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Age-related stimulation by tetragastrin of gastric mucin biosynthesis in rat

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Abstract

The effects of tetragastrin on gastric mucin biosynthesis in middle-aged rats were compared with those in young rats. The incorporation of [^{3}H]glucosamine and [^{35}S]sulfate into mucin was stimulated by tetragastrin in cultured corpus mucosa from 7-week-old rats. In contrast, tetragastrin could not enhance mucin biosynthesis in stomachs from 52-week-old rats. The isosorbide dinitrate-induced stimulation of corpus mucin biosynthesis observed in middle-aged rats was essentially the same as that seen in young rats. Nitric oxide (NO) synthase activity of the corpus was significantly reduced in the middle-aged rats compared to the young rats. NO synthase-immunoreactivity was observed at surface mucous cells in the corpus mucosa of young, but not of middle-aged, rats. These results suggest that aging decreases the effect of gastrin on gastric mucin biosynthesis through the age-related loss of NO synthase function in the surface mucous cell layer of rat stomach.

Keywords: Gastrin; Nitric oxide (NO) synthase; Gastric mucin biosynthesis; Aging

1. Introduction

Extensive research has been devoted to the age-dependent changes in tissue responsiveness in the cardiovascular system and brain (Docherty, 1990; Mollace et al., 1991). Although gastrointestinal symptoms are common in the elderly (Talley et al., 1992), the gastrointestinal tract has received less attention in these experimental studies on aging. Several studies have shown age-related derangements in various functions of the gastrointestinal tract, such as a decrease of gastric mucosal blood flow, an impairment of acid secretion, and an increase of mucosal susceptibility to acid injury (Maitra et al., 1988; Gronbech and Lacy, 1995; Tsukimi and Okabe, 1995; Miyake et al., 1996). Knowledge of which step in the regulatory mechanism is most associated with a progressive decline in these gastric functions is, indeed, very important in the clinical pharmacology of aging.

Mucin, a major component of gastric mucus, is an important mucosal defensive factor. As we have previously reported, the stimulation of gastric mucin biosynthesis is closely related to mucosal protective activity (Ichikawa et al., 1994). Considerable information has been accumulated about the role of gastrin in the regulation of gastric mucin biosynthesis in young rats (Gerard et al., 1968; Scheiman et al., 1992; Ichikawa et al., 1993). Our recent study showed that nitric oxide (NO) mediated gastrin stimulated-mucin biosynthesis in 7-week-old rat stomach (Ichikawa et al., 1998).

The first aim of this study was to compare the effects of gastrin on mucin biosynthesis in the stomachs of young and middle-aged rats. In the second step, we sought to determine whether or not the effects of exogenous NO on mucin production in the stomachs of middle-aged rats were different from those in the stomachs of young rats. In the third part of this study, we compared the activity and immunoreactivity of NO synthase in the stomachs of young- and middle-aged rats.

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2. Materials and methods

2.1. Experimental animals

Male Wistar rats (young: 7-week-old; middle-aged: 52-week-old) were obtained from SLC (Shizuoka, Japan). All rats were fasted for 24 h before the experiments and had free access to water during this time.

2.2. Drugs and chemicals

The following substances were used for this study: tetragastrin (Mect, Tokyo, Japan); isosorbide dinitrate (Sigma, St. Louis, MO, USA); D-[1,6-^{3}H(N)]-glucosamine hydrochloride (1943 GBq/mmol, New England Nuclear, Boston, MA, USA); [^{35}S]sulfate (carrier free, New England Nuclear); L-[2,3,4,5-^{3}H]arginine monohydrochloride (2.04 TBq/mmol, Amersham Pharmacia Biotech, Bucks, UK); N^G-nitro-L-arginine methyl ester (L-NAME; Sigma). Tetragastrin and isosorbide dinitrate were dissolved in phosphate-buffered saline (PBS). Stock solutions of the drugs were freshly prepared, and the concentrations reported are final bath concentrations. The polyclonal antiserum raised against the synthetic peptide derived from the COOH-terminal of the cloned rat cerebellar NO synthase was purchased from Euro-Diagnostica (Malmö, Sweden).

