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STIMULATION BY NITRIC OXIDE OF GASTRIC ACID SECRETION IN BULLFROG FUNDIC MUCOSA *IN VITRO*

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We examined the effect of NO on acid secretion *in vitro* using isolated preparations of Bullfrog stomach. The bullfrog fundic mucosa was bathed in unbuffered Ringer solution gassed with 100% O_2 on the mucosal side and HCO_3^- Ringer's solution gassed with 95% O_2/5% CO_2 on the serosal side, and the acid secretion was measured at pH 5.0 using the pH-stat method and by adding 5 mM NaOH. Serosal addition of a NO donor NOR-3 ($10^{-5} \sim 10^{-3}$ M: (±)-(E)-ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexnamine) caused an increase of acid secretion in a dose-dependent manner, the effect lasting about 1 hr and reaching a maximal level of 2-fold the basal values. The acid stimulatory effect of NOR-3 was mimicked by another NO donor SNAP (10^{-3} mol/L: S-nitroso-O-N--acetyl-penicillamine) and markedly and markedly inhibited by prior administration of cimetidine (10^{-5} mol/L) as well as compound 48/80 (the mast cell degranulator). Likewise, the increased acid response to NOR-3 was significantly mitigatd by pretreatment with carboxy-PTIO (a NO scavenger) or superoxide dismutase (SOD), but not by indomethacin or methylene blue (a guanylyl cyclase inhibitor). Neoither L-NAME, L-arginine nor dibutyryl guanosine-3',5'-cyclic monophosphate (dbcGMP) has any effect on the basal acid secretion. Serosal addition of NOR-3 caused a significant increase in the luminal release of histamine, and this response was inhibited by pretreatment with either compound 48/80, carboxy-PTIO or SOD. These results suggest that the NO donor increases gastric acid secretion in the isolated frog stomach *in vitro*, and this action is mediated by endogenous histamine released from mast cells, the process being cGMP-independent but requiring the presence of superoxide radicals. In addition, it was speculated that the histamine releasing action of NO may be due to peroxynitrite produced by NO and superoxide radicals.

Key words: *nitric oxide, acid secretion, histamine, superoxide radical, bullfrog stomach.*

Abbreviations

PG — prostaglandin
NO — nitric oxide
dbcGMP — dibutyryl guanosine-3',5'-cyclic monophosphate
NOx — NO_2^-/NO_3^-
L-NAME — N^G-nitro-L-arginine methyl ester
SOD — superoxide dismutase
SNAP — S-nitroso-N-acetyl--penicillamine
TRH — thyrotropin-releasing hormone
ECL — enterochromaffin-like
COX — cyclooxygenase

INTRODUCTION

A growing body of evidence suggests that nitric oxide (NO) acts as a transmitter is non-adrenergic and non-cholinergic nerves in the gastrointestinal tissue and modulates various functions, including mucosal blood flow, acid secretion, mucus secretion and bicardonate secretion (1—4). Concerning gastric acid secretion, most studies *in vivo* have found an inhibitory influence of NO on the secretion by both a direct action on the parietal cell and an indirect action via suppression of histamine release (5—8). Barrachina *et al.* (7) reported that acute inhibition by endotoxin of distension-induced action secretion requires the synthesis of NO and the integrity of the peripheral nervous system. More recently, Esplugues *et al.* (8) showed that physiologic inhibition of acid secretion observed during stress is mediated by a nervous reflex involving a neuronal pathway that includes NO synthesis in the brain, specifically in the dorsal motor nucleus of the vagus. We also reported that a NO donor inhibited the acid secretory response to pentagastrin and YM-14673 [an analogue of thyrotropin-releasing hormone (TRH)] but not histamine, suggesting a suppression of histamine release from enterochromaffin-like (ECL) cells (9). In addition, we showed that the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) stimulated gastric acid secretion in response to stomach distension or in the damaged stromach (4, 10). In the former case, the enhanced effect of L-NAME was attenuated by vagotomy, suggesting the existence of regulatory for acid production triggered by a nervous reflex involving NO.

On the other hand, Hasebe *et al.* (11) reported that inhibition of NO production by N^G-nitro-L-arginine (L-NNA) decreased basal acid secretion *in vitro* using the isolated mouse stomach, in an L-arginine-sensitive manner. Furthermore, they also showed that sodium nitroprusside stimulated the acid secretion in the same stomach preparation through histamine release from ECL cells (12). Thus, the influence of NO on gastric acid secretion remains controversial.

