Phosphodiesterase Isozymes Involved in Regulation of HCO_3^- Secretion in Isolated Mouse Stomach *In Vitro*

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Running head: Gastric HCO_3^- secretion and PDE Isozyme

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Abbreviations:
nitric oxide ----------NO
prostaglandins ---------- PGs
cyclooxygenase ---------- COX
prostaglandin E_2 ---------- PGE_2
PGE receptor ---------- EP receptor
3', 5'-cyclic guanosine monophosphate ---------- cGMP
3', 5'-cyclic adenisine monophosphate ---------- cAMP
phosphodiesterase ---------- PDE
EHNA ---------- erythro-9-(2-hydroxy-3-nonyl) adenine
carboxymethylcellulose solution ---------- CMC
dimethyl sulfoxide ---------- DMSO

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Abstract

NOR-3, a nitric oxide (NO) donor, is known to increase HCO_3^- secretion in rat stomachs, intracellulary mediated by 3', 5'-cyclic guanosine monophosphate (cGMP), yet there is no information about the phosphodiesterase (PDE) isozyme involved in this process. We examined the effects of various isozyme-selective PDE inhibitors on the secretion of HCO_3^- in the mouse stomach *in vitro* and the type(s) of PDE isozymes involved in the response to NO. The gastric mucosa of DDY mice was stripped of the muscle layer and mounted on an Ussing chamber. HCO_3^- secretion was measured at pH 7.0 using a pH-stat method and by adding 2 mM HCl. NOR-3, 8-BrcGMP, and various PDE inhibitors were added to the serosal side. Vinpocetine (PDE1 inhibitor) or zaprinast (PDE5 inhibitor) was also added serosally 30 min before NOR-3 or 8-brcGMP. Both NOR-3 and 8-brcGMP stimulated HCO_3^- secretion in a dose- dependent manner, and the response to NOR-3 was significantly inhibited by methylene blue. Likewise, the secretion induced by NOR-3 or 8-brcGMP was significantly attenuated by ONO-8711, the EP1 antagonist, as well as indomethacin and potentiated by both vinpocetine and zaprinast at doses that had no effect by themselves on the basal secretion, while other subtype-selective PDE inhibitors had no effect. NOR-3 increased the mucosal PGE_2 content in a methylene blue-inhibitable manner. These results suggest that NO stimulates gastric HCO_3^- secretion mediated intracellularly by cGMP and modified by both PDE1 and PDE5, and this response is finally mediated by endogenous PGE_2 via the activation of EP1 receptors.

Introduction

The secretion of HCO_3^- from the surface epithelial cells is a key process that aids in preventing acid-peptic injury (Flemstrom and Garner, 1982; Flemstrom, 1987; Allen et al., 1993). The mechanisms that govern HCO_3^- secretion involve neuro- humoral factors and luminal acid (Flemstrom and Garner, 1982; Takeuchi et al., 1991; Montrose et al., 2005), yet both endogenous prostaglandins (PGs) and nitric oxide (NO) play a particularly important role in the local control of this secretion (Heylings et al., 1984; Takeuchi et al., 1986; Sugamoto et al., 2001, Aihara et al., 2005a). The stimulatory effect of PGE_2 on HCO_3^- secretion in the stomach is known to be mediated by the activation of EP1 receptors and coupled intracellularly with Ca^{2+} (Takeuchi et al., 1997a; 2005). On the other hand, NO stimulates the secretion of HCO_3^- in the duodenum via the activation of soluble guanylate cyclase and an increase in the intracellular level of 3', 5'-cyclic guanosine monophosphate (cGMP) (Moncada et al., 1991; Furukawa et al., 1999; Sugamoto et al., 2001). We further showed that NOR-3, a NO donor, increased the secretion of HCO_3^- in the rat stomach, via endogenous PGE_2 in a cGMP-dependent manner (Aihara et al., 2006). It is thus assumed that both PG/Ca^{2+} and NO/cGMP are involved in the local regulation of HCO_3^- secretion in the stomach. Because the HCO_3^- stimulatory action of NO is intracellularly mediated by cGMP and because cGMP is degraded into inactive metabolites via hydrolysis by phosphodiesterase (PDE), it is possible that PDE affects the response to NO by altering the levels of this nucleotide.

At present, the PDE in mammalian tissues has been subdivided into 11 isozymes, each derived from separate gene families and having pharmacologically distinct roles (Francis et al., 2001). PDE1 to PDE5 have been well characterized, and

selective inhibitors of these isozymes are used for the treatment of heart disease, depression, asthma, inflammatory disease, and erectile dysfunction (Thompson, 1991; Xu et al., 2000; Sung et al., 2003). To disclose the profiles of the subtype- selective PDE inhibitors would be helpful for eliminating the adverse influences of these agents in the body, including the gastrointestinal tract. We recently reported using the isolated mouse duodenum *in vitro* that the response of HCO_3^- to PGE_2 is regulated by both PDE1 and PDE3, while the response to NO is modulated by only PDE1 (Hayashi et al., 2007). It remains, however, unexplored which PDE isozyme(s) is involved in the regulation of gastric HCO_3^- secretion. The regulatory mechanism of HCO_3^- secretion differ in many points between the stomach and the duodenum; in the latter, the secretion is mediated intracellularly with both 3', 5'-cyclic adenosine monophosphate (cAMP), cGMP and Ca^{2+}, while that in the stomach is intracellularly mediated by Ca^{2+} and cGMP but not cAMP (Flemstrom, 1977; 1987; Guba et al., 1996; Seidler et al., 1997; Takeuchi et al., 1997b; Furukawa et al., 1999; Rao et al., 2004; Sellers et al., 2005). Thus, there is a possibility that the PDE isozymes involved in the HCO_3^- response are different in these tissues.

In the present study, we examined the effects of subtype-selective inhibitors of PDE1 to PDE5 on the secretion of HCO_3^- in response to NO as well as PGE_2 in the isolated mouse stomach *in vitro* and investigated which isozymes of PDE are involved in the local regulation of gastric HCO_3^- secretion. In addition, we also examined the interactive role of NO and PGE_2 in the stimulation of HCO_3^- secretion in the stomach.