2.3. Tissue culture and measurement of synthesized mucin

Immediately after the rats were euthanized with CO_2, their stomachs were excised and cut along the greater curvature, and the luminal surface was gently washed with PBS. The glandular part was selected, separated into the corpus and antrum and cut into small 2×2 mm sections. Eight tissue fragments were randomly picked out from $6 \sim 8$ different stomachs and then placed, with the mucosal surface facing upward, on a stainless steel grid in the central well of a plastic culture dish (60×15 mm, Falcon, Lincoln Park, USA) containing 0.75 ml of the culture medium and 0.05 ml of test substances. The medium consisted of 90% Eagle's minimum essential medium and 10% dialyzed fetal calf serum, with 370 kBq/ml of [^{3}H]glucosamine hydrochloride, with or without 1.85 MBq/ml [^{35}S]sulfate. Each dish was maintained at 37°C for 5 h in 5% CO_2 and 95% air. Each experimental group consisted of at least 8 dishes. Upon completion of the culture period, the cultured tissues were homogenized in Tris–HCl buffer containing Triton X-100. The extraction and isolation of the mucin were performed as previously described (Ichikawa et al., 1993). The homogenate was centrifuged and the obtained supernatant was applied onto a Bio-Gel A-1.5 m column (1×30 cm). The fractions eluted with the void volume were collected and radioactivity was measured as synthesized mucin (Ichikawa et al., 1993). To compare the synthesis of mucin, the total radioactivity of these fractions was divided by the tissue protein content of each homogenate to give the value relative to that of the control.

2.4. Assay of NO synthase activity

NO synthase activity was measured by the modified method of Bredt and Snyder (1989), using a NO synthase assay kit (Calbiochem, San Diego, CA, USA). Briefly, corpus tissues from 7- and 52-week-old rats were quickly isolated, weighed and homogenized at 4°C in 20 volumes (relative to the sample weight) of buffer containing 1 mM EDTA, 1 mM EGTA, and 25 mM Tris–HCl, pH 7.4. The homogenates were centrifuged at $35{,}000 \times g$ for 5 min. Supernatants were used for the NO synthase assay. Each sample (10 μl) was added to 0.1 μM calmodulin, 0.75 mM $CaCl_2$, 1.25 mM NADPH, 3.75 μM tetrahydrobiopterin, 1.25 μM flavin adenine dinucleotide, 1.25 μM flavin mononucleotide, 0.45 μM L-[^{3}H]arginine and 25 mM Tris–HCl, pH 7.4 (total volume: 50 μl) and incubated for 30 min at 37°C. After incubation, the reactions were stopped with a buffer containing EDTA. NO synthase activity in these supernatants was measured by monitoring the biochemical conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline, using the sodium form of a cation exchange resin column which absorbs L-[^{3}H]arginine. L-[^{3}H]Citrulline was eluted and its radioactivity was measured to determine NO synthase activity. Blank values were determined in the presence of the NO synthase inhibitor, L-NAME.

2.5. Immunohistochemical staining

After being anesthetized with sodium pentobarbital (0.05 mg/g), 7- and 52-week-old rats were perfused through the heart with heparinized (1 IU/ml) 0.1 M PBS, pH 7.4, followed by Zamboni's (Zamboni and de Martino, 1967) fixative solution (4% paraformaldehyde and 0.2% picric acid in 0.1 M PBS, pH 7.4). The stomachs were excised from the body, and the corpus mucosae were selected and immersed in the same fixative for an additional 6 h at 4°C. After a brief wash, the specimens were transferred to 30% sucrose and kept overnight at 4°C. The specimens were then serially cross-sectioned at 10 μm on a cryostat and mounted on poly-L-lysine-coated slides. The sections were immunohistochemically stained with NO synthase antiserum, using the peroxidase-antiperoxidase (PAP) method of Sternberger (1979). The process of immunostaining was detailed in a previous report (Kusakabe et al., 1991). The rabbit polyclonal antiserum against rat NO synthase was diluted to 1:1000. To demonstrate the existence of NO synthase, the section was then incubated with goat anti-rabbit IgG (diluted 1:200, Cappel, Durham, NC, USA). After being washed, the section was overlaid with the rabbit PAP complex (diluted 1:200, Jackson, West Grove, PA, USA). Peroxidase reactivity was demonstrated with the 3,3′-diaminobenzidine method of Graham and

