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Role of Nitric Oxide in Pepsinogen Secretion from Rat Gastric Chief Cells

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Abstract – Nitric oxide (NO), a cellular messenger synthesized from L-arginine by NO synthase (NOS, EC.1.14.13.39), is considered to be a regulator of gastric secretion. In the present study, the role of NO in the regulation of exocrine secretion was investigated in rat gastric chief cells. Treatment of chief cells with carbachol resulted in an increase in the arginine conversion to citrulline, the amount of NO_X, the release of pepsinogen, and the level of cGMP. Especially, carbachol-stimulated increase of arginine to citrulline transformation, the amount of NO_X, cGMP level and the release of pepsinogen were partially reduced by the natural NOS inhibitor, N^G-monomethyl-L-arginine (MMA) and N^G,N^G-dimethyl-L-arginine (DMA). Furthermore, MMA- and DMA-induced decrease of pepsinogen secretion showed dose-dependent pattern. Activation of NOS is one of the early events in receptor-mediated cascade of reactions in gastric chief cells and NO, not completely, but partially mediates gastric secretion. Agonist-stimulated pepsinogen secretion in chief cells has been considered to be mediated in adenosine 3',5'-cyclic monophosphate pathway and/or guanosine 3',5'-cyclic monophosphate (cGMP) pathway. Taken together, the above results suggest that partial decrease of exocrine secretion following treatment of NOS inhibitor may result from the inactivation of NOS and subsequent guanylate cyclase, and NO/cGMP pathway may play a pivotal role in exocrine secretion.

Keywords □ Nitric oxide, Carbachol, cGMP, Gastric secretion.

The exocrine secretion involves a variety of neurohormonal factors and is mediated by multiple regulatory pathways in the acinar cells (Solomon, 1987). Agonists such as carbachol, cholecystokinin (CCK), and bombesin cause changes in cellular Ca^{2+} influx and the secretory response in pancreatic acinar cells (Muallem, 1989). Pandol and Shoeffield-Payne (1990) suggested that cGMP is sufficient to activate Ca^{2+} entry and may mediate the effect of agonists on Ca^{2+} entry in pancreatic acinar cells. Gukovskaya and Pandol (1994) have developed preliminary evidence that NO may play a role in mediating the increase in cGMP caused by the agonist under some conditions. In addition, our recent reports provided evidence that NO might be a putative mediator in pancreatic exocrine secretion (Seo *et al.*, 1995; Ahn *et al.*, 1998). In contrast, Yoshida et al. (1997) demonstrated that NO/cGMP pathways is uncoupled on carbachol and CCK-stimulated Ca^{2+} entry and amylase secretion, and that the NOS system is either not present or not functioning in rat pancreatic acinar cells. Also, a number of studies on exocrine secretion in gastric chief cells producing digestive enzymes have been reported and shown to be mediated by multiple regulatory pathways (Raufman, 1992) as those in pancreatic acinar cells. Relation between NO and exocrine secretion in gastric chief cells is considered to be similar to that in pancreatic acinar cells (Brown *et al.*, 1992; Okayama *et al.*, 1995). In guinea pig gastric chief cells, NO formation may be associated with increased levels of cGMP and induce pepsinogen exocrine secretion (Fiorucci *et al.*, 1995). Interaction of secretin, vasoactive intestinal peptide, cholera toxin, and prostaglandins with receptors linked to the adenylyl cyclase system results in pepsinogen secretion from dispersed chief cells from the guinea pig stomach (Raufman *et al.*, 1992). As described above, the mechanism of exocrine secretion in the stomach have showed multiple pathway pattern. We therefore tried to establish if NO functions as a signaling molecule mediating gastric secretion by determining the change of pepsinogen secretion, NOS activity and cGMP level upon stimulating rat gastric chief cells with carbachol.

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MATERIALS AND METHODS

Materials

[2,3,4,5-^{3}H]-L-arginine (57 Ci/mmol) and cGMP assay kit

were purchased from Amersham Life Science (U.K.), L-arginine, hemoglobin, Percoll and PAS staining kit from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The rest of the chemicals were obtained from various commercial sources and were of the highest purity available.

Animals

Male Sprague-Dawley (SD) rats (200-250 g) were obtained from Ansung Cheil Inc. (Korea) and housed under conditions of controlled temperature (22-24 °C) and illumination (12 h light cycle starting at 8 A.M.) for 1 week in GLP room.

