Differential Development of Aldehyde Dehydrogenase in Fore- and Glandular Stomach in Postnatal Rats

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Many foods contain the unsaturated aldehyde, hexadienal (HX). Human exposure is thus unavoidable. HX feeding to rodents caused cancers only in the forestomach. Aldehyde dehydrogenases (ALDH) are key enzymes in the metabolism of aldehydes. We examined the distribution of ALDH using HX as the substrate (HXDH) along the GI tract of adolescent rats and found that their stomachs have high levels of HXDH activity and the enzyme preferred HX > 9-*cis*-retinal > acetyl aldehyde > formyl aldehyde. We also followed the postnatal development of the stomach. At birth, the forestomach represented 40–50% of the total stomach weight. Both fore- and glandular stomach gained weight, with the glandular portion gaining at a faster rate. By 21 days, the forestomach was 24–28% of the total weight and decreased slightly to an adult level of 22–24%. Gastric HXDH is low from birth to 14 days of age. HXDH activity increased thereafter, reaching higher levels at 21 days and peaking around 30–36 days of age. The activity then decreased to the adult level. The fore- and glandular stomach had the same level of HXDH activity in the newborn and at 7 and 14 days of age. At weaning, HXDH activity was higher (3×) in the forestomach than in the glandular stomach. In adults, the forestomach still had 2× the HXDH activity compared to the glandular stomach. Zymograms showed similar isozyme patterns of HXDH but with different ratios of the three major forms between the forestomach and the glandular stomach. Results indicate a differential development of HXDH between the fore- and glandular stomach that might be related to the higher sensitivity of the forestomach to HX feeding. [Exp Biol Med Vol. 227(7):554–558, 2002]

Key words: hexadienol; aldehyde dehydrogenase; stomach; development

Unsaturated aldehyde 2,4-hexadienal (HX) is formed as a product of *in vivo* or *in vitro* peroxidation of unsaturated lipids. It is a component of numerous vegetable and fish oils, and it is present in oxidized, heated, or cooked fats and oils and in cooked and uncooked meats (1–3). HX is used as a flavoring agent and as an intermediary chemical for various organic syntheses of dyes, antibiotics, and other drugs, and in formulated products (4, 5). It has also been used as the starting material for producing sorbic acid (6), a food preservative.

The presence of HX in a large number of foods and food products, either naturally or as additives, indicates that exposure to low levels of this compound is widespread. According to a recent estimate, the concentration of HX in consumer products can be as high as 0.1% (7). The finding that long-term feeding of HX to young rats and mice induced a high incidence of squamous cell papilloma and carcinoma in their forestomach but not in the glandular stomach is of particular interest and concern (8). Though the mechanism by which HX leads to forestomach carcinogenicity is unknown at present, the observation indicates a difference in the cellular response between the forestomach and the glandular stomach.

Aldehyde dehydrogenase (ALDH) is responsible for the metabolism of aldehydes, and some forms of the enzyme are found in high levels in the rat stomach (9, 10). As such, gastric ALDH should play an important role in determining the metabolic outcome and therefore the reactivity of ingested HX in the stomach. In this study, we characterized the rat stomach ALDH to see whether the enzyme will act on HX as well as other aldehydes. We also examined the ontogeny of the stomach in postnatal rats to determine whether a differential development existed between the fore- and glandular stomach with particular reference to ALDH activity using HX as the substrate (HXDH).

Materials and Methods

Chemicals. Unless otherwise stated, all chemicals were from Sigma Chemical Co. (St. Louis, MO). 2,4-Hexadienal was from Aldrich (Milwaukee, WI). 4-(2-

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Aminoethyl)benzene sulfonyl fluoride hydrochloride (AEBSF) was from ICN Biochemicals, Inc. (Aurora, OH).

Animals. To evaluate the tissue distribution of HXDH activity along the GI tract, three young adults (~8 weeks of age) were sacrificed, and their esophagus, stomach (separated into fore- and glandular portions), liver, and duodenal mucosal scrapings were collected.