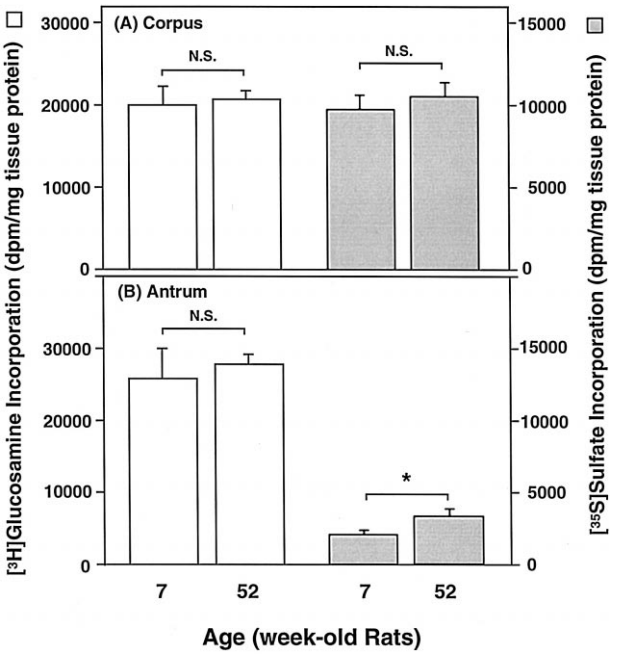

Fig. 1. Effect of age on the incorporation of [^{3}H]glucosamine hydrochloride and [^{35}S]sulfate into mucin in the corpus (A) and the antrum (B) of rat stomach. Values are expressed as dpm/mg tissue protein and represent means ± S.D. for 8 different samples derived from 6–8 different rats. Asterisk indicates statistical significance ($* P < 0.05$). N.S.: not significant.

Karnovsky (1966). The reaction for the immunogen was verified by treating sections with primary antibody which had been inactivated through overnight incubation with 50 μM of its immunogen.

2.6. Protein determination

The protein content of the tissue homogenate was determined by the bicinchoninic acid method (Smith et al., 1985) with a Pierce protein assay kit, using bovine serum albumin as the standard.

2.7. Statistical analysis

Values presented in Figs. 1 and 3 are given as means ± S.D. The differences in mean values between the 7- and 52-week-old rat groups were analyzed by Student's *t*-test. *P* values less than 0.05 were considered to indicate statistical significance. Mucin biosynthesis in the Table and Fig. 2 is expressed relative to the average value of corresponding the control and represent means ± S.D. One-way analysis of variance (ANOVA) with Dunnett's test was used for statistical analysis with $P < 0.05$ being taken as significant.

3. Results

3.1. Influence of tetragastrin on gastric mucin biosynthesis in young and middle-aged rats

Fig. 1 shows the biosynthesis of mucin in the corpus and antrum of stomachs from young and middle-aged rats, as measured by the simultaneous incorporation of [^{3}H]glucosamine hydrochloride and [^{35}S]sulfate. In the corpus, the biosynthesis of mucin was virtually unchanged in young and middle-aged rats. [^{35}S]Sulfate incorporation into antral mucin was significantly increased in the middle-aged rats compared to the young rats, although antral [^{3}H]glucosamine hydrochloride incorporation was similar in the middle-aged and young rats.

The influence of tetragastrin on gastric corpus mucin biosynthesis in the young and middle-aged rats is shown in Table 1. In the control group of young rats without addition of the peptide, ^{3}H- and ^{35}S-radioactivities incorporated into the corpus mucin were 16,989 ± 2545 and 10,263 ± 1945 dpm/mg tissue protein, respectively. The mean biosynthesis of corpus mucin was not significantly different in the two age groups. In the corpus mucosa of young rats, the addition of 10^{-8} and 10^{-7} M tetragastrin

Table 1
Effects of tetragastrin on [^{3}H]glucosamine and [^{35}S]sulfate incorporation into mucin in corpus tissue from young and middle-aged rats

	7-week-old rats		52-week-old rats	
	[^{3}H]GlcN uptake (% of control)	[^{35}S]sulfate uptake (% of control)	[^{3}H]GlcN uptake (% of control)	[^{35}S]sulfate uptake (% of control)
0 M	100 ± 15	100 ± 19	100 ± 16	100 ± 23
10^{-9} M	98 ± 14	102 ± 16	98 ± 20	101 ± 20
10^{-8} M	135 ± 21[a]	149 ± 23[a]	96 ± 25	107 ± 23
10^{-7} M	124 ± 13[a]	145 ± 24[a]	96 ± 22	114 ± 19
10^{-6} M	109 ± 15	103 ± 16	108 ± 4	107 ± 26

