Distinct effects of tetragastrin, histamine, and CCh on rat gastric mucin synthesis and contribution of NO

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Ichikawa, Tatsufumi, Kazuhiko Ishihara, Tatsumi Kusakabe, Makoto Kurihara, Tadashi Kawakami, Toshifumi Takenaka, Katsunori Saigenji, and Kyoko Hotta. Distinct effects of tetragastrin, histamine, and CCh on rat gastric mucin synthesis and contribution of NO. Am. J. Physiol. 274 (Gastrointest. Liver Physiol., 37): G138–G146, 1998.—Although gastrin, histamine, and carbachol (CCh) accelerate gastric mucin metabolism, information about their target cells of mucin production is lacking. To clarify this, we examined the effects of these stimulants, including the possible participation of nitric oxide (NO), on mucin biosynthesis in distinct sites and layers of rat gastric mucosa. Pieces of tissue obtained from the corpus and antrum were incubated in a medium containing radioactive precursors and each stimulant, with or without NO synthase (NOS) inhibitor. Distribution of NOS was compared with that of the specific mucins by immunostaining using specific antisera and monoclonal antibodies. In the full-thickness corpus mucosa, tetragastrin enhanced [3H]glucosamine incorporation into mucin but had no effect on [14C]threonine incorporation. Both histamine and CCh dose dependently increased H- and 14C-labeled corpus mucin. Only CCh stimulated antral mucin biosynthesis. CCh stimulation was noted in the corpus mucosa after removal of surface mucous cells, but stimulation by tetragastrin or histamine disappeared as a result of this pretreatment. Only tetragastrin-induced activation was completely blocked by the NOS inhibitor. NOS immunoreactivity was limited to surface mucous cells. Mucus-producing cells present in the different sites and layers of the gastric mucosa have distinct mechanisms for regulation of mucin biosynthesis. Gastrin-stimulated mucin biosynthesis mediated by NO is limited to surface mucous cells of rat gastric oxyntic mucosa.

Gastrin; nitric oxide; surface mucous cells; carbachol

MUCOSAL IS CONSIDERED to be one of the principal factors in the physiological defense mechanism of the gastrointestinal mucus and is mainly composed of gel-forming, high molecular weight mucus glycoprotein (mucin) (3). Mucus-secreting cells of the mammalian gastric mucosa have been classified mainly into surface mucous and gland mucous cells (21, 42). Mucus from these two types of cells is suggested to have distinct roles in the physiology of the gastric mucosa (31), and the mucins of these two types of cells in a single tissue section are individually characterized by a combination of galactose oxidase-cold thionine Schiff staining and paradoxic concanavalin A staining (32). We have previously shown that the mucins obtained from the distinct regions (corpus and antrum) or particular layers (surface mucosa and deep mucosa) of rat gastric mucosa differ in their subunit structures and the chemical composition of their carbohydrate moieties (10, 16, 30). Our recent studies using newly developed monoclonal antibodies (MAB) that recognize the specific carbohydrate structure of mucin in the gastric mucosa have demonstrated that surface mucous and gland mucous cells from the corpus and antral region produce mucins bearing a particular carbohydrate structure (17–19). The peptide sequence of apomucin derived from surface mucous cells of the human stomach that has recently been identified is MUC5AC. This peptide sequence differs from that of the gland-type mucous cell-derived mucin, which was identified as MUC6 (7, 14). These results suggest that particular types of mucus-producing cells in the different sites and layers of the gastric mucosa may have distinct mechanisms for the regulation of mucin biosynthesis.

Gastrin, histamine, and acetylcholine (ACh), recognized as gastric acid secretagogues, have been reported by several laboratories (12, 13, 36, 37, 49) to stimulate the production and secretion of gastric mucus. We have already reported that gastrin, a gastrointestinal hormone, significantly accelerated mucin biosynthesis in the oxyntic region but yielded no significant change in the antral region of the rat stomach (15). However, information about the different ways in which these agents activate mucin metabolism in the gastric mucosa, particularly with regard to whether or not each stimulant-induced activation is limited to the specific mucus-producing cells, is lacking. The current studies also suggest that nitric oxide (NO), known as a labile free radical and produced by NO synthase (NOS) from L-arginine in various cells and tissues, might have a role in the functioning of gastric mucous cells (4–6, 43, 46), but the contribution of NO to gastric mucin biosynthesis remains to be determined.