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Methods

Animals

Male DDY mice weighing 25-30 g (SLC, Japan) were used in all experiments. The animals, kept in stainless steel cages with raised mesh bottoms, were deprived of food but allowed free access to tap water for 18 hr before the experiments. All experimental procedures used were carried out in accordance with the Helsinki Declaration and have been approved by the Committee for Animal Experimentation established by Kyoto Pharmaceutical University.

Determination of HCO_3^- Secretion

Under deep diethyl ether anesthesia, the mouse was killed and the abdomen opened by a midline incision. The entire stomach was removed and immediately placed in HCO_3^- Ringer's solution containing indomethacin (10^{-6} M) to suppress trauma-induced PG release. The stomach was opened along the greater curvature and stripped off the muscular layers under a microscope (SZ-PT; Olympus). The tissues (the corpus mucosa) were, then, mounted between two halves of a lucite chamber, the exposed area being 12.5 mm^2, and bathed in unbuffered saline (mmol/L: Na^+, 154; Cl^-, 154) gassed with 100% O_2 on the mucosal side and HCO_3^- Ringer's solution (mmol/L: Na^+, 140; Cl^-, 120; K^+, 5.4; Mg^{2+}, 1.2; Ca^{2+}, 1.2; HPO_4^{2-}, 1.4; $H_2PO_4^-$, 2.4; HCO_3^-, 25; glucose 10; indomethacin 0.001) gassed with 95% O_2-5% CO_2 on the serosal side (Seidler et al., 1997; Tuo et al., 2006; Hayashi et al., 2007), and these solutions were warmed at 37ºC and continuously circulated by a gas-lift system. The osmolalities for both solutions were approximately 308 mOsm/kg. The HCO_3^- secretion was measured by the pH-stat method (Comtite-980, Hiranuma industries, Ibaraki, Japan) using 2 mmol/L HCl as the titrant to keep the mucosal pH at 7.0. To unmask HCO_3^- secretion, acid secretion

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had been completely inhibited by prior i.p. administration of omeprazole at a dose of 60 mg/kg. Omeprazole at this dose has been shown to have no influence on gastric HCO_3^- secretion in rats (Flemstrom and Mattsson, 1986). Measurements were made every 5 min starting at least 1 hr after the mounting of the tissues. After the rate of secretion had stabilized for 45 min, the following agents were added to the serosal solution; PGE_2 (10^{-7}~10^{-6} M), NOR-3 (NO donor; 10^{-3}~$3x10^{-3}$ M), 8-bromoguanosine 3',5'-cyclic monophosphate (8-BrcGMP: cGMP analogue; 10^{-5}~10^{-3} M), dibutyryl 3', 5'-cyclic adenosine monophosphate (dbcAMP: 10^{-3} M), forskolin (adenylyl cyclase stimulator: $5x10^{-6}$ M), vinpocetine (PDE1 inhibitor; 10^{-6}~10^{-5} M), EHNA (PDE2 inhibitor; 10^{-5} M), cilostamide (PDE3 inhibitor; 10^{-5} M), rolipram (PDE4 inhibitor; 10^{-5} M), and zaprinast (PDE5 inhibitor; 10^{-6}~10^{-5} M). In some cases, the effects of vinpocetine and zaprinast on the response of HCO_3^- to PGE_2, NOR-3, and cGMP were examined; the PDE inhibitors were added 30 min before the latter agents. In addition, the effects of the following inhibitors or antagonists on the response to PGE_2, NOR-3, and 8-BrcGMP as well as the PDE inhibitors were also examined; methylene blue (guanylate cyclase inhibitor; $1x10^{-4}$ M), L-NAME (non-selective NOS inhibitor; 10^{-3} M), ONO-8711 (EP1 receptor antagonist; 10^{-5} M)(Takeuchi et al., 2006), AE5-599 (EP3 receptor antagonist; $3x10^{-6}$ M)(Aihara et al., 2007), AE3-208 (EP4 receptor antagonist; 10^{-6} M)(Aoi et al., 2004) or indomethacin (non-selective COX inhibitor; 10^{-5} M). These agents were added serosally 30 min before the agonists.

Determination of Nitrite and Nitrate (NOx) Levels

The amount of NO generated in the mucosa was determined indirectly as nitrite/nitrate (NO_2^- and NO_3^-) in the normal mouse stomach and after treatment with L-NAME. After the stomach was rinsed with cold saline, the mucosa was scraped with glass slides and homogenized in 50 mM $KHPO_4$ buffer. This was followed by

centrifugation for 10 min at 10,000 rpm at 4ºC. The supernatant's NOx levels were measured by the Griess reaction-dependent method described by Green et al. (1982), after the reduction of NO_3^- to NO_2^- with nitrate reductase. Nitrites were incubated with Griess reagent (0.1% naphthylene diamine dihydrochloride and 1% sulfanilamide in 2.5% H_3PO_4) for 10 min at room temperature, and the absorbance at 545 nm was measured. For the standard curve, sodium nitrite was used. L-NAME (10^{-3} M) was added to the serosal solution 1 hr before the measurement of NOx.

Measurement of Intracellular Levels of cGMP in Mouse Stomachs

The isolated mouse stomach (the corpus mucosa) mounted between two halves of a lucite chamber was treated with the serosal addition of NOR-3 ($3x10^{-3}$ M) with or without vinpocetine (10^{-6} M) or zaprinast (10^{-6} M) for 10 min. The latter agents were added serosally 30 min before NOR-3. Ten minutes after the addition of NOR-3, the tissue was homogenized in 2 ml of 5% trichloracetic acid in tissue homogenizer on dry ice (0~4ºC). The precipitate was removed by centrifugation at 1500 x g for 10 min, and the supernatant was transferred to a test tube. The supernatant was then extracted 4~5 times with 2 volume of ether, and then the ether fractions were collected and removed from the aqueous layer by heating the sample to 70ºC for 5 min. The levels of cGMP were measured using a cGMP enzyme immunoassay kit (Cayman Chemical Co., Ann Arbor, MI).