The present study was therefore designed to investigate the influence of a NO donor NOR-3 [(±)-(E)-ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexanamine] on acid secretion *in vitro* using isolated bullfrog stomachs, which are devoid of blood supply and vagal innervation, and to analyze its effect in relation to endogenous histamine. NOR-3 is a spontaneous NO releasing drug, which has been shown to generate NO, much faster than sodium nitroprusside (13).

MATERIALS AND METHODS

Animals

Bullfrogs (Rana Catesbelana; Saitama, Japan) were housed at 4°C in 120 mM NaCl containing tetracycline (50 mg/L) and used within one week of purchase. Studies were carried out using 4—6 tissues per group. All experimental procedures described here were approved by the Experimental Aimal Research Committee of the Kyoto Pharmaceutical University.

Determination of Gastric Acid Secretion

The frogs were pithed, the stomachs and duodenums isolated, and the fundic mucosaes were striped from the muscle layer by blunt dissection. The tissues were then mounted between two halves of a lucite chamber (the exposed area: 0.8 mm^2). Tissues were batheed in unbufferred Ringer solution (mM: Na^+, 120; Cl^-, 120) gassed with 100% O_2 on the mucosal side and HCO_3^- Ringer's solution (mM: Na^+, 87; Cl^-, 93; K^+, 5; Mg^{2+}, 1; Ca^{2+}, 1.8; PO_4^-, 1; HCO_3^-, 18; glucose, 2) gassed with 95% O_2/5% CO_2 on the serosal side, and these solutions were warmed at 28°C and circulated by a gas-liftsystem. The acid secretion was measured by the pH-stat method (COMTITE-980, CHIRANUMA Industries, Ibaraki, Japan) using 5 mmol/L NaOH as the titrant to keep the mucosal pH at 7.0. Measurements were made every 10 min starting at least 1 hr after mounting the tissues. After obtaining a stable acid secretion for 30 min, the following agents were added to the serosal solution; NOR-3, a NO donor ($10^{-5} \sim 10^{-3}$ mol/L), S-nitroso-N-acetyl-penicillamine (SNAP: 10^{-3} mol/L), cimetidine (10^{-5} mol/L), L-arginine (10^{-1} mol/L), N^G-nitro-L-arginine methyl ester; a NO synthase inhibitor (10^{-3} mol/L), and dibutyryl guanosine-3',5'-cyclic monophosphate (dbcGMP: 10^{-3} mol/L). In some cases, compound 48/80, a mast cell degranulator (0.1 g/L), indomethacin (10^{-5} mol/L), a cyclooxygenase (COX) inhibitor or methylene blue (5×10^{-6} mol/L), a soluble guanylate cyclase inhibitor (14), was addedto the serosal solution 1 hr before addition of NOR-3 (10^{-3} mol/L), while carboxy-PTIO, a NO scavenger (10^{-3} mol/L) or superoxide dismutase (SOD: 30000 units/L) was added to the serosal solution 30 min before NOR-3.

In a seprarate experiment, stomachs of male guinea pigs (Shimizu, Kyoto, Japan) were isolated and mounted on a lucite chamber, and the acid secretion was measured in the same way as described for bullfrog stomachs. The effect of NOR-3 (10^{-3} mol/L) on the acid secretion was examined by adding the agent to the serosal side.

Determination of Luminal Histamine Release

After stable acid secretion has been obtained at least for 60 min, both serosal and luminal solutions were changed to fresh Ringer solutions every one hour. The amount of histamine in luminal solution was determined by enzyme immunoassay (Histamine EIA kit, Immunotech, Marseille, France) [10]. NOR-3 (10^{-3} mol/L) was added to the serosal solution. In some cases, compound 48/80 (0.1 g/L) or SOD (30000 units/L) was added to the serosal solution 1 hr before addition of NOR-3.