Isolation of rat gastric chief cell

Two male SD rats (200-250 g) were sacrificed by decapitation at the beginning of the experiments. Rat stomach was quickly ligated one at the level of the cardioesophagal junction and the other at the level of the fundipyloric junction. And then, the stomach was removed and the fundus was everted by the use of a glass rod. The mucosa cells were isolated using 4 ml of pronase-EDTA buffer (0.5 mM NaH_2PO_4, 1 mM Na_2HPO_4, 20 mM $NaHCO_3$, 80 mM NaCl, 5 mM KCl, 25 mM HEPES, 11 mM glucose, 2 mM EDTA, 2% BSA and 0.3% pronase, pH 7.4) and gassed with 5% CO_2 and 95% O_2 for 1h at 37 °C in oscillating incubator. The whole mucosa cells were resuspended in 4 ml incubation solution (0.5 mM NaH_2PO_4, 1 mM Na_2HPO_4, 20 mM $NaHCO_3$, 80 mM NaCl, 5 mM KCl, 25 mM HEPES, 11 mM glucose, 1 % BSA, 1 mM $CaCl_2$ and 1.5 mM $MgCl_2$, pH 7.4) and gassed with 5% CO_2 and 95% O_2 for 1 h 30 min at 37°C in oscillating incubator. Isolated cells were centrifuged at 900 rpm for 3 min. Percoll solution (d = 1.065 g/ml) was prepared by adding 46 ml Percoll (d = 1.129 g/ml) to 44 ml of distilled water and 10 ml of 1.5 M NaCl and adjusting the pH to 7.4 with 1 N HCl. The unfractionated cells were resuspended in 4 ml incubation solution and dispersed in 20 ml of Percoll solution. The resulting suspension was centrifuged at 30,000 g for 21 min at 4°C in a ultra-speed centrifuge (Combi Plus, Sorvall, U.S.A.). Fractions (2 ml) were collected from the top of the centrifuge tube. Pepsinogen contents of each fraction was measured to isolate and collect chief cell portions. To measure NOS activity, cGMP level, and pepsinogen release, isolated chief cells containing a large amounts of pepsinogen contents were incubated and divided into media and pellet.

Histological study

For the identification of chief cell Periodic Acid Schiff (PAS) stains were performed using the Sigma PAS staining kit. Chief cells were identified by their smaller size and dark blue-staining cytoplasmic granules. Mucous epithelial cells were identified by their bright red staining in PAS-stained smears (Raufman et al., 1984).

Pepsinogen determination

The pepsinogen content of the sonicated fraction from the Percoll gradient was measured by converting pepsinogen to pepsin and determining the hydrolysis of hemoglobin. Samples (0.1 ml) were incubated for 10 min at 37 °C with 0.1 ml of 50 mM glycine containing 0.01% (w/v) bovine hemoglobin in 0.4 N HCl (pH 1.8). The reaction was terminated by adding 0.5 ml of 6.2% (v/v) trichloroacetic acid and followed by centrifugation at 10,000 g for 1 min. Supernatant (0.5 ml) was taken for monitoring at 280 nm (Raufman et al., 1984).

Effect of carbachol and NOS inhibitor on pepsinogen secretion

Isolated rat gastric chief cells were incubated with the various concentrations of carbachol (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} M) for 15 min to determine optimum concentration releasing maximal amount of pepsinogen, and then, reacted for the indicated times (zero, 3, 7, 10, 20, and 30 min) to inspect agonist-mediated time course effect in the absence or presence of carbachol (10^{-5} M). To determine the relation between carbachol and Ca^{2+}, rat gastric chief cells were incubated with carbachol (10^{-5} M) in extracellular Ca^{2+} free medium containing 2 mM EGTA. The changes of pepsinogen secretion by carbachol-stimulated NO production were measured to confirm the involvement between carbachol-mediated NO and exocrine secretion by pretreatment of N^G-monomethyl-L-arginine (L-MMA) or N^G,N^G-dimethyl-L-arginine (L-DMA), a natural NOS inhibitor in isolated chief cells.