The first aim of this study was to investigate differences among gastrin, histamine, and carbachol (CCh) by comparing their mode of stimulation on mucin biosynthesis, utilizing the [3H]glucosamine and [14C]threonine incorporation method, which indicates biosynthesis of carbohydrates and peptides, respectively. In the second step, the effect of these acid secretagogues on mucin biosynthesis in distinct sites and layers of rat gastric mucosa was compared by using a scraping method to separate the surface mucosal layer from the remaining deep mucosa of the stomach (23). In the
third part of this study, we examined whether endogenous NO release contributes to the stimulation of gastric mucin biosynthesis in rat gastric mcosa.

**MATERIALS AND METHODS**

**Biochemical Studies**

**Animals.** Seven-week-old male Wistar rats (SLC, Shizuoka, Japan), each weighing ~170 g, were deprived of food but allowed free access to water for 24 h before the experiments.

**Materials.** The following substances were used for this study: tetragastrin (Med, Tokyo, Japan); histamine (Wako Pure Chemical, Osaka, Japan); CCh (Sigma Chemical, St. Louis, MO); N^N^-nitro-L-arginine (L-NNA; Wako Pure Chemical Industries); 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazooline-1-oxyl-3-oxide sodium salt [carboxy-PTIO; an antidote that specifically reacts with NO (1, 48); Doping Laboratories, Kumamoto, Japan]; Eagle's minimum essential medium (MEM) and modified Eagle's MEM deficient in L-threonine (GIBCO, Grand Island, NY); d-[1,6-^3H(N)]glucosamine hydrochloride (GlcN) (1,943 GBq/mmol; New England Nuclear, Boston, MA); and [1^4C]-threonine (Thr) (8.9 GBq/mmol, New England Nuclear). All drugs were dissolved in phosphate-buffered saline (PBS), with the exception of L-NNA. L-NNA was prepared in 0.01 N HCl before being neutralized to pH 7.0. Stock solutions of the drugs were prepared and the concentrations reported are final bath concentrations.

**Tissue preparation of different sites and layers.** Rats were euthanized by inhalation of CO₂, and their stomachs were excised immediately and then cut along the greater curvature. The gastric contents were gently rinsed out from the gastric mucosa by immersion in PBS. To obtain the full-thickness layer samples of the glandular tissue, the forestomach was removed from the remaining glandular stomach. The mucosa obtained was separated into the corpus and antrum and then cut into small pieces of 2 × 2 mm. Alternatively, after the stomach was opened, the surface mucosa of the glandular portion was removed from the remaining deep mucosa according to our previously described method (23). Briefly, the glandular part of the stomach was immersed in PBS solution containing 2% (wt/vol) N-acetylcycteine and gently stirred for 5 min at room temperature. The stomach was then gently washed with PBS, and the mucosal surface of the glandular portion was mildly scraped off with forceps having a j-shaped cusp (A-12–2; Natsume, Tokyo, Japan) to peel off the surface mucosal layer from the remaining glandular stomach. The remaining deep mucosal layer of corpus was sliced into small pieces of ~4 mm², similar to the full-thickness layer specimens.

**Tissue culture.** Eight tissue fragments, with the mucosal surface facing up, were placed on a stainless steel grid in the central well of a plastic culture dish (60 × 15 mm; Falcon, Lincoln Park, NJ) and then treated with 0.75 ml of medium neutralized to pH 7.0. Stock solutions of the drugs were prepared and the concentrations reported are final bath concentrations.