Preparation of Drugs

Drugs used were [(±)-(E)-ethyl-2-[(E]-hydroxyimino]-5-nitro-3-hexanamine] (NOR-3), [2-(4--carboxyphenyl)-4,4,4,5-tetramethylimidazoline-1-oxyl-3-oxide] (carboxy-PTIO) (Dojindo, Kumamoto, Japan), S-nitroso-N-acetyl-penicillamine (SNAP), N^G-nitro-L-arginine methyl ester (L-NAME), N2,2'-O-dibutryl guanosine-3', 5'-cyclic monophosphate Na (dbcGMP), cimetidine,

indomethacin, compound 48/80 (Sigma Chemicals, St. Louis, Mo., USA)), methylene blue, L-arginine, superoxide dismutase (SOD) and tetracycline (Nacalai tesque, Kyoto, Japan). NOR-3, SNAP, cimetidine, indomethacin, catrboxy-PTO and dbcGMP were dissolved in dimethyl sulfoxide (>1%) (DMSO: Nacalai tesque) and diluted with distilled water to desired concentrations. Compound 48/80 and SOD was dissolved in distilled water. All agents were prepared immediately before use and added to the serosal solution in a volume of 0.1 ml.

Statistics

Data are presented as the means ± SE for 4—6 tissues from each group. Statistical analyses were performed using a two-tailed Dunnett's multiple comparison test or Student's t-test, and values of $P < 0.05$ were considered as significant.

RESULTS

Effect of NOR-3 on Gastric Acid Secretion

Isolated bullfrog fundic mucosa consistently secreted acid at rates of 0.2~0.3 μEq/10 min/cm^2 as basal secretion. Serosal addition of NOR-3 (10^{-5}~10^{-3} mol/L) caused an increase of acid secretion in a concentration-dependent manner (*Fig. 1 A*). The acid secretion remained unchanged at th concentration of 10^{-5} mol/L and tended to increase in response to 10^{-4} mol/L. At the highest concentration of 10^{-3} mol/L, the acid secretion markedly increased and reached a peak of 1.7 times the basal rates, the action persisting for abour 2 hr after addition of NOR-3. Total acid output at 10^{-5}, 10^{-4} and 10^{-3} mol/L of NOR-3 was 2.77±0.14, 3.28±0.16 and 3.94±0.36 μEq/2 hr, respectively, and the values at 10^{-3} mol/L were significant when compared to those (2.64±0.10 μEq/2 hr) in the control group (*Fig. 1 B*). Similarly, a significant increase of the acid secretion was also observed by another NO donor SNAP at 10^{-3} mol/L, the total acid output being 3.35±0.14 μEq/2 hr.

To confirm the acid stimulatory action of a NO donor, we examined in a preliminary study the effect of NOR-3 on gastric acid secretion in isolated guinea pig stomachs. Serosal addition of NOR-3 (10^{-3} mol/L) caused a clear increase of acid secretion in all tissues tested; the degree of increase was in the range of 190~300%, the mean peak response being 228.4±26.3% of basal values (not shown).

Effects of L-Arginine, dbcGMP and L-NAME on Gastric Acid Secretion

Serosal addition of neither dbcGMP (10^{-3} mol/L), L-arginine (10^{-1} mol/L) nor L-NAME (10^{-3} mol/L) produced an effect on basal acid secretion in bullfrog stomachs (*Fig. 2 A&2 B*).

