Immunolocalization of gastrin-dependent histidine decarboxylase activity in rat gastric mucosa during feeding

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Ohning, Gordon V., Min Song, Helen C. Wong, S. Vincent Wu, and John H. Walsh. Immunolocalization of gastrin-dependent histidine decarboxylase activity in rat gastric mucosa during feeding. Am. J. Physiol. 275 (Gastrointest. Liver Physiol. 38): G660–G667, 1998.—The localization of histidine decarboxylase (HDC) activity in the enterochromaffin-like (ECL) cells of the oxyntic mucosa was studied during fasting and refeeding using monoclonal (CURE no. 44178) and polyclonal (CURE no. 94211) antibodies directed against the COOH terminus of HDC (HDC-CT). Changes in HDC immunostaining were correlated with mucosal HDC enzyme activity. Immunoneutralization of circulating gastrin and atropine treatment during refeeding were used to determine the relative importance of gastrin and cholinergic mechanisms in the regulation of HDC activity and immunostaining. Fasting caused a rapid reduction in the number of ECL cells immunostaining for HDC that was correlated with an almost complete loss of mucosal HDC enzyme activity. Refeeding restored both HDC immunostaining and enzyme activity within 2–4 h, and this response was inhibited by gastrin immunoneutralization but not by atropine treatment. Immunostaining was uniformly decreased and restored in the lower half of the oxyntic mucosa, which corresponds to the predominant area of ECL cells in the gastric gland. Histamine immunostaining and mucosal histamine content were not significantly changed during fasting and refeeding or by gastrin antibody and/or atropine treatment during refeeding. These findings indicate that HDC activity correlates with HDC-CT immunostaining and that both HDC activity and HDC-CT immunostaining are regulated by gastrin during refeeding.

enterochromaffin-like cells; histamine

ENTEROCHROMAFFIN-LIKE (ECL) cells play an important role in the regulation of gastric acid secretion by their ability to synthesize, store, and release l-histamine in response to physiological stimuli (11, 21, 22). Histamine synthesis is regulated by histidine decarboxylase (HDC) (EC 4.1.1.22) (16). The initial report of gastrin-mediated regulation of HDC activity and histamine release in the rat stomach (12) has been confirmed by a variety of studies in which pharmacological, surgical, and meal-stimulated increases in circulating gastrin have been associated with increased gastric mucosal HDC activity (3, 5, 9, 20). Changes in HDC activity are inhibited by surgical antrectomy (19), and stimulation by refeeding was inhibited 60% by pretreatment with the selective CCK-B receptor antagonist L-365,260 (5). Furthermore, a gastrin-responsive promoter has recently been shown to regulate HDC gene expression (10).

Immunocohemical staining of HDC has been localized to the basal half of the oxyntic gland, and gastrin infusion caused an enhancement of HDC immunostaining (3). However, the relative contributions of gastrin and cholinergic mechanisms to meal-stimulated increases in HDC activity and the number of HDC-immunoreactive-like (HDC-IL) ECL cells have not been quantified. In the present study, the effects of fasting and refeeding on HDC activity and histamine content were compared. HDC-IL ECL cells were stained with an anti-HDC COOH-terminal (HDC-CT) antibody, and histamine-immunoreactive ECL cells were stained with an anti-histamine antibody in rat fundic mucosa. Gastrin immunoneutralization and muscarinic-receptor blockade were used to determine the relative contributions of gastrin and cholinergic stimulation to the food-stimulated HDC activity and ECL cell HDC immunostaining.