**Materials and Methods**

**Isolation of labeled mucin and radioactivity measurement.** On completion of the culture period, the tissue fragments in each dish were harvested from the medium after being gently rinsed with PBS and boiled at 100°C for 3 min in 0.4 ml 0.05 M tris(hydroxymethyl)aminomethane (Tris)-HCl buffer, pH 7.2. The extraction and isolation of gastric mucins were performed as previously described (2, 15). The tissue fragments were homogenized with a Physcotron microhomogenizer (Niti-On, Chiba, Japan). After Triton X-100 was added to a 2% (vol/vol) concentration, the homogenate was shaken for 1 h at 37°C and centrifuged at 8,000 g for 30 min to obtain the supernatant. Of the supernatant, 0.4 ml was applied onto a Bio-Gel A-1.5 m column (1 × 30 cm) previously equilibrated with Tris buffer containing 2% Triton X-100, and the column was eluted with this buffer. Finally, fractions of 0.8 ml each were collected, and the radioactivity was measured by scintillation counter (model LS-2800; Beckman, Irvine, CA), using Aquasol-2 (New England Nuclear) as the scintillant. The incorporation of radioactive precursors into the gastric macromolecular fraction corresponding to mucin (29), which was eluted in the void volume of the column, was determined. To compare the synthetic activity of mucin, the total radioactivity of this fraction was divided by the tissue protein content of each homogenate and expressed as disintegrations per minute per milligram of tissue protein.

**Protein determination.** Protein content in the tissue homogenate was determined by the biinchoninic acid method (38) with a Pierce protein assay kit (Pierce, Rockford, IL), using bovine serum albumin as the standard.

**Statistical analysis.** The results were expressed as means ± SD. One-way analysis of variance (ANOVA) with Dunnett’s test was used for statistical analysis, with P < 0.05 considered significant.

**Immunohistochemical Studies**

**Antibodies.** Two MAb (both identified as immunoglobulin M) against rat gastric mucin were produced in our laboratory according to the modified method (9) of Kühler and Milstein (22) as previously described (17–19). The antibodies, designated RGM21 and HIK1083, have been shown to react not only with the purified mucin but also with the specific structure of oligosaccharide obtained from the mucin by alkaline borohydride treatment (17–19). Histochemical observation indicates that RGM21 and HIK1083 react strongly with surface mucous cells of the corpus and with mucous neck and antral gland cells, respectively, on Formalin-fixed, paraffin-embedded sections of rat gastric mucosa (17–19).

The polygonal antiserum raised against the synthetic peptide from the COOH terminus of the cloned rat cerebellar NOS was purchased from Euro-Diagnostica (Malmö, Sweden).

**Immunohistochemical staining.** Male Wistar rats weighing ~170 g were used. Rats were anesthetized with pentobarbital sodium (0.05 mg/g) and perfused through the heart with heparinized (1.1 U/ml) 0.1 M PBS, pH 7.4, followed by 300 ml of Zamboni’s fixative solution (50). The glandular stomachs were then excised and immersed in the same fixative for an additional 6 h at 4°C. After a brief washing with PBS, the corpus specimens were transferred to 30% sucrose in PBS and kept overnight at 4°C. The specimens were then sectioned serially at 10 mm on a cryostat and mounted on poly-L-lysine-coated slides. The sections were immunohistochemically stained for NOS antiserum, RGM21 MAb, and HIK1083 MAb with the peroxidase-antiperoxidase (PAP) method of Sternberger (40), as noted in a previous report (26). Briefly, the sections were dipped in a fresh 0.3% H₂O₂ solution.
in methanol for 30 min at room temperature, followed by washing in several changes of 0.3% Triton X-100 in 0.1 M PBS, treated for 1 h with a protein-blocking agent (Immunon, Stillwater, MN) at room temperature, and incubated at 4°C overnight with the primary antibodies described above. The mouse MAbs against mucin were diluted to 1:10 with 0.2% bovine serum albumin, 1% normal goat serum, and 0.2% sodium azide in PBS, and the antiserum against NOS was diluted to 1:1,000, respectively. To locate antigenic mucins, the sections were transferred to goat anti-mouse immunoglobulin G (diluted 1:100; Biosource, CA). Sections were washed in three changes of PBS and then incubated with mouse PAP complex (diluted 1:200; Jackson, West Grove, PA). To demonstrate the existence of NOS, each section was transferred to goat anti-rabbit immunoglobulin G (diluted 1:200; Cappel, Durham, NC). After washing, the section was overlaid with rabbit PAP complex (diluted 1:200; Jackson). Peroxidase reactivity was demonstrated with the 3,3'-diaminobenzidine method of Graham and Karnovsky (11).