For developmental studies, pregnant Sprague-Dawley rats from an inbred colony maintained at the Medical College of Wisconsin were housed in individual cages and maintained on a 12-hr alternate light-dark cycle. On the expected date of delivery, cages were inspected every 8 hr for birth. The day of birth was designated as day 0. Pups were allowed to suckle freely until 21 days of age and were weaned. For the evaluation of enzyme development, 6–24 animals (depending on age) were killed at various selected ages at 0830 hours on the corresponding dates. Their stomach and esophagus were harvested. The esophagus was cleaned of contents and connective tissue. The stomach was opened and emptied of food contents, rinsed in 0.9% saline, and blotted dry with paper towel before being separated into the fore- and glandular portions. All tissues were frozen immediately over dry ice, weighed, and stored at −80°C until analysis.

The National Institutes of Health Guidelines for the care and use of laboratory animals were followed. All animal protocols were approved by the Animal Research Committee of the Medical College of Wisconsin.

Methods. Thawed tissue samples were used for homogenization. Individual tissue (~100 mg) was homogenized in 0.5 ml of 0.9% saline containing 0.1% Triton X-100, 10 μg aprotinin, 10 μg soy bean trypsin inhibitor, and 5 μg AEBSF with a motorized Teflon pestle homogenizer. The homogenate was centrifuged for 15 min at 4°C and 4,000 rpm. The supernatant fraction was then centrifuged for 1 hr at 4°C at 105,000*g*. The final supernatant fraction was collected for enzyme and protein determination.

Protein concentration was determined by the Lowry method (11) with BSA as the standard.

Unless otherwise stated, the activity of HXDH was determined by the formation of NADH from NAD at 37°C in the presence of glutathione (9 mg/10 ml) and 5 μl of HX in 10 ml of potassium phosphate buffer (100 m*M*, pH 9.0). When other substrates were used their corresponding concentrations for each 10 ml of buffer were as follows: 50 μl of 1:10 aqueous acetyl aldehyde solution, 15 μl of a 37% aqueous formaldehyde solution, and 5 μl of 9-*cis*-retinal stock solution (1 mg/ml). Enzyme activities were expressed as pmol of NADH formed (increase in OD 340 nm) per min per mg protein.

LDH was determined by the formation of NADH from NAD at 37°C using sodium lactate as the substrate in a potassium phosphate buffer (100 m*M*, pH 7.5, with 133 m*M* NaCl and 0.66 m*M* $MgCl_2$). Enzyme activity was expressed as pmol of NADH formed (increase in OD 340 nm) per min per mg protein.

Zymographic analysis was performed in a native 8% polyacrylamide gel electrophoresis. Briefly, tissue samples (100 μg protein/lane for HXDH and 50 μg protein/lane for LDH) were mixed with Laemmli loading buffer—minus SDS, reducing agent, and no boiling—and subjected to electrophoresis. The gel was removed and washed once in corresponding buffer for HXDH or LDH assay for 10 min before incubation in the appropriate substrate buffer. For HXDH, the reaction mixture contained in each 10 ml of phosphate buffer (100 m*M*, pH 9.0); 8 mg NAD, 8 mg glutathione, 3.33 mg nitroblue tetrazolium, 0.33 mg of phenazine methosulfate, and 5 μl of HX (unless otherwise specified). For LDH the reaction mixture contained in each 10 ml of phosphate buffer (100 m*M*, pH 7.5); 2.67 mg of NAD, 133 m*M* NaCl, 0.66 m*M* $MgCl_2$, 3.33 mg nitroblue tetrazolium, and 0.33 mg of phenazine methosulfate. Gels were incubated in a 37°C water bath until distinct color bands appeared, normally 15 min for LDH and 30–60 min for HXDH.

Western blot studies were performed in an attempt to learn about the identity of HXDH. The antibody used was from Dr. James Lipsky's laboratory of the Mayo Clinic. This particular antibody recognizes ALDH1 but not ALDH2. Its reactivity to ALDH3 is still unknown.