MATERIALS AND METHODS

Antibodies

Gastrin monoclonal antibody (CURE no. 051091.1) and control monoclonal antibody (CURE MAb no. 109.21) were characterized and prepared for in vivo use as previously described (15). Anti-histamine antibody was obtained from Incstar (Stillwater, MN). Monoclonal and polyclonal anti-HDC antibodies were prepared using a COOH-terminal peptide antigen conjugated to keyhole limpet hemocyanin (KLH) as previously described (1, 13). The COOH-terminal peptide antigen corresponded to positions 604–615 in the HDC amino acid sequence with the addition of an NH2-terminal tyrosine residue for use in the conjugation to KLH (amino acid sequence: YRARIFSGFPEEM). Monoclonal antibodies were produced and characterized as previously described (24). Briefly, spleen cells from female Robertsonian mice containing the translocated 8–12 chromosome (Jackson Laboratories, ME) immunized with conjugated COOH-terminal peptide antigen and complete Freund’s adjuvant (1:1 emulsion) were fused with FOX-NY myeloma cells that were adenine phosphoribosyltransferase deficient. Ascites fluid was produced in 2,6,10,14-tetramethylpentadecane-primed female BALB/c mice (Charles River, North Wilmington, MA). The ascites fluid contained ~20 mg/ml IgG with a titer of 1:500,000 determined by ELISA. Western blot analysis of enriched rat ECL cells detected a single immunospecific band at a molecular weight of ~60,000. Radial immunodiffusion identified the antibody as an IgG1 subclass. Polyclonal antibodies were produced by multiple intradermal injections in 8-wk-old New Zealand White female rabbits (Irish Farm, Norco, CA), as previously described (23).
Drugs

Atropine sulfate (Sigma Chemical, St. Louis, MO) was dissolved in 0.9% saline before use. All chemicals and reagents used were American Chemical Society grade or the best grade available.

Animals

Male Sprague-Dawley rats (Harlan, San Diego, CA) weighing 220–350 g were maintained ad libitum on Purina laboratory chow and tap water under conditions of controlled temperature (22 ± 4°C) and illumination (12:12-h light-dark cycle) until study. Rats were allowed free access to water during the entire fasting period for all experimental protocols. All animal protocols adhered to the Guide for the Care and Use of Laboratory Animals and were approved by the West Los Angeles Veterans Affairs Medical Center Research Service Animal Committee.

Animal Protocols

Fasting and refeeding. Groups of 4–6 rats were fasted for 6, 12, 24, and 48 h before being killed. Additional groups of rats were fasted for 48 h and refed with solid chow and killed at 0.5, 2, 4, 12, and 24 h after initiation of refeeding. Blood was obtained for gastrin RIA, and the stomach was removed, fixed, and processed for immunohistochemistry as described below.

Gastrin immunoneutralization during refeeding. Groups of four to six rats were fasted for 48 h. Gastrin monoclonal antibody (CURE no. 051091.1) or control monoclonal antibody (CURE MAb no. 105.21) was given by tail vein injection (3 mg, iv) 30 min before initiation of refeeding. Rats were killed at 4 h after the initiation of refeeding, and blood and tissue samples were obtained as described above.

Atropine treatment during refeeding. Groups of four to six rats were fasted for 48 h. Atropine (1 mg/kg) or vehicle was given by intraperitoneal injection 30 min before initiation of refeeding, and additional doses were given at 60-min intervals for the remainder of the experiment. Additional groups of rats received concomitant gastrin monoclonal antibody and atropine treatment. Rats were killed at 4 h after the initiation of refeeding, and blood and tissue samples were obtained as described above.

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Assay of Gastrin

Blood was collected in ice-chilled tubes containing 0.04 ml of 7.5% EDTA solution. Samples were centrifuged at 2,000 g for 10 min, and the plasma was removed and stored at −20°C until assay. Gastrin RIA was performed using gastrin polyclonal antibody (CURE no. 1611) as previously described (7).

Determination of Histamine Content

The stomach was opened along the greater curvature, and the oxyntic mucosa was removed, weighed, and stored at −70°C until analysis. Samples were thawed and homogenized in ice-cold 0.2 N perchloric acid. Samples were centrifuged at 10,000 g for 5 min at 4°C, and the supernatant was collected and neutralized by addition of an equal volume of 1 M potassium tetraborate, pH 9.25. A commercial RIA kit (AMAC; Immunootech, Paris, France) was used for histamine measurement according to the manufacturer’s instructions.

Assay of HDC Activity

HDC activity was assayed by modification of the technique of Beaven et al. (2). Oxyntic mucosa was homogenized in ice-cold 0.05 M sodium phosphate, pH 7.0, and centrifuged at 10,000 g for 15 min at 4°C. All samples were kept on ice until initiation of the assay. Replicate 80-µl aliquots of supernatant were incubated with 50 nCi L-[1-14C]histidine (NEN, Boston, MA), 5 × 10–3 M L-histidine, 10 × 10–5 M pyridoxal-5-phosphate, and 10 × 10–7 M diithothreitol in a final volume of 160 µl for 60 min at 37°C. Assays were performed in 16 × 100 mm culture tubes (Baxter Scientific, Irvine, CA) with well-fitting stoppers (Kontes, Vineland, N.J.) containing a filter paper strip wetted with 50 µl of Solvable (Packard Instrument Company, Downers Grove, IL) suspended in a center well (Kontes). The [14C]CO2 formed by the reaction was trapped in filter paper by addition of 2 M citric acid and incubation for 50 min at 37°C. Radioactivity retained in the filter paper was measured by liquid scintillation counting, and HDC activity was expressed as picomoles of CO2 formed per milligram of protein per hour. Buffers blank values were subtracted from sample values before activity calculation.