Analyses for Gene Expression of PDE Isozymes by RT-PCR

Mice were killed under deep ether anesthesia, and the stomachs were removed. Gastric tissue samples were pooled from 3 mice for extraction of total RNA, which was achieved by a single-step acid phenol-chloroform extraction procedure using TRIZOLE (GIBCO BRL, Gaithersburg, MD). Total RNA primed by random hexadeoxy ribonucleotide was reverse-transcribed with the SUPERSCRIPT pre-

amplification system (GIBCO BRL). The sequences of sense and antisense primers for mouse PDE1~5, including the splicing variants in PDE1, 3 and 4, are listed in **Table 1**. An aliquot of the RT reaction product served as a template in 35 cycles of PCR with 1 min of denaturation at 94ºC, 0.5 min of annealing at 58ºC and 1 min of extension at 72ºC on a thermal cycler. A portion of the PCR mixture was electrophoresed in 1.8% agarose gel in TAE buffer (Tris buffer 40 mM, EDTA 2 mM and acetic acid 20 mM; pH 8.1), and the gel was stained with ethidium bromide and photographed.

Preparation of Drugs

Drugs used were prostaglandin E_2 (PGE_2: Funakoshi, Tokyo, Japan), NOR-3 ((±)-(E)-Ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexeneamine: Doujindo, Tokyo, Japan), dibutyryl adenosine 3',5'-cyclic monophosphate (dbcAMP), 8-bromoguanosine 3',5'-cyclic monophosphate (8-brcGMP), isobutylmethylxanthine (IBMX), vinpocetine, EHNA ((erythro-9-(2-Hydroxy-3-nonyl)-adenine hydrochloride), cilostamide, rolipram, zaprinast (Aldrich, Milwaukee, WI), indomethacin, N^G-nitro-L-arginine methylester (L-NAME)(Sigma Chemicals, St. Louis, MO), methylene blue, forskolin (Nacalai Tesque, Kyoto, Japan), ONO-8711, AE5-599, AE3-208 (Ono Pharmaceutical Co., Osaka, Japan) and omeprazole (Astra Zeneca, Möndal, Sweden). Omeprazole was suspended in 0.5% carboxymethylcellulose solution (CMC: Osaka, Japan), while other agents were dissolved in dimethyl sulfoxide (DMSO: Wako) and diluted with distilled water to desired concentrations. All agents were prepared before use and added to the nutrient solution.

Statistical Analysis

Data are expressed as the mean±SE for 4~6 mice. Statistical analyses were performed with a one-way analysis of variance (ANOVA) followed by the Dunnett multiple comparison test or, when appropriate, Student-*t* tests, and values of $P< 0.05$

were considered significant.

Results

Effects of NOR-3 and PGE_2 on Gastric HCO_3^- Secretion

Under inhibition of acid secretion caused by pretreatment with omeprazole, the isolated mouse stomach consistently secreted HCO_3^- at rates of 0.15~0.2 μEq/hr as basal secretion in the absence or presence of 0.1% DMSO, a solvent for the agents used. The isolated mouse stomach responded to the serosal addition of NOR-3 (10^{-3} and $3x10^{-3}$ M) with an increase of HCO_3^- secretion in a concentration-dependent manner, and the effect at $3x10^{-3}$ M reached a maximal level of about 2 times greater than basal values, the $\bullet HCO_3^-$ output being 0.14±0.03 μEq/hr **(Figure 1)**. This secretion induced by NOR-3 ($3x10^{-3}$ M) was totally attenuated by prior addition of methylene blue (10^{-4} M), the inhibitor of soluble guanylate cyclase, the inhibition being 87.9%. Furthermore, this response was significantly mitigated by indomethacin (10^{-5} M), a cyclooxygenase (COX) inhibitor, or the EP1 antagonist, ONO-8711 (10^{-5} M), the inhibition being 71.4% or 61.2%, respectively.

Likewise, the application of PGE_2 (10^{-7}~10^{-6} M) caused a gradual increase of HCO_3^- secretion in a concentration-dependent manner, the $\bullet HCO_3^-$ output at 10^{-6} M being 0.15±0.01 μEq/hr **(Figure 2)**. The HCO_3^- stimulatory effect of PGE_2 (10^{-6} M) was all but totally attenuated by pretreatment of the tissue with the EP1 antagonist ONO-8711 (10^{-5} M), the $\bullet HCO_3^-$ output being 0.012±0.019 μEq/hr, which is almost equivalent to that observed in normal animals without PGE_2 treatment. However, neither indomethacin, AE5-599 (EP3 antagonist) nor AE3-208 (EP4 antagonist) had any effect on the response to PGE_2.

The serosal application of 8-brcGMP (10^{-3} M: N=6) significantly increased the rate of HCO_3^- secretion in the isolated mouse stomach, the $\bullet HCO_3^-$ output being

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0.11±0.03 μEq / hr. By contrast, neither dbcAMP (10^{-3} M: N=5) nor forskolin ($5x10^{-6}$ M: N=5) had any effect on the secretion, the $\bullet HCO_3^-$ output being 0.021±0.015 μEq / hr and -0.021±0.024 μEq / hr, respectively, both of which remained in the range observed in control stomachs.

Effects of IBMX on Gastric HCO_3^- Stimulatory Action of NOR-3 or PGE_2

The non-selective PDE inhibitor IBMX (10^{-5} and 10^{-4} M) dose-dependently increased HCO_3^- secretion in the isolated mouse stomach, and the effect at 10^{-4} M was significant, the $\bullet HCO_3^-$ output being 0.085±0.016 μEq / hr **(Figure 3)**. This agent at 10^{-5} M did not by itself increase HCO_3^- secretion, yet markedly potentiated the response to NOR-3 at 10^{-3} M; the $\bullet HCO_3^-$ output increased to approximately 6 times greater than that of the corresponding control **(Figure 4A)**. However, the response of HCO_3^- to PGE_2 (10^{-7} M) was not affected by IBMX **(Figure 4B)**. Neither PGE_2 (10^{-7} M) nor NOR-3 (10^{-3} M) by itself significantly stimulated HCO_3^- secretion in the isolated mouse stomach.

Effects of Various PDE Inhibitors on Gastric HCO_3^- Secretion

The nonselective PDE inhibitor IBMX significantly increased HCO_3^- secretion and potentiated the stimulatory action of NOR-3 in the mouse stomach. To determine which PDE isozyme(s) are involved in the regulation of gastric HCO_3^- secretion, we examined the effects of various PDE inhibitors on the response induced by NOR-3.