Corpus tissue fragments obtained from the stomachs of 7- and 52-week-old rats were incubated in medium with [^{3}H]glucosamine(GlcN) and [^{35}S]sulfate for 5 h.
Tetragastrin was added to the culture medium at final concentrations of 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} M.
Values are expressed as percentages of control and represent means ± S.D. from 8 different samples derived from 6–8 different rats.
[a] $P < 0.01$ as compared with the control value. Radiolabeled precursor uptake into mucin in the control group (0 M) as dpm/mg tissue protein was as follows: young[^{3}H]; 16,989 ± 2545, young[^{35}S]; 10,263 ± 1945, middle-aged[^{3}H]; 17,028 ± 2642, middle-aged[^{35}S]; 11,326 ± 2621.

enhanced both [^{3}H]glucosamine hydrochloride and [^{35}S]sulfate incorporation into mucin, but mucin biosynthesis was not affected by the addition of 10^{-9} and 10^{-6} M. In contrast to the results for the young rats, in the middle-aged rats there was no significant change in mucin biosynthesis in the corpus mucosa after the addition of tetragastrin (Table 1). In the antrum of both 7- and 52-week-old rats, no significant change could be detected in mucin biosynthesis after the addition of tetragastrin (data not shown).

3.2. Influence of isosorbide dinitrate on gastric mucin biosynthesis in young and middle-aged rats

Fig. 2 shows the change in the biosynthesis of mucin after the addition of isosorbide dinitrate to stomachs from young and middle-aged rats as measured by [^{3}H]glucosamine hydrochloride incorporation. In the stomachs of young rats, the addition of isosorbide dinitrate increased [^{3}H]glucosamine hydrochloride incorporation into mucin in a concentration-dependent manner. The biosynthetic response noted in the stomachs of 52-week-old rats was essentially the same as that in the stomachs of the young rats (Fig. 2).

3.3. NO synthase activity in corpus tissue from young and middle-aged rats

Fig. 3 shows the change in total NO synthase activity of the corpus mucosa with age, as determined by measuring the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline. The total NO synthase activity of the corpus mucosa was significantly lower in the middle-aged rats than in the young rats.

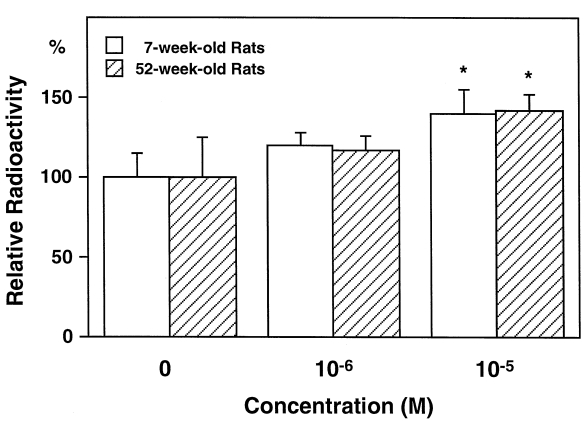

Fig. 2. Influence of isosorbide dinitrate on [^{3}H]glucosamine hydrochloride incorporation into corpus mucins in the stomachs of 7-week-old and 52-week-old rats. Values are expressed as percentages of the control and represent means ± S.D. for 8 different samples derived from 6–8 different rats. $* P < 0.05$ as compared with the control value. Mucin biosynthesis in the control (0 M) expressed as dpm/mg tissue protein was as follows: 7-week-old rat; 21,086 ± 2011, 52-week-old rat; 20,880 ± 4176.