Morphological Measurements and Immunohistochemistry

Tissue strips were taken from the oxyntic part of the stomach and fixed in 4% paraformaldehyde, 2% picric acid, and 0.01 M PBS, pH 7.4, for 4 h at room temperature. Sections were subsequently incubated in 20% sucrose and 0.01 M PBS, pH 7.4, overnight at 4°C. The tissue strips were infiltrated with OCT compound embedding medium (Miles, Elkhart, IN) and frozen on dry ice. Cryostat sections (8 µm) were obtained and mounted on Superfrost Plus slides (Fisher, Tustin, CA).

HDC immunostaining was performed using anti-HDC mouse monoclonal (CURE no. 44178) and rabbit polyclonal (CURE no. 94221) antibodies that were raised against the COOH-terminal fragment of rat HDC as described above. Histamine immunostaining was performed using a commercially available rabbit polyclonal antibody against histamine (Instar). Working dilutions were 1:1,000 for the monoclonal and polyclonal anti-HDC antibodies and 1:20 for the anti-histamine antibody.

Primary antibodies were incubated on rehydrated tissue sections for 18 h at 4°C immediately followed by a 30-min incubation at 37°C. Sections were washed three times in PBS and FITC-conjugated goat anti-rabbit antibody (Jackson Laboratories) or FITC-conjugated horse anti-mouse antibodies (Vector Laboratories, Burlingame, CA) were applied to the appropriate sections and incubated for 60 min at room temperature. Double staining to determine the colocalization of histamine and HDC immunoreactivity was performed by incubating sections with a 1:1 mixture of mouse monoclonal anti-HDC and rabbit polyclonal anti-histamine antibodies with subsequent incubation with a 1:1 mixture of FITC-horse anti-mouse and tetramethylrhodamine isothiocyanate (TRITC)-goat anti-rabbit antibodies. Immunofluorescent cells were counted in three randomly selected 2.4-mm2 fields in each section using a Nikon Labophot-2 fluorescent microscope (Nikon Instrument Group, Garden City, NY). Cell counts were expressed as the number of immunoreactive positive cells per square millimeter. Nonspecific immunostaining (negative control immunostaining) was identified by using primary antibodies preincubated with excess antigen before use.

Statistics

Results are expressed as means ± SE and were evaluated by one-way ANOVA. When ANOVA revealed significant differences (P < 0.05), a nonparametric Kruskal-Wallis test was performed to compare groups.
RESULTS

Fasting and Refeeding: Effect on HDC Immunostaining and HDC Activity

Immunostaining of HDC with either monoclonal or polyclonal HDC-CT antibody colocalized with histamine immunostaining in ECL cells (Fig. 1). Furthermore, the HDC immunostaining localized predominantly to ECL cells in the lower half of the oxyntic mucosa (Fig. 2). During fasting, the number of ECL cells detected by HDC-CT immunostaining markedly decreased during the first 12 h to ~10% of the freely fed level and remained at this low level for the remaining 48 h of fasting (Figs. 3A and 4A). Refeeding caused a rapid increase in HDC-CT immunostaining in the oxyntic ECL cells back to prefasting levels within the first 2 h of feeding (Figs. 3B and 4A). Similarly, HDC enzyme activity markedly decreased during the first 12 h and reached a barely

Fig. 1. Photomicrographs of enterochromaffin-like (ECL) cell immunostaining. Immunostaining of rat gastric mucosa with histidine decarboxylase (HDC) polyclonal antibody (A) and histamine polyclonal antibody (B). Colocalization is seen in ECL cells with FITC secondary antibody against mouse monoclonal antibody and tetramethylrhodamine isothiocyanate secondary antibody against rabbit polyclonal antibody. Original magnification, ×1,000.