Statistics. Results are reported as means ± SD. ANOVA was used to evaluate the difference between multiple groups. If significance was observed between groups, then a post-hoc *t*-test was used to compare the means of the two specific groups, with $P < 0.05$ considered as significant.

Results

Developmentally, the stomach shows a differential growth rate relative to the body (Fig. 1). A decrease in gastric weight per body weight was seen soon after birth, reaching a low value around 14 days of age. The stomach weight then abruptly increased relative to the body weight by 21 days of age. This higher stomach weight relative to body weight was maintained up to around 40 days of age and then declined to an adult level comparable to that of the 14-day-old pups.

More interesting is the differential growth of the forestomach compared to the glandular part of the stomach during postnatal development. Figure 2 summarizes the findings. At birth the forestomach weight represented about 45% of the total stomach weight. This forestomach/total stomach ratio rapidly decreased postnatally to reach a low of 22% around 21 days of age and maintained at that level until adulthood.

Preliminary studies indicated no difference in HXDH activities between males and females of various tissues in all age groups examined. The results of HXDH activities were therefore from mixed females and males. Figure 3 summarizes the relative level of HXDH activity in various parts of the GI tract and the liver. The esophagus has the

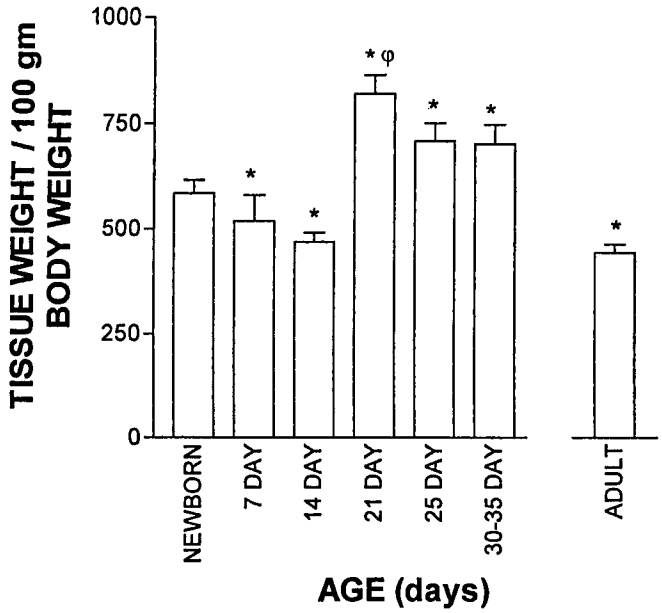

Figure 1. Developmental profile of stomach weights in postnatal rats. The date of birth was considered as day 0. Values represent the means ± SD from all animals killed at each time point. Vertical lines above the bars represent SD of the means. *Values significantly different from the newborn value. φValue significantly different from the adult value. The number of animals varies from 6 to 24 for each age. Stomach weight was normalized against body weight for each animal.

highest level of HXDH, followed by the stomach, with the forestomach in the young adults having higher HXDH activity than the glandular stomach. The liver and duodenum have much lower HXDH activity compared to the esophagus or either part of the stomach.