NOS assay and analysis

Activity of NOS was determined using an assay based on the conversion of ^{3}H-L-arginine to ^{3}H-L-citrulline as described (Gukovskaya and Pandol, 1994). Rat gastric chief cells were preincubated with ^{3}H-L-arginine (L-[2,3,4,5-^{3}H]-L-arginine HCl, 3 μ Ci : Amersham Life Science, U.K.) for 15 min and then incubated in the absence or presence of carbachol (10^{-5} M) from zero to 15 min at 37 °C. The reaction was terminated with ice-cold buffer containing 5 mM arginine and 4 mM EDTA. This mixture was centrifuged at 10,000 g for 30 sec. One ml of 1 M trichloroacetic acid was added to the pellet, and the suspension was sonicated and centrifuged at 12,000 g for 20 min. Supernatants were extr-

acted by ethyl ether for elimination of trichloroacetic acid three times and neutralized by HEPES buffer (pH 6.0). Neutralized mixture was eluted with Dowex-50W column (50X8-400, 200-400 mesh), Na^+ form (prepared from the H^+ form). The eluent was collected into a suitable water-miscible scintillant and the radioactivity was counted. In order to confirm the validity of assay method for NOS activity, the formation of NOx was directly determined by NO analyzer (Model 7020, ANTEK instruments, U.S.A.). This assay method is based on the reduction of NO_2^-, and NO_3^- to NO by vanadate at high temperature, the formed NO reacts with internally produced ozone to produce metastable nitrogen dioxide, and the metastable nitrogen dioxide chemiluminesces as it returns to the ground state. Thus, the light emitted at a specific wavelength is directly proportional to the concentration of NO in the original sample. The amounts of NO produced was calculated using $NaNO_2$ as a standard.

Determination of cGMP

Isolated rat chief cells were incubated alone or with carbachol (10^{-5} M) and pretreated with L-MMA in the presence of carbachol (10^{-5} M) to inspect the effect of L-MMA on cGMP content. The enzymatic reactions were stopped by adding 0.5 ml of ice-cold ethanol and carried out sonication. After sonication, the lysates were centrifuged at 12,000 g for 20 min. The content of cGMP was measured in 0.1 ml of supernatants using commercially prepared kit from Amersham Life Science. Values for each experimental sample were expressed as a ratio to the control value (experimental to control).

Statistical analysis

The results were expressed as mean plus or minus the standard error. Statistical analysis was performed by using a two-tailed Student's *t*-test. A difference with a p value of < 0.05 was considered statistically significant.

RESULTS

Isolation and purification of gastric chief cells

Isolated gastric whole cells applied to Percoll gradient were separated each 12 (each 2 ml) fractions. Cell counts revealed that 50-60 % of the cells applied to the Percoll gradient migrated to fractions 3 and 4. Red blood cells were visible in fraction 12. Separation of mucosal cells in the Percoll gradient yielded a peak of pepsinogen activity distributed in fractions 10 and 11 (Fig. 1). To characterize further the cell populations in the fractions where most of the cells banded in

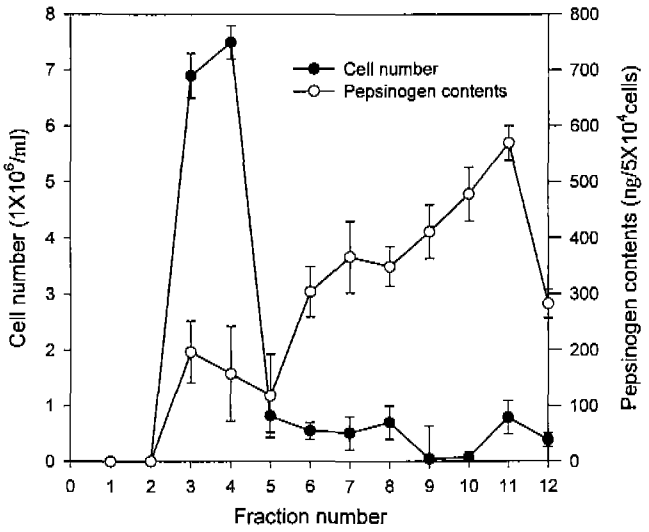

Fig. 1. Cell counts and pepsinogen contents of 12 (each 2ml) fractions from Percoll gradient. Cell counts were obtained on indicated fractions which were then sonicated to measure pepsinogen contents. The detailed experimental conditions are described as under "Materials and Methods". Data are the mean ± SE of three separate experiments.