RESULTS
Effects of Tetragastrin, Histamine, and CCh on \([3H]\text{GlcN}\) and \([14C]\text{Thr}\) Incorporation into Gastric Mucins in Full-Thickness Corpus Mucosa

Figure 1 shows the biosynthetic activity of mucin in the full-thickness corpus mucosa as measured by the simultaneous incorporation of \([3H]\text{GlcN}\) and \([14C]\text{Thr}\) with or without acid secretagogues. In the controls of the corpus tissue, \(^{3}H\) and \(^{14}C\) radioactivities incorporated into the gastric mucin were 20,698 ± 2,178 and 3,864 ± 502 disintegrations per minute (dpm)/mg tissue protein, respectively. The addition of \(10^{-8}\) M tetragastrin enhanced \([3H]\text{GlcN}\) incorporation into mucin by 34% (\(P < 0.01\)) but had no significant effect on \([14C]\text{Thr}\) incorporation. The biosynthetic response to \(10^{-7}\) M tetragastrin was essentially the same as the response to \(10^{-8}\) M tetragastrin, but the addition of \(10^{-9}\), \(10^{-6}\), and \(10^{-5}\) M tetragastrin had no effect on the incorporation of either \([3H]\text{GlcN}\) or \([14C]\text{Thr}\) into the mucin molecule (Fig 1 A). In contrast to tetragastrin, \(^{3}H\)- and \(^{14}C\)-labeled mucins were significantly increased with the addition of \(10^{-6}\) and \(10^{-5}\) M histamine and CCh. The effects of histamine and CCh on the incorporation of \([3H]\text{GlcN}\) and \([14C]\text{Thr}\) into the mucins were expressed in a concentration-dependent manner (Fig 1 B and C).

Effects of Tetragastrin, Histamine, and CCh on \([3H]\text{GlcN}\) and \([14C]\text{Thr}\) Incorporation into Gastric Mucins in the Antral Mucosa

No significant change could be detected in mucin biosynthesis of the antrum after the addition of either tetragastrin or histamine (sp act 30,246 and 33,246 dpm/mg, respectively; Fig 2, A and B). In contrast, the biosynthetic responses to CCh in the antrum were essentially the same as the response to CCh in the corpus mucosa (Fig 2 C).

Effects of Tetragastrin, Histamine, and CCh on \([3H]\text{GlcN}\) Incorporation into Gastric Mucins in the Deep Layer of Corpus Mucosa

To examine whether the stimulatory effects of acid secretagogues on corpus mucin biosynthesis are limited to specific mucous cells of the rat gastric mucosa, the deep layer tissues of the corpus, including the mucous

**Fig. 1. Effects of acid secretagogues on mucin biosynthesis in full-thickness corpus mucosa. A: tetragastrin. B: histamine. C: carbachol (CCh). Tissue fragments obtained from full-thickness corpus mucosa were incubated in medium 1 (described in MATERIALS AND METHODS) with \(^{3}H\)-labeled D-[1,6-\(^{3}H\)(N)]glucosamine hydrochloride (\([3H]\text{GlcN}\)) and \([14C]\text{Thr}\) for 5 h. Each acid secretagogue was added to the culture medium at final concentrations of \(10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}\), and \(10^{-5}\) M. On completion of culture, fragments were harvested, and newly synthesized radiolabeled mucin was extracted and isolated by Bio-Gel A-1.5 m column chromatography. To compare synthetic activity of mucin, incorporation of \([3H]\text{GlcN}\) (open bars) and \([14C]\text{Thr}\) (solid bars) into mucins was determined and divided by tissue protein content. Values are means ± SD from 8 samples, given as % of control. Control groups (C) in A–C had no acid secretagogues. **P < 0.01 compared with each control value.**
neck cells but removing a large portion of the surface mucous cells, were cultured in the presence of $[^3H]$GlcN. Figure 3 shows the biosynthetic activity of mucin in the deep corpus mucosa with or without the addition of each agent at the maximal condition noted in the full-thickness tissue model. Each agent was added to the culture medium at final concentrations of $10^{-8}$, $10^{-7}$, $10^{-6}$, and $10^{-5}$ M. Values are means ± SD from 4 different samples, given as % of control. Both $[^3H]$- and $[^14C]$-labeled mucins were significantly increased (***P < 0.01, analysis of variance) by CCh at concentrations of $10^{-6}$ and $10^{-5}$ M, whereas no significant change in mucin biosynthesis of the antrum could be detected after stimulation by either tetragastrin or histamine.