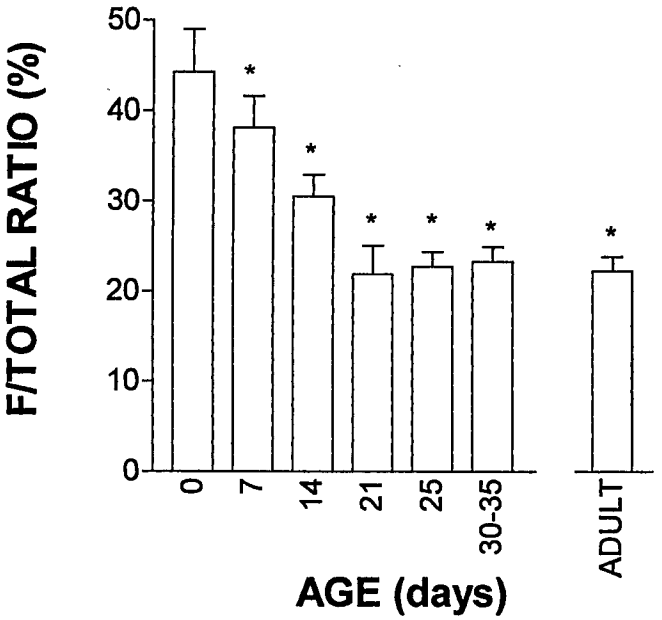

Figure 2. Developmental changes of the ratio of forestomach (F) to total stomach weight in postnatal rats. The date of birth was considered as day 0. Values represent the means ± SD from all animals killed at each time point. Vertical lines above the bars represent SD of the means. *Values significantly different from the newborn value.

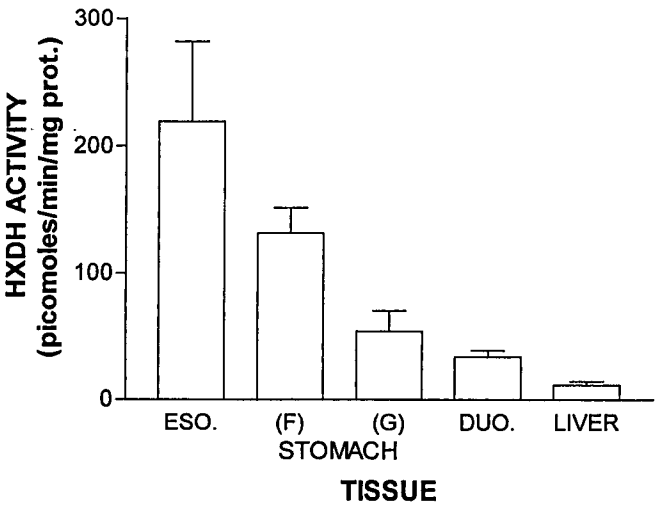

Figure 3. Distribution of HXDH activity along the GI tract and liver in young adult rats (8 weeks of age). Tissues were obtained from 3 separate animals. Values represent means from separate measurements of the corresponding tissues in all animals. The vertical lines above the bars represent SD of the corresponding means. F and G denote the forestomach and glandular stomach, respectively. Esophagus (ESO); duodenum (DUO).

In terms of substrate specificity, the stomach homogenate was most active against HX, followed by 9-*cis*-retinal, acetyl aldehyde, and the least for formyl aldehyde (Table I). Zymographic analysis using these substrates showed differences in staining intensity that confirmed the ranking preference of the corresponding substrates (data not shown).

The development of HXDH activity in the two parts of the stomach (fore- and glandular) and esophagus was followed. Figure 4 summarizes the results. Newborns had low HXDH activity in the forestomach, glandular stomach, and esophagus. Increase in HXDH activity was not seen until the time of weaning, i.e., 21 days of age, at which time the HXDH activities sharply increased to a level several times that of the newborn in the forestomach, glandular stomach, and esophagus. In both the fore- and glandular stomach, HXDH activity peaked around 30–36 days and then gradually declined to the adult level. In contrast, the HXDH activity in the esophagus kept rising to reach an even higher level in adults. More intriguing, the relative level of HXDH reached in the forestomach was higher (3×) than those

Table I. Substrate Specificity of the Forestomach HXDH[a]

Substrate	Enzyme activity (pmol/min/mg protein)
Hexadienal	95.6 ± 11.3
9-*cis*-Retinal	43.3 ± 9.4
Acetyl aldehyde	7.5 ± 2.6
Formyl aldehyde	2.9 ± 1.0

[a] Values are means ± SD from 3 separate determinations. All activity measurements were done in the presence of glutathione. Equivalent concentrations of each substrate were used (~5 μg/10 ml of reaction mixture).