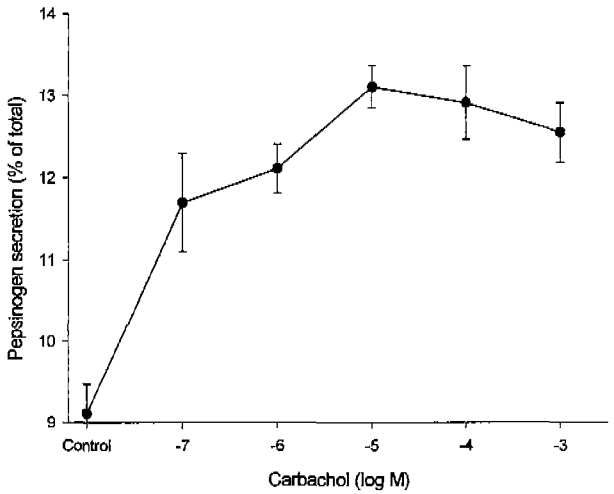

Fig. 2. Dose-response curve of carbachol on pepsinogen secretion in rat gastric chief cells. Isolated chief cells were suspended in incubation solution and 0.1 ml aliquots were incubated with the indicated concentration of carbachol for 15 min at 37 °C. The amount of pepsinogen released into medium was measured as described in "Materials and Methods". Data are the mean ± SE of three separate experiments.

the Percoll gradient, we performed PAS stains on both the unfractionated mucosal cells and the cells in fractions 10 plus 11 using commercially available PAS staining kit (Sigma Chemical Co., St. Louis, MO, U.S.A.). This staining results

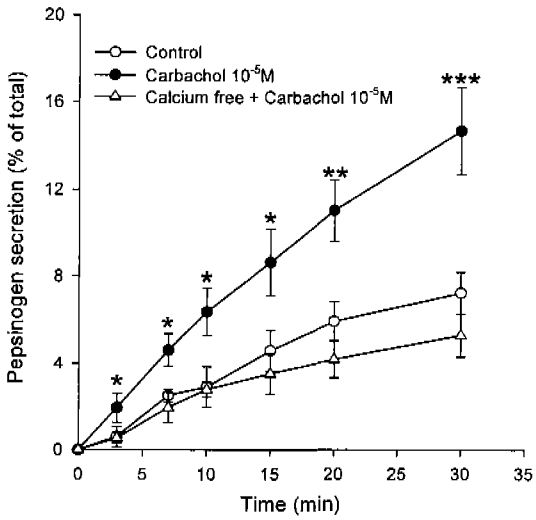

Fig. 3. The relationships among carbachol, Ca^{2+} and pepsinogen secretion in isolated chief cells. Pepsinogen secretion was determined as described in "Materials and Methods" and expressed as percentage of pepsinogen in cells at the beginning of incubation that was released into extracellular medium during incubation. Data are the mean± SE six separate experiments. $^{*}p<0.05$ *vs* control, $^{**}p<0.01$ *vs* control, $^{***}p<0.001$ *vs* control (two-tailed Student's *t*-test)