Among the subtype-selective PDE inhibitors examined, both the selective PDE1 inhibitor vinpocetine and the selective PDE5 inhibitor zaprinast by themselves significantly increased the basal rate of HCO_3^- secretion, the $\bullet HCO_3^-$ output at a dose

of 10^{-5} M being 0.10±0.01 μEq/hr and 0.09±0.02 μEq/hr, respectively **(Figure 5)**. Neither EHNA (the selective PDE2 inhibitor), cilostamide (the selective PDE3 inhibitor) nor rolipram (the selective PDE4 inhibitor) at 10^{-5} M had any effect on HCO_3^- secretion in the stomach**.**

To further investigate the involvement of endogenous PGs and NO in these responses, we examined the effects of indomethacin and L-NAME on the HCO_3^- stimulatory action of these PDE inhibitors at 10^{-5} M. Both vinpocetine and zaprinast at 10^{-6} and 10^{-5} M increased the basal rate of HCO_3^- secretion in a concentration-dependent manner **(Figures 6 and 7)**. The response to vinpocetine (10^{-5} M) was significantly abrogated by the co-treatment with indomethacin (10^{-5} M) or L-NAME (10^{-3} M), the inhibition being 90.1% and 88.3%, respectively **(Figure 6)**. Likewise, the response of HCO_3^- to zaprinast (10^{-5} M) was also significantly attenuated in the presence of indomethacin or L-NAME, the inhibition being almost complete, 95.3% and 95.0%, respectively **(Figure 7)**. The HCO_3^- output caused by vinpocetine or zaprinast in the presence of L-NAME or indomethacin was almost equivalent to that obtained in control stomachs treated with vehicle alone (DMSO).

Effect of Various PDE Inhibitors on Gastric Stimulatory Action of NOR-3

The non-selective PDE inhibitor IBMX significantly potentiated the stimulatory effect of NOR-3 on HCO_3^- secretion. In addition, both vinpocetine and zaprinast were found to significantly stimulate the basal secretion. Then, we further examined the effects of various PDE inhibitors on the response to NOR-3 in order to determine which PDE isozyme(s) are responsible for this phenomenon.

As shown in **Figure 8**, the response of HCO_3^- to NOR-3 was significantly increased by both vinpocetine and zaprinast at a dose (10^{-6} M) that did not by itself

affect the basal secretion in the mouse stomach. The $\bullet HCO_3^-$ output induced by NOR-3 in the presence of vinpoccetine or zaprinast was 0.140±0.005 μEq/hr and 0.143±0.010 μEq/hr, respectively, both of which are about 4 times greater than the output induced by NOR-3 alone (0.034±0.008 μEq/hr). Neither EHNA, ciostamide nor rolipram, at even 10^{-5} M, had any effect on the response induced by NOR-3. On the other hand, the response of HCO_3^- to PGE_2 was not significantly affected by pretreatment with either of these PDE inhibitors **(Figure 9)**.

Effect of 8-BrcGMP on Gastric HCO_3^- Secretion

It was demonstrated in this study that NOR-3 stimulated HCO_3^- secretion in the mouse stomach via cGMP and that the process was, at least in part, mediated by endogenous PGs. Furthermore, the response to NOR-3 was potentiated by PDE inhibitors through intracellular accumulation of cGMP. In addition, we also found that both the PDE1 inhibitor vinpocetine and the PDE5 inhibitor zaprinast by themselves stimulated the secretion mediated by endogenous NO and PGs. To further substantiate these points, we repeated the same experiments examining the effect of 8-BrcGMP on HCO_3^- secretion in the isolated mouse stomach.

The serosal application of 8-brcGMP (10^{-5}~10^{-3} M) increased HCO_3^- secretion in a concentration-dependent manner, and the $\bullet HCO_3^-$ output at 10^{-3} M was 0.10±0.03 μEq/hr, almost equivalent to that induced by NOR-3 at $3x10^{-3}$ M **(Figure 10)**. This response was significantly mitigated by co-addition of either ONO-8711 (10^{-5} M) or indomethacin (10^{-5} M), the inhibition being 78.2% and 70.0%, respectively. On the other hand, the response to 8-brcGMP (10^{-5} M) was markedly increased by both vinpocetine and zaprinast at 10^{-6} M, a dose that did not by itself affect the HCO_3^- secretion **(Figure 11)**. Although 8-brcGMP alone at 10^{-5} M did not stimulate HCO_3^-

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secretion, this agent in the presence of the above PDE inhibitors significantly increased the secretion to 5~7 times greater than the control, the $\bullet HCO_3^-$ output being 0.068±0.014 μEq/hr and 0.085±0.023 μEq/hr, respectively.

Effects of L-NAME on NOx Levels in Mouse Stomachs

The concentration of NOx in the normal stomach was 421.3±12.0 μM/g tissue. When the stomach was incubated for 2 hr with L-NAME (10^{-3} M), the NOx content significantly decreased to 249.0±7.2 μM/g tissue, approximately 59.1% of control values. The addition of DMSO as a vehicle had no effect on NOx levels in the stomach, the value being 422.5±23.2 μM/g tissue.

Intracellular Generation of cGMP in Mouse Stomachs

Levels of cGMP in the isolated mouse stomachs were 0.34±0.14 pmol/g tissue (N=4) under basal conditions and significantly increased to the values of 1.65±0.32 pmol/g tissue (N=5) after the serosal addition of NOR-3 ($3x10^{-3}$ M). This response to NOR-3 was further enhanced by the co-addition of the PDE1 inhibitor vinpocetine or the PDE5 inhibitor zaprinast at 10^{-6} M, the values being 3.4±1.2 pmol/g tissue (N=5) or 4.2±1.0 pmol/g tissue (N=5), respectively, both of which are significantly greater than those obtained by NOR-3 alone. Neither vinpocetine nor zaprinast by itself at the dose used had any effect on intracellular cGMP contents of mouse stomachs (data not shown).

Gene Expressions of PDE Isozymes in Mouse Stomachs

Since it was found that both PDE1 and PDE5 were involved in the regulation of gastric HCO_3^- secretion, we examined the gene expression of PDE isozymes

(PDE1~5), including the splicing variants of PDE1, 3 and 4, in the mouse stomach. As shown in **Figure 12**, the expression of all PDE isozymes (PDE1~PDE5) was observed in the entire gastric mucosa, although some differences were observed in the intensity of their expressions.