Fig. 2. Effect of gastrin immunoneutralization on ECL cell immunostaining during refeeding. HDC polyclonal antibody immunostaining of rat gastric mucosa 4 h after refeeding (A). Pretreatment with gastrin monoclonal antibody markedly reduced HDC immunostaining (B). Original magnification, ×200.
detectable level at 48 h (Fig. 4B). Refeeding increased HDC enzyme activity, but prefasting levels were not achieved until 24 h after the initiation of feeding (Fig. 4B).

**Fasting and Refeeding: Effect on Histamine Immunostaining and Histamine Content**

Immunostaining for histamine and histamine content in the oxyntic mucosa did not significantly change during the 48-h fasting period or during the 24-h period after initiation of feeding (Fig. 5).

**Fasting and Refeeding: Effect on Circulating Gastrin**

Fasting and refeeding caused a marked decrease in circulating gastrin during the period of fasting and rapid restoration of the prefasting level after initiation of feeding (Fig. 6).

**Effect of Gastrin Immunoneutralization and Atropine Treatment During Refeeding**

Immunoneutralization of circulating gastrin with monoclonal antibody during refeeding reduced HDC-CT immunostaining by 70% (Figs. 2 and 7A) and inhibited the expected increase in HDC enzyme activity by >90%.
compared with control antibody treatment (Fig. 7B). Histamine immunostaining and histamine content were not significantly affected by gastrin immunoneutralization (Fig. 8). Atropine treatment during refeeding did not significantly change HDC-CT or histamine immunostaining and did not significantly affect HDC enzyme activity or histamine content (Figs. 7 and 8). Concomitant treatment with gastrin monoclonal antibody and atropine during refeeding caused a reduction in HDC-CT immunostaining that was significantly greater than the reduction produced by gastrin monoclonal antibody alone (Fig. 7A). The expected increase in HDC enzyme activity during refeeding was markedly reduced during combined gastrin monoclonal antibody and atropine treatment, but it was not significantly lower than with gastrin monoclonal antibody alone (Fig. 7B).

**DISCUSSION**

This study demonstrates the strong correlation between immunohistochemical detection of HDC using HDC-CT antibodies and the measurement of mucosal HDC enzyme activity in the oxyntic ECL cells in the rat stomach. Immunoreactive HDC-containing cells and HDC enzymatic activity demonstrated parallel decreases during fasting, parallel increases during subsequent refeeding, and similar inhibition of the refeeding response by immunoneutralization of circulating gastrin. Immunostaining of histamine colocalized to the oxyntic ECL cell; however, it did not change during fasting or refeeding and it was not affected by gastrin immunoneutralization or atropine treatment. This finding was supported by a similar lack of change in oxyntic mucosal histamine content under the same experimental conditions.

Both mouse monoclonal (CURE no. 44178) and rabbit polyclonal (CURE no. 94211) anti-HDC antibodies demonstrated immunostaining of ECL cells. Double staining with mouse monoclonal HDC antibody and rabbit polyclonal histamine antibody demonstrated colocalization in ECL cells throughout the mucosa with a predominant localization in the lower half of the gastric gland (Fig. 2). During fasting, HDC immunostaining in ECL cells was uniformly reduced, and refeeding caused a uniform increase throughout the lower gastric gland, suggesting the absence of a particular subpopulation of ECL cells responsive to refeeding (Fig. 2). The marked reduction in HDC immunostaining in the ECL cells was temporally correlated with a near total loss of HDC enzymatic activity. This rapid loss of immunoreactivity likely reflects rapid degradation of the enzyme. The HDC amino acid sequence has several PEST sites [regions within a protein that are enriched with proline (P), glutamic acid (E), serine (S), and/or threonine (T) amino acid residues within a hydrophilic fragment and flanked by cationic amino acids], including one near the COOH-terminal end (6). The truncation of HDC at this COOH-terminal PEST site would result in the loss of the peptide region used to generate the antibodies used in this study. Although the COOH-terminal PEST-containing region is not essential for mammalian HDC activity (6), PEST sites are correlated with...
rapid degradation of proteins (17, 18). The initiation of HDC degradation within the COOH-terminal PEST region may signal the accelerated subsequent degradation of critical regions required for enzymatic activity and would explain the high degree of correlation in the time course between changes in HDC-CT immunoreactivity and enzymatic activity.