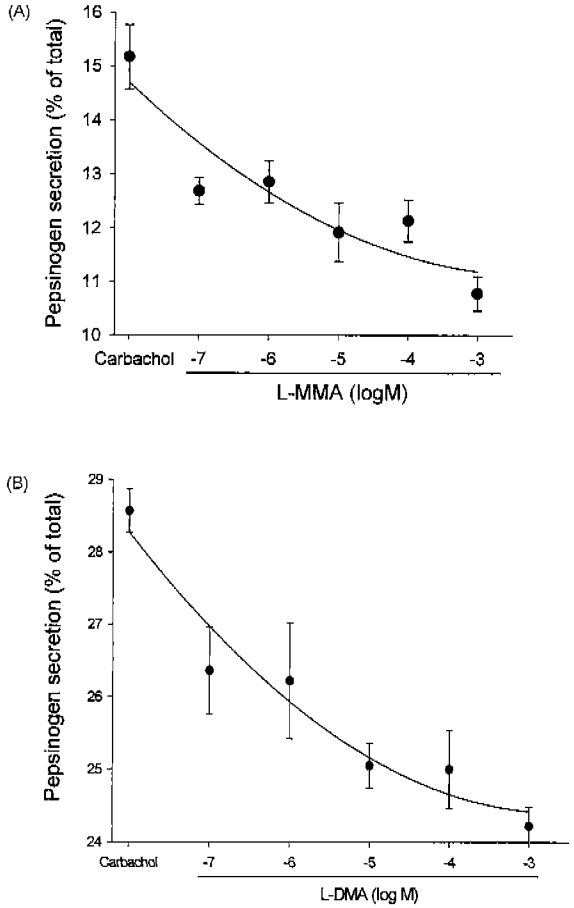

Fig. 4. Concentration-response of two NOS inhibitors on pepsinogen secretion in carbachol-treated chief cells. The det-ailed experimental conditions were determined as described under "Materials and Methods". Rat gastric chief cells were pretreated with L-MMA (10^{-3}-10^{-7}M) (A) and L-DMA (10^{-3}-10^{-7}M) (B) for 15 min and stimulated for 15 min with 10^{-5} M carbachol. Data are the mean± SE of three separate experiments.

indicated that unfractionated cells were moderately enriched with mucous epithelial cells shown red color. Also, in fractions 10 plus 11 high-pepsinogen-content cells were enriched with chief cells shown dark blue color (>85 %) (data not shown). Trypan blue was almost totally excluded by chief cells for indicated incubation time.

Effect of Ca^{2+} and NOS inhibitors on pepsinogen secretion in carbachol-stimulated chief cells

To determine the optimal concentration of carbachol for pepsinogen secretion in isolated chief cells, dose response of carbachol (10^{-3} - 10^{-7} M) was carried out as described in "Materials and Methods". The concentration causing maximal increase of pepsinogen secretion was the 10^{-5} M (Fig. 2). During 30 min incubation at 37°C, pepsinogen release from chief cells was approximately 15%. The increase of pepsinogen secretion by 10^{-5} M carbachol was reduced to basal level in Ca^{2+} free medium containing 2 mM EGTA (Fig. 3). To establish whether NO is involved in gastric exocrine secretion, the change of carbachol-induced pepsinogen secretion was determined by pretreatment of N^{G}-monomethyl-L-arginine (L-MMA) or N^{G},N^{G}-dimethyl-L-arginine (L-DMA) for 15 min (10^{-7}-10^{-3} M). Increase of pepsinogen secretion by carbachol was dose-dependently reduced by L-MMA or L-DMA (Fig. 4A and 4B). Therefore, these results suggest that Ca^{2+}-mediated NO may be involved in pepsinogen secretion in isolated chief cells.

The Changes of NO in carbachol-stimulated chief cells

The rate of NO production by carbachol was determined by detecting NO released into medium for indicated times with NO analyzer (Model 7020, ANTEK instruments, U.S.A.). NO secretion by carbachol increased at the peak level 10 min but decreased gradually to basal level following pretreatment of 1 mM L-MMA (Fig. 5A). Also to confirm the relation between the change of NO produced and increase in pepsinogen release by carbachol, NOS activity was measured using [^{3}H]citrulline accumulation method upon stimulating chief cells with carbachol. Carbachol activated NOS activity transiently with maximum at 10 min (113.16 ± 15.8 *vs* 130.98 ± 9.42, % basal), which was sig-