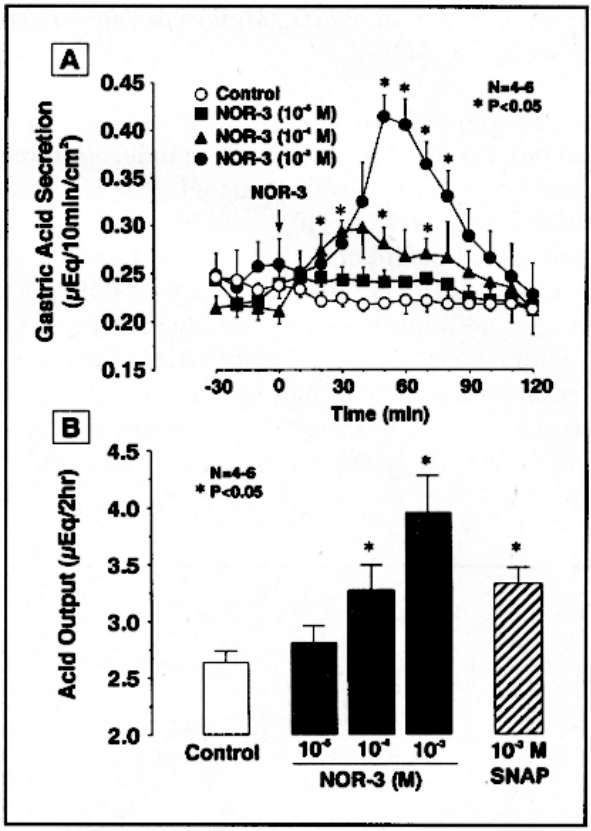

Fig. 1. Effects of NOR-3 ($10^{-5} \sim 10^{-3}$ mol/L) and SNAP (10^{-3} mol/L) on acid secretion in *in vitro* preparations of Bullfrog stomach. NOR-3 or SNAP was added to the nutrient solution. Values in B show the total acid output for 2 hr after addition of NOR-3 or SNAP. Data are presented as the means ± SE from 4 ~ 6 tissues per group. * Significant difference from control at $P < 0.05$.

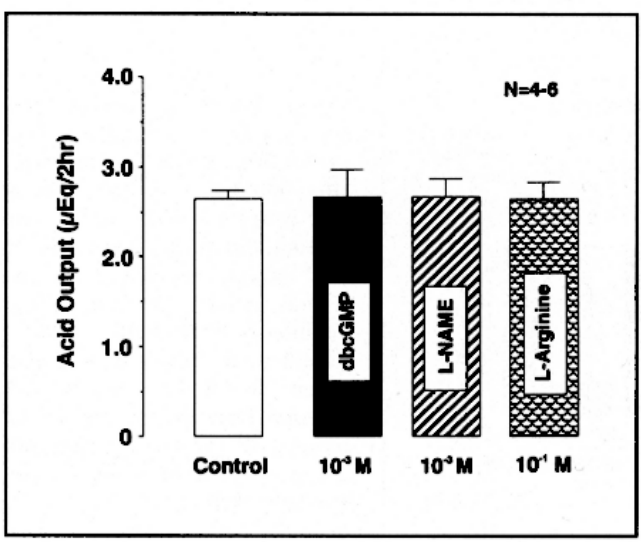

Fig. 2. Effects of dbcGMP (10^{-3} mol/L), L-NAME (10^{-3} mol/L), and L-arginine (10^{-1} mol/L) on acid secretion in *in vitro* preparations of Bullfrog stomach. Each drug was added to the nutrient solution. Data are presented as the means ± SE from 4 ~ 6 tissues per group.

Effects ofcarboxy-PTIO, Methylene blue and Indomethacin on Acid Secretion Induced by NOR-3

Serosal addition of carboxy-PTIO (10^{-3} mol/L) did not have any influence on basal rates of acid secretion in bullfrog stomachs (not shown). However, this agent almost totally attenuated the acid secretion in response to serosal addition of NOR-3 (10^{-3} mol/L), and the rate of acid secretion remained unchanged before and after NOR-3 treatment (*Fig. 3 A&3 B*). By contrast, the acid secretory response induced by NOR-3 was not significantly affected by serosal addition of methylene blue at the dose (5×10^{-5} mol/L) that inhibits soluble guanylate cyclase by over 50% (14). Similar to methylene blue, indomethacin (10^{-5} mol/L) had no effect on the acid secretory response induced by NOR-3. Neither methylene blue nor indomethacin at the dose had any effect on basal rates of acid secretion, similar to carboxy-PTIO (not shown).