Refeeding after a 48-h fast has been shown to increase HDC mRNA (4). Ding et al. (5) reported that...
fasting caused a reduction in HDC enzymatic activity and refeeding caused a rapid increase in HDC activity. The HDC activity correlated with circulating gastrin, and treatment with the selective CCK-B/gastrin receptor antagonist L-365,260 and antrectomy inhibited the feeding-induced increase in mucosal HDC activity by 60% and 70%, respectively. In the present study, similar responses of HDC activity to fasting and refeeding were observed. Immunoneutralization of circulating gastrin with a gastrin-selective antibody inhibited 93% of the expected increase in HDC enzyme activity in response to refeeding, emphasizing the importance of gastrin in HDC regulation. The higher efficacy of antibody in this inhibitory response may reflect the advantage of using high-affinity monoclonal antibody to trap gastrin within the circulating compartment compared with the competitive inhibition by receptor antagonists at the cell surface. In the present study, HDC-CT immunostaining also demonstrated a marked decrease (70%) in refeed rats treated with gastrin antibody. Atropine treatment alone during refeeding did not significantly change either HDC enzyme activity or HDC-CT immunostaining, indicating that cholinergic stimulation does not have a substantial role in the refeeding response. The dose of atropine administered (1 mg/kg) has previously been shown to completely block stimulation of gastric acid secretion by exogenously administered bethanecol in urethane-anesthetized rats (14). Concomitant atropine treatment did not cause a further significant decrease in HDC activity over gastrin antibody treatment alone but did significantly decrease HDC-CT immunostaining. This quantitative difference in the HDC enzyme activity and HDC-CT immunostaining may reflect a modest difference in turnover of enzymatically inactive but immunologically detectable HDC protein during cholinergic inhibition. This discrepancy in immunostaining vs. enzymatic activity is likely due to the loss of the COOH-terminal region of the HDC protein required for immunostaining with the antibodies used in the present study by rapid PEST site cleavage before the subsequent degradation of the active site responsible for the HDC enzymatic activity (6).

The apparent absence of significant changes in ECL cell histamine content by both chemical measurement and immunostaining with histamine antibodies in this study suggests that only a small fraction of histamine stores is responsive to the physiological events of fasting and feeding or the inhibition of gastrin and/or atropine. Chen et al. (3) have shown that pharmacological stimulation with gastrin-17 caused a significant decrease in oxyntic mucosal histamine content and caused a reduction in the number and size of cytoplasmic vesicles in ECL cells consistent with exocytosis. The dose of gastrin utilized resulted in sustained levels of circulating gastrin of >700 pmol/l, which is at least sixfold higher than physiologically achieved in the postprandial period in the present study. In the rat oxyntic mucosa, HDC and histamine are present in ECL cells and mast cells (9, 22). Mast cells contain large amounts of histamine, but relatively small amounts of HDC. Furthermore, the turnover rate for histamine is rapid in ECL cells and slow in mast cells (8, 20). Fasting decreases the rate of histamine turnover in the ECL cell, allowing for conservation of histamine content. This effect is likely mediated by decreased circulating gastrin, leading to a marked reduction in gastrin-stimulated histamine release. Refeeding leads to a rapid increase in circulating gastrin and should cause a release in histamine release with a concomitant reduction in mucosal histamine content and immunostaining. Although the histamine content time course demonstrates a decrease after the initiation of feeding, these results did not achieve statistical significance. Furthermore, the recovery of HDC enzymatic activity after the first 2 h may be sufficient to replenish histamine stores. Histamine immunostaining is similarly unaffected by fasting or refeeding. The amount of cellular histamine necessary to give a positive staining result may be sufficient in the ECL cells at all times during the 48-h period of fasting and subsequent refeeding. Hence, the sensitivity of histamine immunostaining may be insufficient to detect the changes that occurred in this study.

In summary, immunostaining with antibodies directed toward HDC-CT colocalized with histamine immunostaining in the rat oxyntic mucosa. HDC-CT, but not histamine, immunostaining correlated with changes in HDC enzyme activity observed during fasting and refeeding and in response to immunoneutralization of circulating gastrin. Immunostaining of ECL cells with HDC-CT antibodies was predominantly localized to the lower half of the gastric gland; however, changes in HDC immunostaining in response to fasting and refeeding occurred uniformly within this zone. These data demonstrate that all ECL cells within the gastric gland respond to changes in circulating gastrin in response to fasting and refeeding and argue against a gastrin-responsive subpopulation of ECL cells.

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