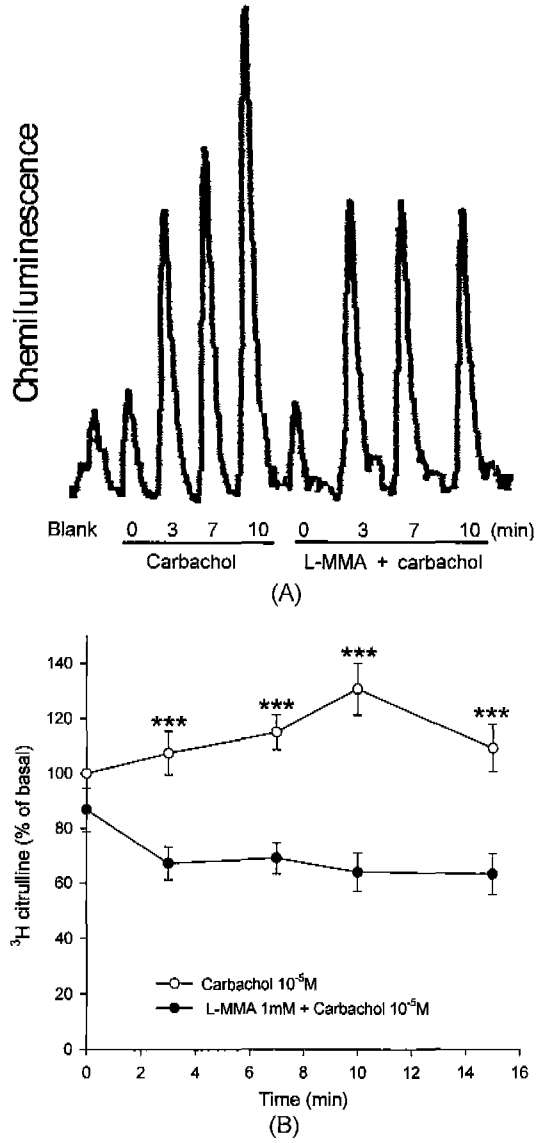

Fig. 5. Effect of time course on the release of NO from isolated chief cells in the presence of carbachol. Time course of NO secretion by carbachol (10^{-5} M) from isolated chief cells was determined using NO analyzer in the absence or presence of 1 mM L-MMA (A) and [^{3}H]citrulline formation (B) was also carried out as the same condition. NO contents of the peak at 10 min was 710 pmole/10^7 cells (A). ***$p<0.001$ *vs* L-MMA plus carbachol (two-tailed Student's *t*-test)

nificantly decreased to 65% of basal level by the inhibitor of NOS, L-MMA as the incubation time changed (Fig. 5B). However, as described above, pepsinogen secretion increased continuously for the indicated incubation times (Fig. 3) and was dose-dependently reduced by NOS inhibitors (Fig. 4A and 4B). These results indicate that the increase of pepsinogen might be regulated by NOS inhibitor and that early NO increase by carbachol treatment might trigger the effect of Ca^{2+}-mediated agonist on pepsinogen release in isolated chief cells. However, NO is likely to partially modulate the pepsinogen release by carbachol due to different pattern of activation between the change of NOS activity and pepsinogen release.

Time course on cGMP level in chief cells

Exocrine signal transduction pathway in chief cells has been reported to be involved in cGMP pathway and cAMP pathway. To determine the relationships among pepsinogen secretion, NO generation and cGMP generation, we performed cGMP assay in the presence of carbachol. Carbachol-induced cGMP increased from 1 to 3 min and then it returned to the basal level at 5 min. We also carried out inhibtor study to examine effect of L-MMA on carbachol-induced cGMP generation. Pretreatment of 1 mM L-MMA in gastric chief cells for 15 min abolished basal cGMP rise as well as the stimulatory effect of carbachol on cGMP generation as the incubation time changed. This strong inhibition against cGMP production may be due to inhibition of constitutive NO generation by high concentration of L-MMA. Therefore, NO production in chief cell is considered to play an important role in cGMP generation (Fig. 6).

DISCUSSION

During the last several years, a number of discoveries from many different research have revealed the major biological role of NO as a neurotransmitter in the nervous system and other parts of the body (Moncada *et al.*, 1991), a potent vasodilating and cytoprotective substance, a mediator of endotoxin-induced cytotoxicity, and a substance involved in various disorders. In addition, NO plays an important role in paracrine and autocrine regulation of neurotransmitter, protein, and ion secretion (Wrenn *et al.*, 1994). NO can lead to an increase or decrease in the secretory response. In pancreatic acinar cells, NO formation is associated with increased levels of cGMP and endocrine/exocrine secretion (Seo *et al.*, 1995; Ahn *et al.*, 1998). The exocrine pancreatic secretion involves a variety of neurohormonal factors and is mediated by multiple regulatory pathways in the acinar cells (Palmer *et al.*, 1987; Gukovskaya and Pandol, 1994). As like acinar cells, some agonists such as carbachol, cholecystokinin-pancreozymin and bombesin causing changes in cellular Ca^{2+} induce exocrine secretion of gastric chief cell. Agonist-induced Ca^{2+} influx occurs as a result of the agonist