Discussion

The secretion of HCO_3^- from the surface epithelium is one of the mucosal defensive mechanisms and plays an important role in protecting the gastroduodenal mucosa against acid (Flemstrom and Garner, 1982; Flemstrom, 1987; Allen et al., 1993; Montrose et al., 2005). This secretion is controlled by various neuro-humoral factors as well as luminal acid (Flemstrom, 1987; Takeuchi et al., 1991), endogenous PGs and NO being particularly important in its local regulation (Heylings et al., 1984; Sugamoto et al., 2001; Takeuchi et al., 1999; Aihara et al., 2005a). In the present study, we confirmed in the isolated mouse stomach *in vitro* that PGE_2 stimulates HCO_3^- secretion through EP1 receptors and that NO also increases the secretion in a cGMP-dependent manner, though the action of NO is mediated, in large part, by endogenous PGs through EP1 receptors. Furthermore, we demonstrated for the first time that both PDE1 and PDE5 are involved in the regulation of gastric HCO_3^- secretion by modifying the response to NO.

We previously reported that PGE_2 stimulates HCO_3^- secretion via different EP receptor subtypes in the stomach and duodenum; the former process is mediated by EP1 receptors and coupled with Ca^{2+}, while the latter is mediated by EP3/EP4 receptors and coupled with both cAMP and Ca^{2+} (Takeuchi et al., 1997a; 1999; 2006; Aihara et al., 2007). Consistent with previous findings *in vivo* (Takeuchi et al., 1997a; 2006), the present study showed that the PGE_2-stimulated HCO_3^- secretion in the isolated mouse stomach was inhibited only by the selective EP1 antagonist ONO-8711 but not the EP3 or EP4 antagonist, confirming the involvement of EP1 receptors in this response.

NO also contributes to the regulation of gastroduodenal HCO_3^- secretion

(Guba et al., 1996; Aihara et al., 2005a; 2005b). We previously reported that the acid-induced secretion of HCO_3^- in the stomach was inhibited by the prior administration of L-NAME, an inhibitor of NO production as well as indomethacin (Aihara et al., 2005b; 2006), suggesting the involvement of endogenous NO in the response, in addition to PGs. Indeed, HCO_3^- secretion was stimulated by the NO donor NOR-3 in the rat stomach and duodenum, and these actions were significantly attenuated by indomethacin (Sugamoto et al., 2001; Aihara et al., 2005a; 2006). In the present study, we showed that the response to NOR-3 in the mouse stomach was significantly attenuated by the prior addition of methylene blue, an inhibitor of guanylate cyclase as well as indomethacin, suggesting the mediation of this response locally by PGs and intracellularly by cGMP. This contention was further supported by the finding that 8-brcGMP, a membrane permeant analogue of cGMP, stimulated gastric HCO_3^- secretion in the indomethacin- and EP1 antagonist-inhibitable manners. Several studies have shown that NO stimulates PG production in various organs and cells (Uno et al., 1997; Furukawa et al., 1999; 2000; Watkins et al., 1997). Uno et al. (1997) reported that *S*-nitroso-*N*-acetyl penicillamine, a NO donor, stimulates PGE_2 production in gastric epithelial cells. Watkins et al. (1997) showed that cGMP, either alone or in combination with SNAP or interleukin 1β increased the production of PGE_2 in airway epithelial cell line (A549) through activation of COX. Furukawa et al. (1999; 2000) showed that the secretion of HCO_3^- in isolated duodenum *in vitro* was stimulated by NOR-3, at least partly, via production of PGE_2 through activation of COX-1. We also showed that NOR-3 also increased increased PGE_2 production in the rat stomach, resulting in stimulation of HCO_3^- secretion (Aihara et al., 2005b). The levels of cGMP in the isolated mouse stomach was also significantly increased by NOR-3. These all results together with the present findings strongly suggest that NO

stimulates gastric HCO_3^- secretion mediated intracellulaly by cGMP through the activation of guanylate cyclase. Furthermore, it is also assumed that the stimulatory action of NO in the mouse stomach is partially mediated by endogenous PGs via EP1 receptors.

Since PDE inactivates both cAMP and cGMP by converting them into 5'-AMP and 5'-GMP, respectively, the physiological responses mediated by these nucleotides are expected to be augmented by inhibitors of PDE. PDE is genetically subdivided into 11 isozymes, five of which, PDE1 to PDE5, have been well characterized pharmacologically (Thompson, 1991). PDE1 is activated by Ca^{2+}/ calmodulin and PDE2 by cGMP, yet both of them catalyze the conversion of cAMP and cGMP into inactive metabolites (Francis et al., 2001). By contrast, both PDE3 and PDE4 selectively bind to cAMP as the substrate, while PDE5 catalyzes cGMP's conversion to 5'GMP (Xu et al., 2000; Sung et al, 2003). In general, the fundamental properties of PDE isozymes are well preserved among species. We recently demonstrated in isolated mouse duodenum *in vitro* that the response of HCO_3^- to PGE_2 is regulated by both PDE1 and PDE3, while the response to NO is modulated by only PDE1 (Hayashi et al., 2007). We also examined in this study the gene expression of PDE isozymes (PDE1~PDE5), including their splicing variants, in the mouse stomach and confirmed that they all clearly expressed in this tissue. At present, since reliable anti-PDE antibodies are not available, it remains unknown which cell type expresses each PDE isozyme.

Because the stimulatory action of NO in the stomach is intracellularly mediated by cGMP and because this nucleotide is degraded into inactive metabolites via hydrolysis by PDE, it is possible that PDE affects the response of HCO_3^- to NO by altering the levels of this nucleotide. PGE_2 stimulates duodenal HCO_3^- secretion

intracellularly mediated by Ca^{2+}/cAMP via the activation of EP3/EP4 receptors (Takeuchi et al., 1997a; Aoi et al., 2004; Aihara et al., 2007). Although the secretion in the stomach is also stimulated by PGE_2, the action is intracellularly mediated by Ca^{2+} but not by cAMP. Indeed, neither forskolin, the adenylate cyclase stimulator, nor a pituitary adenylate cyclase activating polypeptide had any effect on HCO_3^- secretion in the stomach (Takeuchi et al., 1997b), some of them being confirmed in the present study, though these agents potently stimulate duodenal HCO_3^- secretion. Thus, there is no doubt that cGMP, but not cAMP, plays a role in the local regulation of gastric HCO_3^- secretion.