Figure 3. Effects of acid secretagogues on mucin biosynthesis in deep corpus mucosa. Tissue fragments of deep corpus mucosa were obtained using a mucus scraping method (23) to separate mucous gel layer and surface mucosal layer from remaining deep mucosa containing mucous neck cells as mucous-producing cells. Tissues were incubated in medium 2 (described in MATERIALS AND METHODS) with $[^3H]$GlcN for determination of mucin synthesis. Each acid secretagogue was added to culture medium at maximal effective concentration noted using full-thickness tissue model (tetragastrin, $10^{-8}$ M; histamine, $10^{-5}$ M; and CCh, $10^{-5}$ M). Values are means ± SD from 6 different samples, given as % of control. Tetragastrin and histamine had no effect on $[^3H]$-labeled mucin in corpus deep mucosa, whereas addition of CCh significantly increased (***P < 0.01, analysis of variance) $[^3H]$GlcN incorporation into mucin in deep corpus layer.

Effects of an NOS Inhibitor or an NO Antidote on Tetragastrin- and Histamine-Stimulated Mucin Biosynthesis

The effects of the l-arginine analog l-NNA, an NOS inhibitor, on tetragastrin-, histamine-, and CCh-stimulated mucin biosynthesis in full-thickness and deep corpus mucosa. Tissue fragments of full-thickness corpus or deep corpus mucosa were incubated for times indicated with $[^3H]$GlcN in absence (C, black bars) or presence (A, white bars) of $[^3H]$GlcN incorporation into mucin in deep layer as well as the full-thickness corpus region. Removing the surface mucous cells caused the stimulatory effects of tetragastrin and histamine on $[^3H]$GlcN incorporation into corpus mucin to disappear (Fig. 3). The difference in the susceptibility to tetragastrin of the full thickness and the deep layer corpus mucosa on the incorporation of $[^3H]$GlcN into the mucin was essentially the same, even when the incubation time was extended up to 9 h (Fig. 4).

Effects of an NOS Inhibitor or an NO Antidote on Tetragastrin-, Histamine-, and CCh-Stimulated Mucin Biosynthesis

The effects of the l-arginine analog l-NNA, an NOS inhibitor, on tetragastrin-, histamine-, or CCh-stimu-
lated incorporation of $[3H]GlcN$ into the mucin in the full-thickness corpus mucosa are shown in Fig. 5. Addition of $10^{-4} M$ L-NNA had no significant effect on mucin biosynthesis in either the corpus or the antrum (Fig. 5, A and C). Neither histamine- nor CCh-induced activation of mucin synthesis was significantly suppressed by the addition of $10^{-4} M$ L-NNA (Fig. 5, B and C). On the other hand, the tetragastrin-induced increase in $^3$H-labeled mucin in the corpus mucosa was completely blocked by the addition of $10^{-4} M$ L-NNA. Concurrent pretreatment with L-arginine ($5 \times 10^{-3} M$) entirely reversed the stimulation of mucin biosynthesis to the level observed after tetragastrin alone (Fig. 5A), whereas p-arginine ($5 \times 10^{-3} M$) had no significant effect (radioactivity relative to the control level, 98 ± 12%).

For further clarification of the participation of NO, we studied the effects of carboxy-PTIO on gastrin-stimulated incorporation of $[3H]GlcN$ into mucin in full-thickness corpus mucosa (Fig. 6). Addition of carboxy-PTIO ($10^{-5} M$) had no significant effect on mucin biosynthesis. Concurrent addition of carboxy-PTIO at a concentration of $10^{-5} M$ completely suppressed the $10^{-7} M$ tetragastrin-stimulated mucin biosynthesis of corpus mucosa. Values are means ± SD from 5 different samples, given as % of control. **P < 0.01. Asterisks just above SD bar show significance vs. control value.