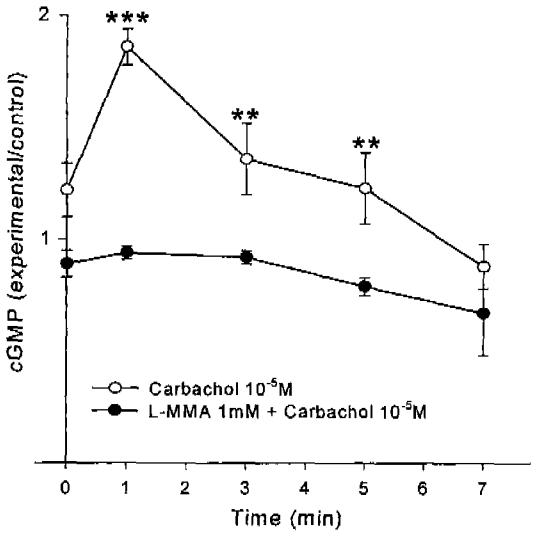

Fig. 6. Changes in cGMP generation in response to carbachol from isolated chief cells preincubated with or without carbachol. cGMP was measured using a specific enzyme immunoassay kit. Basal level for cGMP was 4 pmole/tube. Results were expressed as experimental per control. $^{**}p<0.01$ *vs* L-MMA plus carbachol, $^{***}p<0.001$ *vs* L-MMA plus carbachol (two-tailed Student's *t*-test) Data are the mean±SE of five separate experi-ments.

ability to release Ca^{2+} from inositol-1,4,5-triphosphate-sensitive stores (Raufman *et al.*, 1986; Chew and Brown, 1986). Increase of cGMP has been associated with stimulation of pancreatic acinar cells, but its direct role as a mediator in this process has remained to be controversial. Pandol and Shoeffield-Payne (1990) reported that cGMP mediates the agonist-stimulated increase in plasma membrane calcium entry. In addition, Gukovskaya and Pandol (1994) suggested that NO production regulate cGMP formation and calcium influx in pancreatic acinar cells and Xu *et al.* (1994) also reported that depletion of intracellular Ca^{2+} stores activates NOS to generate cGMP and regulate Ca^{2+} influx. These ideas indicated that NO/cGMP system is coupled with enzyme secretion in pancreatic acinar cells. However, Yoshida *et al.* (1997) demonstrated that, in the rat pancreatic acinar cells, the NOS system is either not present or not functioning, that exogenous NO is capable of increasing endogenous cGMP by activating the soluble guanylate cyclase system, which results in modest Ca^{2+} transients and increases in amylase secretion, and that the NO/cGMP system is not linked to the signal transduction pathway activated by carbachol and CCK-OP. The reason why it is difficult to explain the connection between NO/cGMP system and enzyme secretion in pancreatic acinar cells is that the amount of NO produced by NOS in pancreatic acinar cells is very small and rapidly oxidized, diffused to other cells. The majority of NO effects under physiological conditions appears to be mediated primarily by the activation of the intracelluar NO receptor guanylate cyclase (Garbers, 1992) concomitant with a cGMP increase. In the present study, treatment of carbachol resulted in increasing pepsinogen secretion, NO production, and cGMP generation from isolated chief cells. Carbachol, an agent increasing intracellular Ca^{2+} induced increase of pepsinogen secretion in a concentration- and time-dependent fashion and NOS activity. These effects were inhibited by preincubating the cells with L-MMA or L-DMA, a natural inhibitor of NOS. Furthermore, exclusion of extracellular Ca^{2+}, an essential factor on NOS activation, decreased in pepsinogen secretion to basal level. Therefore, Ca^{2+}- mediated NO produced by carbachol is considered to be an intracellular mediator of carbachol-induced soluble guanylate cyclase activity and pepsinogen secretion. But time discrepancy between increase of NO and cGMP (Fig. 5 and 6) might result from the detection limit of small amount of NO which is produced within 1-2 min following carbachol treatment and is sufficient to activate guanylate cyclase. Pretreatment of L-MMA inhibited the cGMP generation and accumulation in carbachol-stimulated chief cells by which multiple pathways may regulate gastric exocrine secretion. In conclusion, it is thought that carbachol stimulates pepsinogen release by activating cholinergic receptor, triggering Ca^{2+} mobilization, NO generation, and cGMP accumulation. However, together those results suggest that NO might partially mediate gastric pepsinogen release and multiple pathways exist in gastric exocrine secretion. Further studies should clarify the more precise role and mechanism of NO in the overall scheme of secretory regulation in the gastric chief cells.

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