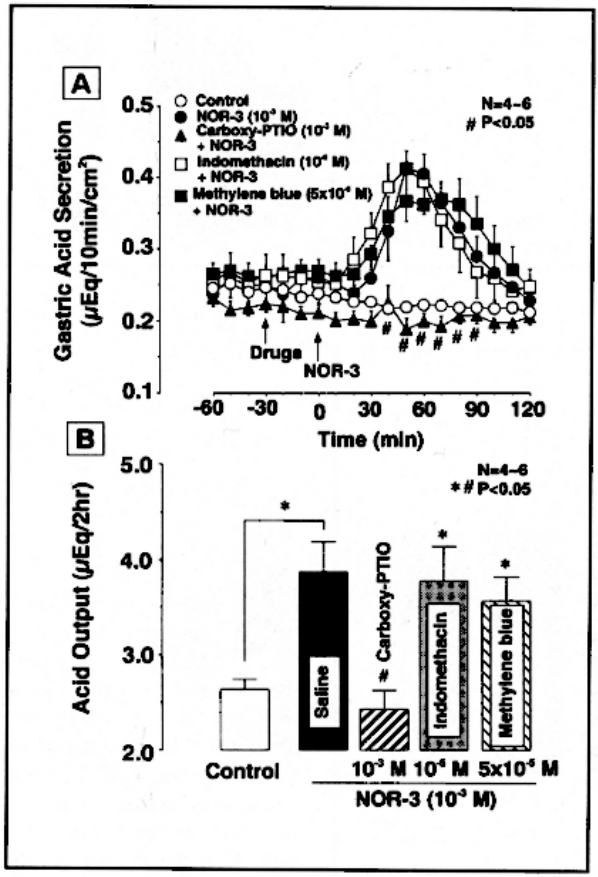

Fig. 3. Effects of carboxy-PTIO (10^{-3} mol/L), methylene blue (5×10^{-5} mol/L) and indomethacin (10^{-5} mol/L) on the acid stimulatory action of NOR-3 in *in vitro* preparations of Bullfrog stomach. Each agent was added to the serosal solution 30 min before addition of NOR-3 (10^{-3} mol/L). Values in B show the total acid output for 2 hr after NOR-3 addition. Data are presented as the means ± SE from 4~6 tissues per group. Significant difference at $P < 0.05$; * from control; # from NOR-3 alone.

Effects of Cimetidine, Compound 48/80 and SOD on Acid Secretion Induced by NOR-3

NOR-3 (10^{-3} mol/L) added to the serosal solution increased the acid secretion from 0.25 ± 0.02 μEq/10 min/cm^2 to a peak value of 0.42 ± 0.02 μEq/10 min/cm^2 in isolated bullfrog stomachs. Serosal addition of cimetidine (10^{-5} mol/L) by itself slightly decreased the rates of basal acid secretion and almost the increase of acid secretion in response to NOR-3; the total acid output after addition of NOR-3 was 1.32 ± 0.14 μEq/hr, which is significantly lower than that (3.87 ± 0.31 μEq/2 hr) in control tissues (*Fig. 4 A&B*). Likewise, the acid secretory response to NOR-3 was significantly attenuated when the tissue was pretreated by serosal addition of compound 48/80 (0.1 g/L). After addition of compound 48/80, the basal acid secretion decreased gradually with time, and did not increase any further after subsequent addition of NOR-3; the total acid output after NOR-3 was 2.05 ± 0.29 μEq/2 hr. On the other hand, the

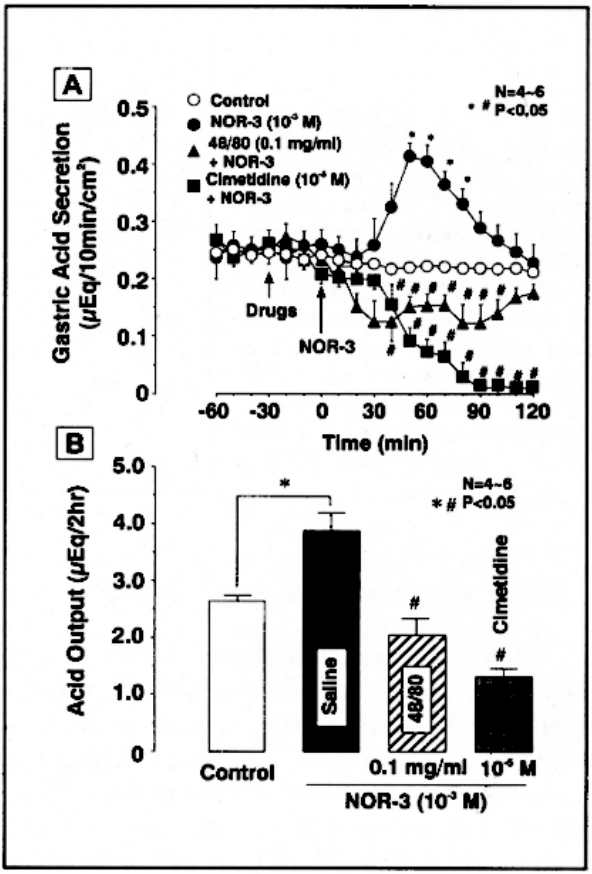

Fig. 4. Effects of compound 48/80 (0.1 g/L) and cimetidine (10^{-5} mol/L) on the acid stimulatory action of NOR-3 in *in vitro* preparations of Bullfrog stomach. Each agent was added to serosal solution 1 hr before addition of NOR-3 (10^{-3} mol/L). Values in B show the total acid output for 2 hr after NOR-3 addition. Data are presented as the means ± SE from 4~6 tissues per group. Significant difference at $P < 0.05$; *from control; #from NOR-3 alone.

increased acid response to NOR-3 was also significantly mitigated by the concurrent addition of SOD (30000 units/L), scavenging superoxide radicals (*Fig. 5*). The rate of basal acid secretion was not altered by serosal addition of SOD, but the acid secretory response to NOR-3 was suppressed in the presence of SOD, the total acid output after NOR-3 being 2.64 ± 0.21 μEq/2 hr, which is significantly lower than that in control tissues given NOR-3 alone.