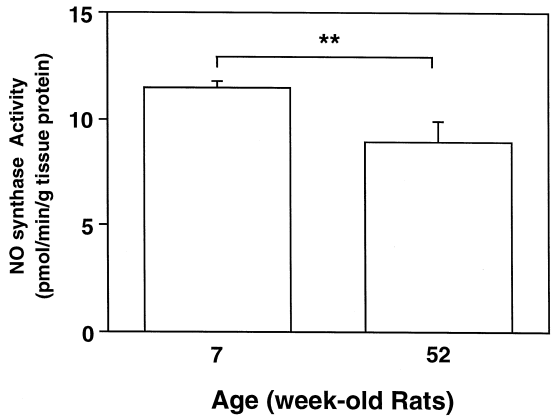

Fig. 3. Total NO synthase activity of the corpus mucosa from 7-week-old and 52-week-old rats. Values are expressed as pmol min^{-1} g^{-1} tissue protein and represent means ± S.D. for different samples derived from 4 different rats. Asterisk indicates statistical significance ($** P < 0.01$).

3.4. Immunohistochemical staining with NO synthase antiserum in corpus mucosa of young and middle-aged rats

Fig. 4 shows the microphotographs of the corpus sections from young and middle-aged rats stained with NO synthase antiserum. NO synthase immunoreactivity was detected in the corpus of both 7- and 52-week-old rats. In the young rats, the immunoreactivity was detected in both the surface mucous cell layer and the nerve fibers distributed in the muscle layer. In contrast, in the middle-aged

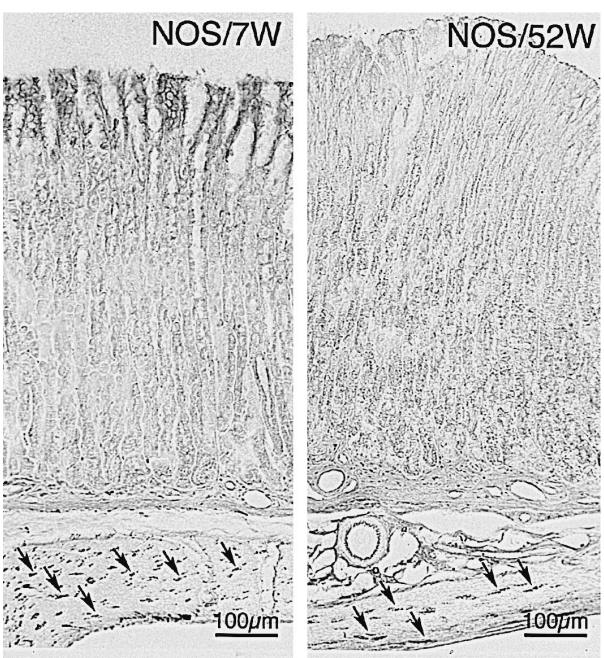

Fig. 4. Photomicrographs of rat corpus mucosa immunostained for NO synthase. NO synthase immunoreactivity was found in the surface epithelial layer of the stomach in 7-week-old rats (NOS/7W) but not in 52-week-old rats (NOS/52W). Note also NO synthase-immunoreactive nerve fibers in the muscle layers (arrows). Original magnification, ×60. Bar = 100 μm.

rats, the immunoreactivity was detected mainly in the nerve fibers and less or none was detected in the surface epithelial layer. No NO synthase immunoreactivity was found in the corpus sections incubated with preabsorbed antiserum (result not shown).

4. Discussion

The results of the present study show that the effects of tetragastrin on corpus mucin biosynthesis in young adult rats are distinct from those on mucin biosynthesis in middle-aged rats. In young rats, tetragastrin exerted stimulatory effects on mucin biosynthesis in the oxyntic region. The optimal concentrations of tetragastrin were 10^{-8} and 10^{-7} M, 10^{-9} and 10^{-6} M tetragastrin failed to stimulate corpus mucin synthesis in young rats. In contrast, tetragastrin had no significant effect on mucin biosynthesis in the corpus mucosa from middle-aged rats, although the mean biosynthetic activity was not significantly different in young and middle-aged rats in the control situation.

Recently, tetragastrin-induced activation of mucin biosynthesis in the stomachs of young rats was shown to be completely blocked by both the NO synthase inhibitor, N^{G}-nitro-L-arginine, and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide sodium salt (carboxy-PTIO), an NO antidote that can react only with NO, indicating that NO plays an important role in mediating the activation of gastric mucin synthesis elicited by gastrin (Ichikawa et al., 1998). In the present study, the stimulant response of gastric mucosa to isosorbide dinitrate, an exogenous NO-donor, was essentially the same in young and middle-aged rats. Smits and Lefebve (1995) showed that the relaxant response to sodium nitroprusside, another exogenous NO-donor, in longitudinal muscle strips of gastric fundus was not significantly different between 3- and 12-month-old rats, but was significantly decreased in 24-month-old rats. Taken together, the results reported here suggest that the cyclic GMP pathway for NO is not affected by aging up to about 1 year.