We previously reported that IBMX, a nonselective PDE inhibitor, markedly increased duodenal HCO_3^- secretion induced by not only PGE_2 but also NOR-3 (Takeuchi et al., 1997a; Hayashi et al., 2007). Likewise, the duodenal response to PGE_2 was also enhanced by vinpocetine, the PDE1 inhibitor, and cilostamide, the PDE3 inhibitor, while the response to NOR-3 was potentiated only by vinpocetine (Hayashi et al., 2007). In the present study, we observed that IBMX increased by itself HCO_3^- secretion and significantly potentiated the response to NOR-3 as well as 8-brcGMP in the isolated mouse stomach. The same effects were obtained with both the PDE1 inhibitor vinpocetine and the PDE5 inhibitor zaprinast, suggesting the involvement of different PDE isozymes in the regulatory mechanism of HCO_3^- secretion in the stomach and duodenum. Certainly, none of the PDE inhibitors tested, including IBMX, significantly affected the response of HCO_3^- to PGE_2 in the stomach. These results would be reasonable because the PGE_2-induced response in the stomach is intracellularly mediated by Ca^{2+} but not cyclic nucleotide (Takeuchi et al., 1997a; 2005). These results suggest that both PDE1 and PDE5 are involved in the local regulation of HCO_3^- secretion in the mouse stomach through modulation of the NO/cGMP system.

This idea was supported by the findings that the intracellular generation of cGMP in response to NOR-3 was markedly increased by the co- addition of PDE1 and PDE5 inhibitors.

It should be noted in the present study that IBMX, vinpocetine, and zaprinast at higher doses increased by themselves the rate of HCO_3^- secretion in the isolated mouse stomach. These responses were significantly mitigated by the co-addition of L-NAME or indomethacin. It is assumed that these PDE inhibitors increase the accumulation of cGMP produced by endogenously generated NO in the stomach. Indeed, we observed that the isolated stomach generated NO under basal conditions without any treatment and that the production was significantly reduced by the serosal addition of L-NAME. Consistent with our previous findings (Aihara et al., 2005b), we further observed in this study that NOR-3 stimulated HCO_3^- secretion in the stomach, partly mediated by endogenous PGs and through the activation of EP1 receptors. Thus, it is assumed that the inhibition of PDE1 and PDE5 first increases intracellular levels of cGMP and then stimulates gastric HCO_3^- secretion in two ways, directly via cGMP and indirectly mediated by PGE_2/EP1 receptors.

Given the findings in the present study, we concluded that NO stimulates gastric HCO_3^- secretion essentially mediated intracellularly by cGMP and modified by both PDE1 and PDE5, and this response is, in large part, mediated by endogenous PGE_2 via the activation of EP1 receptors **(Figure 13)**. In addition, since NO promotes the secretion of HCO_3^- via endogenous PGE_2, it is possible that both PG/Ca^{2+} and NO/cGMP are involved in the local regulation of gastric HCO_3^- secretion.

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Footnotes

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Legends for Figures

Figure 1. Effect of NOR-3 on HCO_3^- secretion in isolated mouse stomachs. NOR-3 (10^{-3}~$3x10^{-3}$ M) was added to the serosal solution. Methylene blue (10^{-4} M), indomethacin (10^{-5} M) or ONO-8711 (10^{-5} M) was added to the serosal solution 30 min before NOR-3 ($3x10^{-3}$ M). Data show the total net HCO_3^- output for 1 hr after addition of DMSO or NOR-3 and are presented as the mean±SE from 4-6 mice. Significant difference at $P<0.05$; *from DMSO; #from Control (NOR-3 alone at $3x10^{-3}$ M).

Figure 2. Effect of PGE_2 on HCO_3^- secretion in isolated mouse stomachs. PGE_2 (10^{-7}~10^{-6} M) was added to the serosal solution. ONO-8711 (10^{-5} M), AE5-599 ($3x10^{-6}$ M), AE3-208 (10^{-6} M) or indomethacin (10^{-5} M) was added to the serosal solution 30 min before PGE_2 (10^{-6} M). Data show the total net HCO_3^- output for 1 hr after addition of DMSO or PGE_2 and are presented as the mean±SE from 4-6 mice. Significant difference at $P<0.05$; *from DMSO; #from Control (PGE_2 at 10^{-6} M alone).

Figure 3. Effect of IBMX on HCO_3^- secretion in isolated mouse stomachs. IBMX (10^{-5}~10^{-4} M) was added to the serosal solution. **A:** Data are presented as the mean±SE of values determined every 10 min from 4-6 mice. **B:** Data show the total net HCO_3^- output for 1 hr after addition of IBMX and are presented as the mean±SE from 4-6 mice. *Significant difference from DMSO in the corresponding group, at $P<0.05$.

Figure 4. Effect of IBMX on the stimulatory action of NOR-3 (A) and PGE_2 (B) in isolated mouse stomachs. NOR-3 (10^{-3} M) and PGE_2 (10^{-7} M) were added to the serosal solution. IBMX (10^{-5} M) was added to the serosal solution 30 min before NOR-3 or

PGE_2. Data show the total net HCO_3^- output for 1 hr after addition of IBMX or NOR-3 or PGE_2 and are presented as the mean±SE from 4-6 mice. Significant difference at $P<0.05$; *from DMSO; #from NOR-3 alone (10^{-3} M). N.S.: Not Significant.

Figure 5. Effects of various subtype-selective PDE inhibitors on HCO_3^- secretion in isolated mouse stomachs. Vinpocetine (10^{-5} M), EHNA (10^{-5} M), cilostamide (10^{-5} M), rolipram (10^{-5} M) or zaprinast (10^{-5} M) was added to the serosal solution. Data show the total net HCO_3^- output for 1 hr after addition of each agent and are presented as the mean±SE from 4-6 mice. *Significant difference from DMSO in the corresponding group at $P<0.05$.