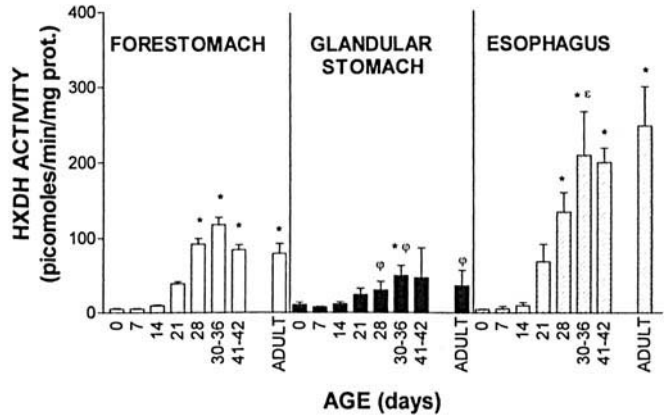

Figure 4. Developmental profiles of HXDH activity in forestomach and glandular stomach in comparison to esophagus. The date of birth was considered as day 0. Values represent the means ± SD from all animals killed at each time point. Vertical lines above the bars represent SD of the means. *Values significantly different from the newborn value in the same tissue. ᵠValues significantly different from corresponding values from the forestomach of animals from the same age group. ᵋValue significantly different from the adult value in the esophagus.

reached in the glandular stomach at weaning and during adolescence. In adults, the forestomach still has 2× the HXDH activity compared to that in the glandular stomach.

Zymographic analysis of HXDH isozymes during different postnatal ages showed multiple forms in the forestomach and glandular stomach (Fig. 5, left panel). At least four major bands were detected in the forestomach of the weaned animal (arbitrarily labeled as I, II, IIIA and IIIB). HXDH activity (as depicted by the intensity of staining) changed from low to high in the weaned animal and dropped back in adults to a level lower that the value seen in the 21-day-old animal. Band II was absent or very low in suckling rats and only appeared in weaned and adult animals. The glandular stomach showed a somewhat different

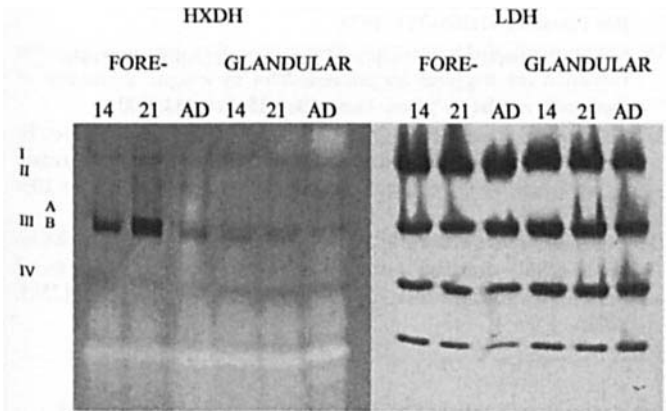

Figure 5. Zymographic analysis of HXDH from selected age groups of rats. Zymograph showing a comparison of forestomach and glandular stomach from 14-day-old, 21-day-old, and adult animals. The left-hand panel was stained for HXDH activities, and the right-hand panel was stained for LDH activities. Isozyme bands for HXDH are arbitrarily assigned Roman numerals (I, II, IIIA, B, and IV) on the left-hand side, for identification purposes only.

picture. The overall intensity of staining was much less than those from the forestomach even though the same amount of protein was loaded to each lane. Band II was also absent in suckling rats but appeared in weaned and adult animals. Band IIIB was found in suckling animals but was undetectable in weaned and adult animals. In addition, band IV, not detectable in the forestomach, was present in the glandular stomach at all ages. For comparison, a corresponding gel was stained for LDH activity. Figure 5 (right panel) shows the zymographic pattern of LDH. Both fore- and glandular stomach showed 4 LDH isozyme bands. There were no measurable differences when comparing forestomach or glandular stomach among different age groups. In contrast to the HXDH zymography, however, the glandular stomach has higher LDH activity relative to the forestomach.