Mollace et al. (1995) reported that the total NO synthase activity of the hippocampus and lower brain stem was lower in aged rats than in young rats. This observation suggests that the decrease in NO synthase activity could contribute to the age-related change in cerebral functions. To clarify whether or not aging affects the activity of NO synthase in the gastric mucosa, we measured the NO synthase activity in the corpus mucosa of 7- and 52-week-old rats. Total NO synthase activity in the corpus mucosa was moderately, but significantly reduced with age. The immunohistochemical findings of this study showed that NO synthase-immunoreactivity in the corpus mucosa of young rats was detected in both the surface mucous cell layer and the nerve fibers distributed in the muscle layer. In contrast, the immunoreactivity in the corpus mucosa of middle-aged rats was detected mainly in the nerve fibers, and less or none was detected in the surface epithelial layer. A similar result for the young rat stomach was obtained by Price et al. (1996), using a specific antiserum or a monoclonal antibody reacting with the neuronal NO synthase. Although NO functions as a diffusible messenger molecule, calculations using models of the diffusional spread of NO have indicated that the physiological sphere of influence of a point source of NO has a diameter of up to about 200 μm (Wood and Garthwaite, 1994). NO released from the muscle layer would not therefore influence the function of surface mucous cells. Recently, we found that the stimulant effects of tetragastrin on corpus mucin biosynthesis in the stomachs of young rats disappeared with the removal of the surface mucosal cell layer in which NO synthase immunoreactivity is found (Ichikawa et al., 1998). These data indicate that NO synthase localized in the surface mucous cells, not in the nerve fibers, plays an important role in mediating the activation of gastric mucin synthesis elicited by gastrin.

We have already shown that tetragastrin stimulates the process of mucin production through the gastrin/cholecystokinin-B receptor in young rat stomach (Ichikawa et al., 1993), in a similar manner as other actions such as gastric acid secretion and trophic action. Recently, gastrin/cholecystokinin-B receptor gene expression in the rat stomach has been shown to increase with age (Waki et al., 1998). It is possible that the membrane changes, such as the decrease in receptor-binding activity, partly participate in the age-related loss of the gastrin-induced stimulation of mucin synthesis. Majumdar and Wahby (1993) reported that the age-related loss of responsiveness of the gastric mucosa to the growth-promoting action of gastrin was due in part to the inability of the hormone to activate tyrosine kinase and tyrosine phosphorylation of membrane proteins. Measurement of some variables in surface mucous cells, such as the biosynthesis of mucin, NO synthase activity, and receptor binding activity, will help us to gain a profounder understanding of the results obtained in this paper.

In contrast to its effect in the corpus mucosa, tetragastrin had no significant effect in the antral region of the stomach of young and middle-aged rats. In the previous studies, mucins obtained from the corpus and antrum of rat gastric mucosa were shown to differ in their subunit structures and in the chemical composition of their carbohydrate moieties (Ohara et al., 1988; Goso and Hotta, 1989). These results, together with our present finding of the difference in the synthesis of the sulfated mucin in the corpus and antrum, strongly indicate that there are different types of mucus-producing cells in which there is a distinct regulatory mechanism for mucin biosynthesis in the corpus and antrum of the gastric mucosa.

In this study, [^{3}H]glucosamine hydrochloride incorporation into antral mucin of young rats was similar to that of middle-aged rats, but the biosynthesis of sulfated mucin in the antral mucosa significantly increased with age. This

finding is consistent with the result of our previous study (Goso et al., 1985) using human gastric mucosa. In the human study, gastric resection specimens that had an increased [^{35}S]sulfate incorporation into mucin tended to be associated with intestinal metaplasia. Further studies are needed to clarify whether the increase in the biosynthesis of sulfated mucin in the antrum of middle-aged rats is due to metaplasia of the gastric mucosa.

In conclusion, the present observations show that the effects of tetragastrin on mucin production in the stomachs of middle-aged rats were different from those for young rats. The decreased NO synthase activity and immunoreactivity in the corpus mucosa of middle-aged rats may be a principal cause for the lack of regulation of mucin biosynthesis by gastrin.

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