Fig. 5. Effect of N^{6}-nitro-L-arginine (L-NNA) on acid secretagogue-stimulated mucin biosynthesis. Incorporation of $[3H]GlcN$ into mucins in entire mucosa of corpus (A, B) and antrum (C) was measured in groups of tissues exposed to each acid secretagogue with or without $10^{-4} M$ L-NNA. L-NNA treatment started 20 min before incorporation study. Addition of L-NNA ($10^{-4} M$) did not show any significant change in mucin biosynthesis and completely inhibited tetragastrin ($10^{-8} M$)-induced increase in $^3$H-labeled mucin in all tissues of corpus. Concurrent pretreatment with L-arginine ($5 \times 10^{-3} M$) entirely reversed mucin biosynthetic stimulation to the level observed after tetragastrin alone (A). On the other hand, neither histamine ($10^{-6} M$)- nor CCh ($10^{-6} M$)-induced activation of mucin synthesis was significantly suppressed by pretreatment with L-NNA (B, C). Values are means ± SD from 5 different samples, given as % of control. **P < 0.01. Asterisks just above SD bar show significance vs. control value.

Fig. 6. Influence of 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide sodium salt (carboxy-PTIO) on tetragastrin-stimulated mucin biosynthesis in full-thickness corpus mucosa. Incorporation of $[3H]GlcN$ into mucins in all tissues of corpus was measured in groups of tissues treated with or without concurrent addition of $10^{-5} M$ of carboxy-PTIO and $10^{-7} M$ of tetragastrin. Addition of carboxy-PTIO ($10^{-5} M$) did not show any significant change in mucin biosynthesis and completely suppressed $10^{-7} M$ tetragastrin-stimulated mucin biosynthesis of corpus mucosa. Values are means ± SD from 5 different samples, given as % of control. **P < 0.01. Asterisks just above SD bar show significance vs. control value.

**Immunolocalization with Antibodies Against Distinct Types of Mucin and NOS**

As shown in Fig. 7a, immunoreactivity with RGM21 MAb was detected only in the surface epithelial layer of the corpus mucosa. In contrast, immunoreactivity with HIK1083 MAb was found selectively in the mucus neck cells of the corpus mucosa and was not entirely observed in the surface mucous cells (Fig. 7c). No immunoreactivity of RGM21 and HIK1083 was detected in sections of the corpus mucosa using the media preabsorbed with purified rat gastric mucin. NOS immunoreactivity was detected in the corpus mucosa (Fig. 7b). The distribution of the cells with NOS immunoreactivity was in agreement with that of RGM21-positive cells but not with cells positive to...
HIK1083. NOS immunoreactivity was also recognized in the nerve fibers distributed in the submucosa and the smooth muscle layers (Fig. 7b). No NOS immunoreactivity was found in the corpus sections incubated with preabsorbed antiserum.

**DISCUSSION**

In our previous study, the high molecular weight $[^3H]GlcN$-labeled substances isolated by chromatography on a Bio-Gel A-1.5 m column formed a single peak by equilibrium centrifugation in a CsCl density gradient at the typical density of mucin-type glycoproteins (15). Most of the radiolabeled oligosaccharides obtained from the high molecular weight glycoconjugates distributed within the molecular size corresponding to the oligosaccharides obtained from the purified rat gastric mucin. Moreover, treatment with either hyaluronidase or amylase did not change the profile of the radiolabeled oligosaccharides in a TSK gel HW-50S chromatogram, indicating the negligible contamination of polysaccharides such as hyaluronic acid and glycogen. Goso and Hotta (10) reported that $[^35S]$sulfate-labeled mucin prepared by a procedure similar to that used in this study did not contain sulfated proteoglycans such as chondroitin sulfate, heparin, or keratan sulfate. These observations demonstrate that most of the $[^3H]GlcN$-labeled substances obtained from the void volume peak of Bio-Gel A-1.5 m gel-filtration column chromatography are mucin-type glycoproteins (39), and the radioactivity measurement of this peak is thought to be valid for estimation of the biosynthetic activity of gastric mucin. Using this method, we examined the radioactivity distribution of $[^3H]GlcN$-labeled mucin in tissue and that excreted into the medium. Of the total $[^3H]$ radioactivity incorporated into mucin, 10–15% was found in the medium, and this amount was independent of drug addition (15). In our experimental model, in which most of the secreted mucins were retained in the adherent mucus gel attached on the epithelium, the proportion of radiolabeled mucin recovered from the medium to the total radioactivity corresponding to mucins was not changed with the addition of CCh, although cholinergic stimulation has previously been shown to increase mucin secretion (37).