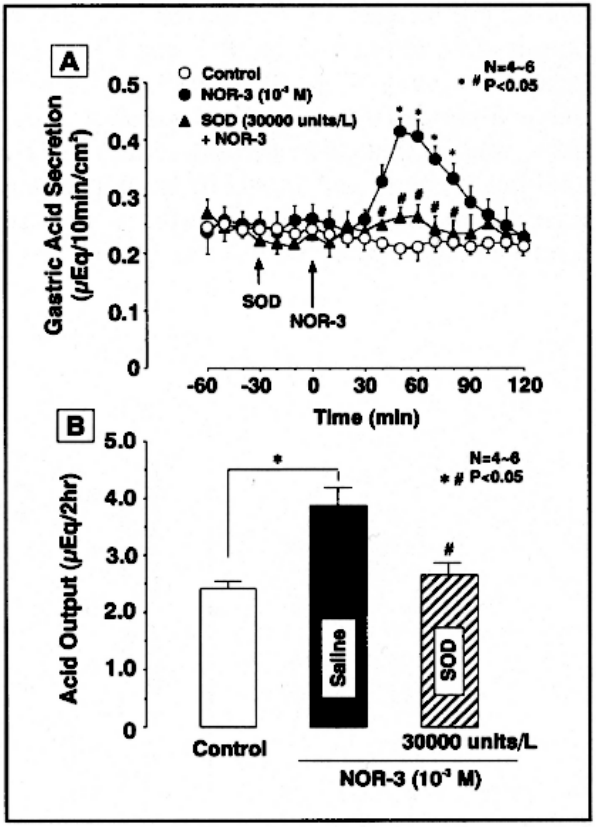

Fig. 5. Effect of SOD (30000 units/L) on the acid atimulatory action of NOR-3 in *in vitro* preparations of Bullfrog stomach. SOD was added to the serosal solution 30 min before addition of NOR-3 (10^{-3} mol/L). Values in B show the total acid output for 2 hr after NOR-3 addition. Data are presented as the means ± SE from 5 tissues per group. Significant difference at $P < 0.05$; *from control; #from NOR-3 alone.

Luminal Histamine Release by NOR-3

Under normal conditions, bullfrog stomachs spontaneously released histamine into the luminal solution, the values being 276.9 ± 42.6 pmol/hr. The luminal release of histamine was significantly increased following serosal addition of NOR-3 (10^{-3} mol/L), reaching the value of 600.8 ± 124.8 pmol/hr 1 after the treatment (*Fig. 6*). The increased release of histamine by NOR-3 was significantly suppressed by prior addition of compound 48/80 (0.1 g/L) and carboxy-PTIO (10^{-3} mol/L) as well as SOD (30000 units/L), the values for the

initial 1 hr after NOR-3 treatment being 292.5±42.5, 324.7±49.5 and 328.6±23.6 pmol/hr, respectively, either of which was significantly lower than those obtained by NOR-3 alone.

