increase (60% increase by Northern blot analysis), whereas the ODC activity at day 24 showed a 20-fold increase compared to day 10. Western blot analysis of ODC protein showed a similar expression pattern as that of the enzyme activity (i.e., a sharp increase at day 19, peak at day 24, and decline at day 30). Both results suggest that the induction of intestinal ODC activity during postnatal development is most likely a post-transcriptional event.

In mammalian cells, the expression of ODC gene is under the control of a variety of regulatory mechanisms including, but not limited to, transcriptional [25–27] and post-transcriptional [18,25,28,29] regulation. In the digestive system, the expression of ODC gene appears to be post-transcriptional modification in IEC-6 cells [29], pancreatic AR42J cells [30] and pancreatic tissue [18]. Whether the expression of ODC gene is tissue-specific remains unknown. In our study, the 40% increase in ODC mRNA at postnatal day 24 might not account completely for the concurrent 20-fold increase in ODC activity. An increase in ODC transcription and/or mRNA stabilization is probably not the major cause of the increase in intestinal ODC activity. The exact mechanisms by which intestinal mRNA levels remain practically unchanged during postnatal development have not been investigated in the current study. One of the possible mechanisms is the role of an exogenous supply of polyamines during the weaning period, and their negative feedback on ODC mRNA and S-adenosylmethionine decarboxylase (SAM-DC) mRNA. Dietary polyamines are important in cell regeneration and growth [31]. It has been suggested that polyamines contained in rat food could play an important role in postnatal maturation of the rat intestine [32]. The study by Romain et al. [32] showed rat food was much richer in polyamines than the rat milk (about 150 times for putrescine and spermine, about 30 times for spermidine). Both ODC and SAM-DC are under negative feedback control by polyamines [24]. Kameji and Pegg [33] showed that in vitro synthesis of ODC, as well as of SAM-DC, was substantially inhibited by physiological concentrations of spermidine or spermine in a reticulocyte lysate system. It is conceivable that the exogenous supply of polyamines from rat food during the weaning period may have contributed to the inhibition of ODC mRNA increases.

Hormones [4] have been implied to stimulate postnatal growth in rats. Different hormones (thyroxine and insulin) may have potential effects on the post-transcriptional steps of ODC synthesis. Thyroxine has been shown to induce pancreatic ODC activity in suckling rats and the regulatory mechanisms may be at the post-transcriptional levels [18]. Insulin has been shown to induce intestinal ODC activity in postnatal days 18 and 20 in rats [6]. In cells treated by actinomycin C, insulin is still able to activate the ODC activity, suggesting a post-transcriptional regulation of the ODC by the insulin [34]. The exact mechanisms involving post-transcriptional regulation of intestinal ODC during postnatal development are subjects of further investigation.

Our results also demonstrated a decline in ODC activity (45%) and ODC protein level (58%) at postnatal day 30 when compared to the peak of ODC at day 24. However, the ODC mRNA only showed a slight decrease (18%) during the same period. Given the fact that ODC activity is tightly related to the amount of ODC protein, as shown in our study and others [35], this data suggests that there are negative translational effects on ODC from surge to decline during postnatal development. One possibility could be the effect of ODC antizyme. Increased ODC activity led to increases in cellular polyamines, which have been shown to inhibit ODC both directly and via the induction of a protein inhibitor (ODC antizyme) [36]. In mammalian cells, ODC antizyme is involved in the degradation of ODC both in vivo and in vitro [37,38]. The ODC antizyme binds to ODC, inactivates it, and targets it for degradation by 26S proteasome [38,39]. It is possible that the surge in ODC activity at day 24 leads to the increase of polyamines and stimulates the production of ODC antizyme, which in turn degrades the ODC protein at day 30.

In summary, the ontogenic increase in sucrase activity, a marker for intestinal maturation, occurs at the same time to that of the induction of intestinal ODC activity. The measurement of mRNA levels reveals that the induction of intestinal ODC activity during postnatal development is the result of post-transcriptional events or other cellular mechanisms. A better understanding of the regulation of polyamine biosynthesis during postnatal development of the small intestine will provide the insights contributing to the maturation of the small intestine. This information may be helpful in the development of therapeutic strategy in the management of intractable diarrhea of infancy.

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