Discussion

The stomach in the newborn rats is immature and undergoes rapid postnatal development. The responsiveness of acid and pepsinogen secretion to stimulation is acquired during postnatal development (12–14). Increased pepsinogen with age was seen both in the developing rat gastric mucosa (15) and gastric glands (14). Changes in other gastric variables have, however, rarely been studied.

The present results show that the stomach weight relative to body weight changes with age, suggesting a more rapid rate of stomach growth than body growth just before weaning (21 days of age). This pre-weaning period was also marked by differential growth between the fore- and glandular stomach. The rapid drop of the ratio of forestomach to total stomach weight from 45% in newborns to around 22% in weaned animals suggests a far higher growth rate of the glandular stomach relative to the forestomach during this period.

The stomach has been shown to have relatively high levels of ALDH activity compared to other tissues (9, 10). Our results showed that, in mature rats, the esophagus has the highest activity of HXDH followed by the stomach, small intestine, and liver. The forestomach had a much higher level (closer to the level found in esophagus) than the glandular stomach. Examination of gene markers during development of the GI tract has shown that the forestomach and regions more anterior to it (presumed embryonic esophagus) express different sets of regulatory genes (sox2 and pdx-1) than the posterior of the stomach and intestine (16). The similarity in the level of HXDH expression by the forestomach and esophagus may relate to their common embryonic origin.

The present results show that the neonatal stomach contains little HXDH activity at birth and during the early suckling period and that this activity rapidly increases just before weaning at 21 days of age. The developmental profile of HXDH activity found was very similar to that recently reported for retinal dehydrogenase development in rats (10). It

is unlikely that the enzyme is aldehyde oxidase since the enzyme is NAD dependent. Further, Moriwaki *et al.* have shown by histochemical techniques that aldehyde oxidase, though present in abundance in the tongue, renal tubules, bronchioles, and liver, is absent in the esophagus, stomach, and intestine in rats (17). Similar studies in human tissues also failed to detect aldehyde oxidase in the stomach (18). However, Western blot analysis using a similar antibody that recognized the proteins reported by the author did not react with the enzymatic proteins of our stomach homogenate, suggesting that the HXDHs we measured are different gene products. On the basis of the known reactivity of the antibody used, we can safely conclude that none of the HXDHs is ALDH1, but the exact identity of these HXDHs will require further characterization, particularly with the enzymes from the forestomach.

Two observations on the developmental profile of gastric HXDH might be of physiological importance. One is that the extent of HXDH rise in the forestomach was far greater than that in the glandular stomach such that by 21 days of age, the forestomach HXDH activity had 3× that found in the glandular stomach. The difference in the level of HXDH activity was quite specific, as the glandular stomach had LDH activity levels comparable to those of the forestomach. The other is the difference in HXDH isozyme patterns in the fore- and glandular stomach as development progressed. These together suggest that the forestomach behaves differently than the glandular stomach, at least where the handling of HX is concerned.

One more finding in our study is the substrate preference of the stomach homogenate, particularly from the forestomach, on various aldehydes used. Of the different forms of aldehydes used in the study, the stomach homogenate used HX much better than 9-*cis*-retinal, acetyl aldehyde, or formyl aldehyde. This may have important implications in terms of HX metabolism in the rat stomach. Although the mechanism of action of HX on gastric carcinogenesis is not known, it was shown that oral feeding of HX to rodents specifically induced cancer in the forestomach (8). Differences in HXDH activity levels and isozyme patterns in the forestomach might play a role in the metabolic fate of HX within the cells of the forestomach, thus leading to biochemical and cellular changes that are prerequisite to cancerous changes. This remains to be explored. Also, because of the ubiquitous presence of HX in food, further characterization of HXDH and its actions particularly on HX become important issues.

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