The findings of this study show that gastrin, histamine, and CCh exert stimulatory effects on mucin biosynthesis in rat gastric corpus mucosa, but each of these agents has a distinct mode of action. Histamine and CCh are shown to stimulate biosynthesis of the mucin peptide as well as the glycosylation step in the corpus in a dose-dependent manner. On the other hand, tetragastrin at 10$^{-8}$ M increased the incorporation of $[^3H]GlcN$ into the corpus mucin but failed to change $[^14C]$Thr incorporation (15). Even if the incubation time was extended to 9 h, tetragastrin had no significant effect on the $[^14C]$Thr incorporation (15). Because threonine is one of the major amino acids of the backbone peptide of mucin (39), these observations indicate that gastrin stimulates the process of glycosylation but not backbone peptide elongation in corpus mucin biosynthesis. The present data for tetragastrin reconfirmed that 10$^{-6}$ and 10$^{-5}$ M of gastrin family peptides failed to stimulate corpus mucin synthesis (15). Similar results for histamine (12, 13) and gastrin (36) were obtained by Heim et al. (12, 13), using isolated pig gastric mucosal cells, and Scheiman et al. (36), using highly enriched canine gastric mucous cells, respectively. The present data and those of Scheiman et al. (36) indicate that a higher dose of gastrin might have an inhibitory action competing with the stimulatory signal-transduction pathways generated by a lower dose of the agonist.

In this study, the antral tissue separated from the corpus was also cultured. Tetragastrin and histamine had no significant effect on mucin biosynthesis in this region of the rat stomach. In contrast to these two agents, CCh stimulates the biosynthesis of the mucin...
peptide as well as the glycosylation step, both in the corpus and the antrum, suggesting the presence of a cholinergic stimulation mechanism for mucin biosynthesis throughout the glandular stomach. In previous studies using rabbit and canine antral explants, ACh was thought to be responsible for increasing antral mucin synthesis (37, 49). These and our own results also support evidence that mucin production is differently regulated in the corpus and antrum of the gastric mucosa. This might be related to the results indicating that different types of mucus are present in the corpus and antrum of rat gastric mucosa (10, 17, 18, 30).

It has been histologically characterized that surface mucous cells and gland mucous cells of the gastric mucosa produce a distinct type of mucin, having a different stainability (20, 32). We have already demonstrated that the surface mucosal layer of the corpus, chiefly composed of surface mucous cells, can easily be removed mechanically from the remaining deep mucosa of the rat without causing any notable damage to the underlying gland mucous cells (23). In this study, incorporation of $^{3}$H GlcN into the deep corpus mucin linearly increased with the elapse of time up to 9 h, and the ratio of $^{3}$H radioactivity in the deep corpus mucin to that in the full-thickness corpus mucosa (75%) was well maintained, indicating that it is feasible to estimate the biosynthetic activity of mucin in the deep corpus layer by peeling off the surface mucous cells, as described above. Using this method, we surveyed the effects of the three acid secretagogues on mucin biosynthesis in the deep corpus mucosa rich in mucous neck cells. Unlike the results obtained from full-thickness corpus mucosa, both tetragastrin and histamine failed to stimulate mucin synthesis in the deep mucosa. These findings strongly suggest that the stimulant effects of these agents are limited to the surface mucous cells in the rat corpus mucosa. By the subtraction of radiolabeled mucin synthesized in the deep mucosa, it can be calculated that the rate of mucin synthesis in the surface mucous cells is more than doubled by the addition of these two stimulants. The uptake of $^{3}$H and $^{14}$C was unexpectedly small in the mechanically scraped surface mucous cell layer, using the culture system applied in this study. Thus the surface mucous cell-rich layer peeled from the corpus mucosa was not suitable for assessing the biosynthesis of surface mucous cell-derived mucin. In contrast to gastrin and histamine, the biosynthetic response to CCh in the deep corpus mucosa was essentially the same as that in the full-thickness corpus tissue. Further studies are needed to clarify whether CCh-induced activation mechanisms extend to surface mucous cells of corpus mucosa and to clarify the effect of CCh on mucin synthesis in the distinct layers of the antral mucosa by developing a novel separation method to obtain the surface mucous cell-rich layer of the corpus and antrum. The present evidence, together with our previous results (22a, 24), strongly indicates that a distinct control mechanism underlies the biosynthesis and accumulation of a mucin present in a specific region and layer of the gastric mucosa.