Figure 6. Effect of vinpocetine on HCO_3^- secretion in isolated mouse stomachs. Vinpocetine (10^{-6} and 10^{-5} M) was added to the serosal solution. L-NAME (10^{-3} M) or indomethacin (10^{-5} M) was added 30 min before vinpocetine (10^{-5} M). **A:** Data are presented as the mean±SE of values determined every 10 min from 4-6 mice. **B:** Data show the total net HCO_3^- output for 1 hr after addition of vinpocetine and are presented as the mean±SE from 4-6 mice. Significant difference at $P<0.05$; *from DMSO; #from vinpocetine (10^{-5} M) alone.

Figure 7. Effect of zaprinast on HCO_3^- secretion in isolated mouse stomachs. Zaprinast (10^{-6} and 10^{-5} M) was added to the serosal solution. L-NAME (10^{-3} M) or indomethacin (10^{-5} M) was added 30 min before zaprinast (10^{-5} M). **A:** Data are presented as the mean±SE of values determined every 10 min from 4-6 mice. **B:** Data show the total net HCO_3^- output for 1 hr after addition of zaprinast and are presented as the mean±SE from 4-6 mice. Significant difference at $P<0.05$; *from DMSO; #from zaprinast (10^{-5} M)

alone.

Figure 8. Effects of various subtype-selective PDE inhibitors on the stimulatory action of NOR-3 in isolated mouse stomachs. NOR-3 (10^{-3} M) was added to the serosal solution. Vinpocetine (10^{-6} M), EHNA (10^{-5} M), cilostamide (10^{-5} M), rolipram (10^{-5} M), or zaprinast (10^{-6} M) was added to the serosal solution 30 min before NOR-3. Data show the total net HCO_3^- output for 1 hr after application of NOR-3 and are presented as the mean±SE from 4-6 mice. Significant difference at $P<0.05$, *from DMSO, #from Control.

Figure 9. Effects of various subtype-selective PDE inhibitors on the stimulatory action of PGE_2 in isolated mouse stomachs. PGE_2 (10^{-7} M) was added to the serosal solution. Vinpocetine (10^{-6} M), EHNA (10^{-5} M), cilostamide (10^{-5} M), rolipram (10^{-5} M), or zaprinast (10^{-6} M) was added to the serosal solution 30 min before PGE_2. Data show the total net HCO_3^- output for 1 hr after application of PGE_2 and are presented as the mean±SE from 4~6 mice. N.S.: Not Significant.

Figure 10. Effect of 8-BrcGMP on HCO_3^- secretion in isolated mouse stomachs. 8-BrcGMP (10^{-5}~10^{-3} M) was added to the serosal solution. ONO-8711 (10^{-5} M) or indomethacin (10^{-5} M) was added to the serosal solution 30 min before 8-BrcGMP. Data show the total net HCO_3^- output for 1 hr after addition of 8-Br-cGMP and are presented as the mean±SE from 4-6. Significant difference at $P<0.05$; *from DMSO; # from Control (8-BrcGMP alone at 10^{-3} M).

Figure 11. Effects of vinpocetine and zaprinast on the stimulatory action of 8-BrcGMP

in isolated mouse stomachs. 8-Br-cGMP (10^{-5} M) was added to the serosal solution. Vinpocetine (10^{-6} M) and zaprinast (10^{-6} M) were added to the serosal solution 30 min before 8-BrcGMP. **A:** Data are presented as the mean±SE of values determined every 10 min from 4-6 mice. **B:** Data show the total net HCO_3^- output for 1 hr after addition of 8-Br-cGMP and are presented as the mean±SE from 4-6 mice. Significant difference at $P<0.05$; *from DMSO; #from Control (8-BrcGMP alone at 10^{-3} M).

Figure 12. Gene expressions of PDE1~5 in the mouse stomach. M: marker; 1A~1C: PDE1A~1C; 3A and 3B: PDE3A and 3B; 4A~4D: PDE4A~4D

Figure 13. The interactive roles for NO, PGE_2 and PDEs in the regulation of gastric HCO_3^- secretion. NO stimulates gastric HCO_3^- secretion essentially mediated intracellularly by cGMP and modified by both PDE1 and PDE5, and this response is mediated by endogenous PGE_2 via the activation of EP1 receptors. GC: guanylate cyclase, COX; cyclooxygenase.

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Table 1.

Sequences of Sense and Antisense Primers for Mouse PDE1~PDE5

Gene		Sequences	Product size (bp)
PDE1A	Sense	5'-GGGAAACTACAGTGCCATCTTC-3'	549
	Anti sense	5'-CGCAATCCCTGAACTGTATGTA-3'	
PDE1B	Sense	5'-CTCATCCGACCAATGTCTGTAA-3'	356
	Anti sense	5'-TACAAGAGAGGAGGAGGCAGTC-3'	
PDE1C	Sense	5'-CCTGAGCAGATCGAGAAAATCT-3'	378
	Anti sense	5'-CCGTCTGTACATTCTTTCCACA-3'	
PDE2A	Sense	5'-ACCTGGATGATGTCTCTGTCCT-3'	548
	Anti sense	5'-ATCGTGGAGAAGCTTGGTGTAT-3'	
PDE3A	Sense	5'-ACTCCGATTCTGACAGTGGATT-3'	669
	Anti sense	5'-CCACGATGTGTGAGATAAAGGA-3'	
PDE3B	Sense	5'-TAGTCCCTTCATGGATCGTTCT-3'	660
	Anti sense	5'-GAGTCAACTGCAGCAAGCTCTA-3'	
PDE4A	Sense	5'-CAACACGTTCCTAGACAAGCAG-3'	620
	Anti sense	5'-TCTCAAGCACAGACTCATCGTT-3'	
PDE4B	Sense	5'-ATGAGGATCATCTAGCCAAGGA-3'	575
	Anti sense	5'-GAGGCTCATGTGTTTGGACATA-3'	
PDE4C	Sense	5'-AACCAGGTGTCGGAGTACATCT-3'	378
	Anti sense	5'-GTGGTAGTGCCCCTCTAGTGTC-3'	
PDE4D	Sense	5'-TGATGTACTCTCTCGGAGCAAA-3'	388
	Anti sense	5'-GTCAGATCGGTACAGGAAGGAC-3'	
PDE5E	Sense	5'-GATCGCTATACCCTGTTCCTTG-3'	624
	Anti sense	5'-TTCCTCACACTCCATGTGAAAC-3'	