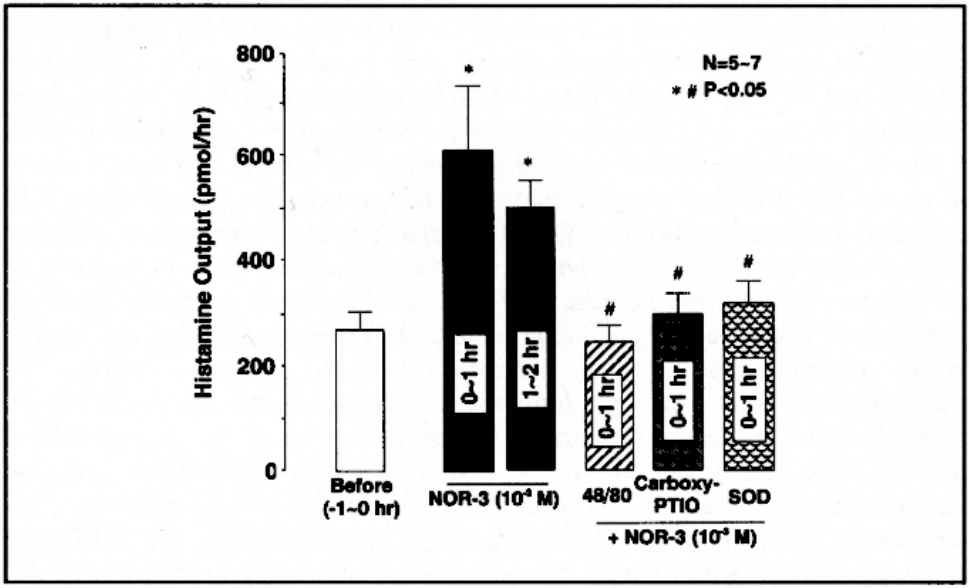

Fig. 6. Effect of NOR-3 on luminal histamine release in *in vitro* preparations of bullfrog fundic mucosa. NOR-3 was added to the serosal solution at a concentration of 10^{-3} mol/L. The release of histamine was measured every 1 hour for 3 hr, 1 hour before and 2 hr after the addition of NOR-3. Compound 48/80 (0.1 g/L) was added to serosal solution 1 hr before addition of NOR-3, while carboxy-PTIO (5×10^{-5} mol/L) or SOD (30000 units/L) was added 30 min before addition of NOR-3. Data are presented as the means±SE from 7 different tissues per group. * Significant difference at $P < 0.05$ * from Before; # from NOR-3 alone (1 hr).

DISCUSSION

In the present study, we found that NOR-3, a NO donor, stimulated gastric acid secretion in isolated preparations of bullfrog stomach *in vitro*. This action was independent of cGMP and mediated by endogenous histamine released from mast cells.

A number of studies have investigated the effects of NO synthese inhibitors on gastric acid secretion in various species of animals such as rats, dogs and mice [2, 4—6, 7—12, 15], although the results remain controversial. Pique *et al.* [2] reported that the NO synthase inhibitor L-NMMA did not affect basal or pentagastrin-stimulated acid secretion in rats. Martinez-Cuesta *et al.* [5] showed that the NO synthase inhibitor L-NAME antagonized the inhibitory action of lipopolysaccharide on acid secretion induced by gastric distension or

pentagastrin in rats. We also showed that the NO donor NOR-3 suppressed the acid secretory response to pentagastrin and TRH analogue but not histamine in rats, suggesting a negative effect of NO on histamine release from ECL cells (9). In addition, we recently reported that the acid secretory response to stomach distension was markedly potentiated by L-NAME, accompanied by an increase of histamine release (10]. Under *in vitro* conditions, Brown *et al.* (6) found that a high concentration of NO donor, S-nitroso-N-acetyl-penicillamine (SNAP), inhibits acid secretion using rat isolated parietal cells, suggesting a direct inhibitory action at the parietal cell. These results together suggest that NO has a negative influence on acid secretion. In contrast, Bilski *et al.* (15) reported that the NO synthase inhibitor did not affect basal acid secretion but reduced the acid secretion in response to feeding or pentagastrin in dogs, probably because of a decreased mucosal blood flow. More recently, Hasebe *et al.* (11) showed using isolated mouse whole stomach that L-NNA decreased the acid secretion induced by pentagastrin or vagal electrical stimulation. Since NO-containing neurons have been identified in the central nervous system as well as in the gastrointestinal mucosa (16), and since NO plays a role as a neuromodulator in some non-adrenergic non-cholinergic neurons in the gut (3), it is possible that NO decreases vagally-mediated acid secretion by suppressing neuronal activity of the vagus nerves, even if NO has a stimulatory influence on acid production.