Brown et al. (4, 5) reported that NO generators increased the thickness of the mucous layer of the rat stomach and induced mucus secretion by gastric mucosal cells without evidence of cellular damage. This observation suggests that NO could promote the mucus metabolism in gastric mucosa. To clarify the participation of endogenous NO in acid secretagogue-induced increases in gastric mucin synthesis, we examined the susceptibility of the effect of each agent to the NOS inhibitor L-NNA (27). The tetragastrin-induced increase in $^{3}$H-labeled corpus mucin was completely suppressed by addition of L-NNA, and this blockade was reversed with the coaddition of L-arginine. Our present results on the effects of carboxy-PTIO, a new class of NO antidotes that can react only with NO (1, 48), also indicated that NO itself plays an important role in mediating the activation of the corpus mucin synthesis elicited by gastrin. Recently, L-NNA has been shown to inhibit the gastroprotective effect of gastrin against ethanol-induced gross and histological damage (41). Similarly, inhibition of NO biosynthesis abolished the increase in gastric mucosal blood flow that accompanies pentagastrin-stimulated acid secretion (33). Neither histamine nor CCh-induced activation of mucin synthesis was significantly suppressed by the addition of L-NNA. Studies in vivo in the rabbit and rat demonstrated that the NO pathway played an important role in the endothelium-dependent vasodilatory response to ACh (35, 47). Tanaka et al. (44) have shown that another NOS inhibitor, $^{N}$O-nitro-L-arginine methyl ester (L-NAME), blocked the increase in gastric mucosal blood flow induced by central vagal stimulation without influencing gastric acid secretion. Taken together, results reported here suggest that the response mechanism of gastric mucus-producing cells to cholinergic stimulation differs from that of endothelial cells. From the results of this study, it can be concluded that the gastrin-induced activation of mucin biosynthesis critically involves NO synthesis, indicating that gastrin and the other two agents might have a distinct function in the regulatory mechanisms of mucin biosynthesis in the entire layer of rat corpus mucosa.

Morphological location of NOS in the gastric mucosa is significant for understanding the participation of NO in the regulatory mechanism of gastric mucin production. The antiserum against NOS used in this study clearly stained the surface mucous cells but not the mucous neck cells of rat corpus mucosa fixed with Zamboni’s solution. Price et al. (34) reported that antiserum raised against the COOH-terminal peptide of rat brain NOS recognizes rat neuronal NOS (nNOS; also known as type I) but not endothelial NOS (eNOS; type III); thus we might have demonstrated the presence of nNOS in the surface mucous cells. Staining of the serial section with RGM21 MAb (18), which reacts selectively with the surface mucous cell-derived mucin of rat corpus mucosa, confirmed that NOS immunoreactivity actually existed in the surface mucous cells and not in the mucous neck cells recognized by HIK1083 MAb (19) staining of rat gastric corpus mucosa. These histochemical findings confirmed and extended the
recent work of Price et al. (34), who reported that the immunoreactivities of the nNOS antiserum and the MAb directed against nNOS localized at the surface layer of gastric mucosa, except for the myenteric plexus.

In contrast to the the corpus mucosa, the anti-NOS antiserum used in this study rarely reacted with the antral mucous cells (<10% of the total surface mucous cells of antrum). This finding further supports our result that mucin biosynthesis stimulated by gastrin does not occur in the antral region. Our previous study indicated that gastrin stimulated the process of mucin production through the gastrin/cholecystokinin-B receptor (GR) in rat stomach (15). In a recent immunohistochemical study using the polyclonal antiserum to the GR, Tarasova et al. (45) showed that the GRs were expressed exclusively in the oxyntic mucosa of guinea pig stomach, especially in parietal cells, chief cells, and endocrine cells, and are rarely found in the antral region. It is thus inconceivable that gastrin may directly react with surface mucous cells. Further studies are needed to clarify how the GR-mediated stimulatory action on nonmucous cells induces NO production in the surface mucous cells of corpus mucosa.

In summary, the present findings demonstrate that acid secretagogues have distinct effects on mucin biosynthesis in a specific region and layer of rat gastric mucosa, suggesting different regulatory mechanisms underlying the mucus metabolism of distinct mucus-producing cells. The mucin biosynthesis stimulated by gastrin and mediated by NO occurs in surface mucous cells, and not in gland mucous cells, of gastric oxyntic mucosa.

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