In the present study, we found that the NO donor NOR-3 dose-dependently increased acid secretion accompanied by an increase of luminal histamine release in isolated bullfrog stomachs. Similar results were obtained by NOR-3 in guinea pig stomachs and by another NO donor SNAP in the bullforg stomach *in vitro*. In addition, we also found that the acid secretory response to NOR-3 was almost totally attenuated by both cimetidine and compound 48/80, a mast cell degranulator, strongly suggesting that the NO induces acid secretion under *in vitro* conditions, mediated by endogenous histamine. These results are in agreement with the findings by Horie *et al.* (12), who showed that the NO donor SNP stimulated acid secretion in isolated mouse stomach with an enhanced release of histamine from ECL cells. In the present study, the acid stimulatory action of NOR-3 was totally blocked by carboxy-PTIO, a NO radical scavenger, but not affected by methylene blue, an inhibitor of guanylate cyclasem suggesting that the NOR-3 action is accounted for by NO generated from this compound, but is not mediated by cGMP. Indeed, we noted that dbcGMP even at 10^{-3}mmol/L did not stimulate acid secretion in the present study. Since this action of NOR-3 was also totally blocked by compound 48/80, it is assumed that NOR-3 stimulates acid secretion mediated by endogenous histamine but not by acting directly on the parietal cell. Horie *et al.* (12), however, reported that dbcGMP stimulated acid secretion in isolated mouse stomach similar to SNP, suggesting the

involvement of cGMP in the acid stimulatory action of NO. At present, the reason for these different results is unknown, but they may bedue to different experimental conditions, including species differences or the doses used.

We observed a significant increase of histamine release after addition ofa NO donor. Salvemini *et al.* (17), however, showed that exogenous NO inhibits the release of histamine in rat mast cells mediated by a cGMP-dependent mechanism. Wallace *et al.* (18) showed that interleukin-1β exhibited an antisecretory action against pentagastrin by suppressing histamine release, in an L-NAME-sensitive manner. We also reported that NOR-3 inhibited pentagastrin-induced acid secretion by suppressing histamine release from ECL cells in rats (9). The reason for these different results also remains unexplained.

Is endogenous NO involved in the occurrence of basal acid secretion? To answer this question, we examined the effects of L-arginine and L-NAME on basal acid secretion in bullfrog stomachs. As evidenced in *Fig. 2*, neither L-arginine (0.1 mol/L) nor L-NAME (10^{-3} mol/L) had any effect on the basal rate of acid secretion. Thus, it is assmed that NO generated endogenously is not involved in the regulation of basal acid secretion. Since the acid stimulatory action of NOR-3 was observed at high concentration, over 10^{-4} mol/L, and since this NO action is not mediated by cGMP, it is likely that the acid response to NOR-3 is a nonspecific action of this molecule. NO stimulates soluble guanylyl cyclase to produce cGMP and also reacts with other free radicals. Since gastric surface mucous cells possess a phagocyte NADPH oxidase-like system and secrete abundant superoxide anion (O_2) (19), and since the reaction of NO with O_2^- results in production of the toxic species, peroxynitrite (ONOO) (20), it may be speculated that this molecule damages mast cells to result in a release of histamine. This contention is supported by the fact that both acid secretory and histamine releasing effects of NOR-3 were significantly mitigated in the presence of SOD, a scavenger of superoxide radicals. In the present study, the isolated stomach is devoid of blood supply and perfused with oxygen in place of blood circulation. It may be possible that the tissue metabolism would favor the production of superoxide radicals under such conditions. Thus may also explain why NO affects the acid secretion in opposite directions between *in vivo* and *in vitro* conditions. In any case, further studies including the direct measurement of peroxynitrite after NOR-3 treatment should be done to verify this speculation, concerning the role of peroxynitrite in the acid stimulation action of NO *in vitro*.

Several studies have shown that NO orNO donors stimulate prostaglandin E_2 (PGE_2) production in various organs and cells (21—24). Uno *et al.* (24) demonstrated that the NO donor, SNAP, stimulates PGE_2 generation in rat gastric epithelial cells. We also reported that NOR-3 stimulates HCO_3^- secretion in the bullfrog duodenum, mediated by PGE_2 (25). These studies

suggest that NO directly activate cyclooxygenase, independent of the cGMP pathway. However, in the present study, the acid stimulatory action of NOR-3 was not affected by indomethacin, excluding a possible mediated of this action by endogenous PGs. These results suggest no interaction between NO and PG generation, at least, in the acid stimulated action of NOR-3.

In summary, the present study suggests that the NO donor NOR-3 increase gastric acid secretion in the isolated frog stomach *in vitro*, and this action is mediated by endogenous histamine released from mast cells, the process being cGMP-independent but dependent on the presence of superoxide radicals. Although we did not provide any direct evidence, it is speculated that the histamine releasing action of NO may be due to peroxynitrite produced by NO and superoxide radicals.

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