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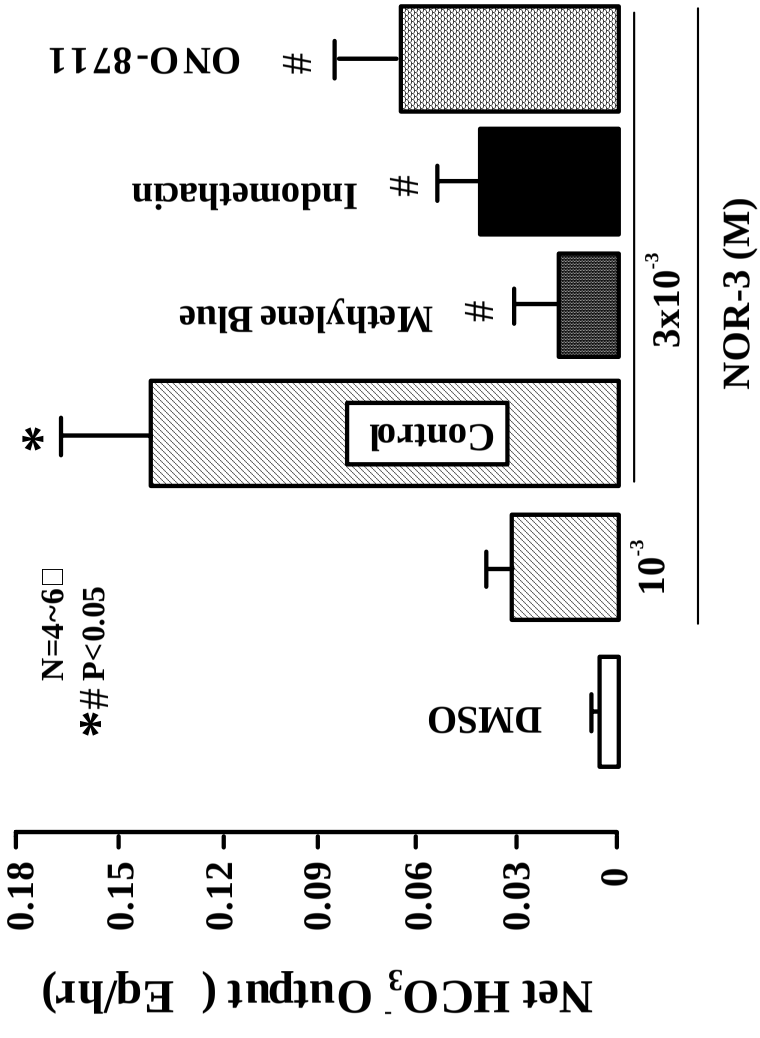

Figure 1

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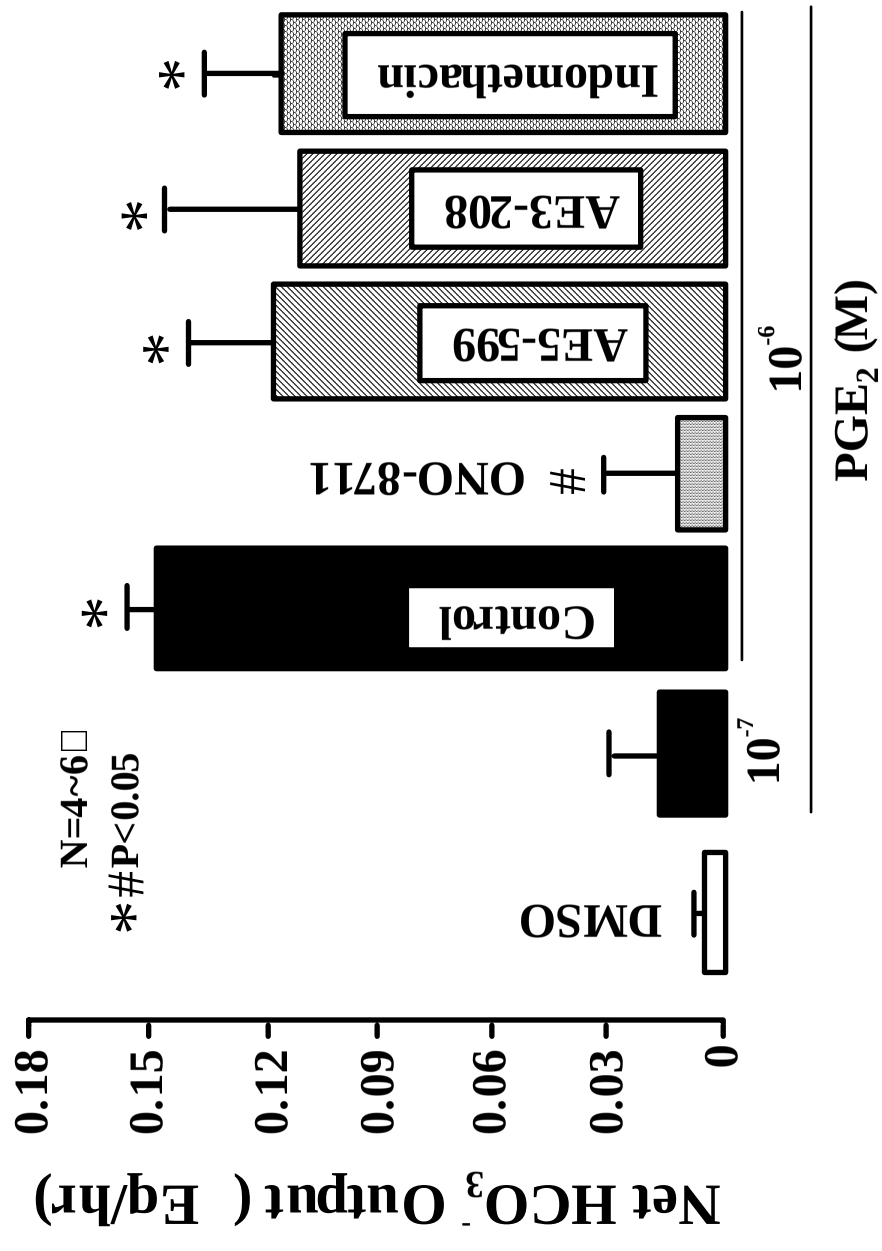

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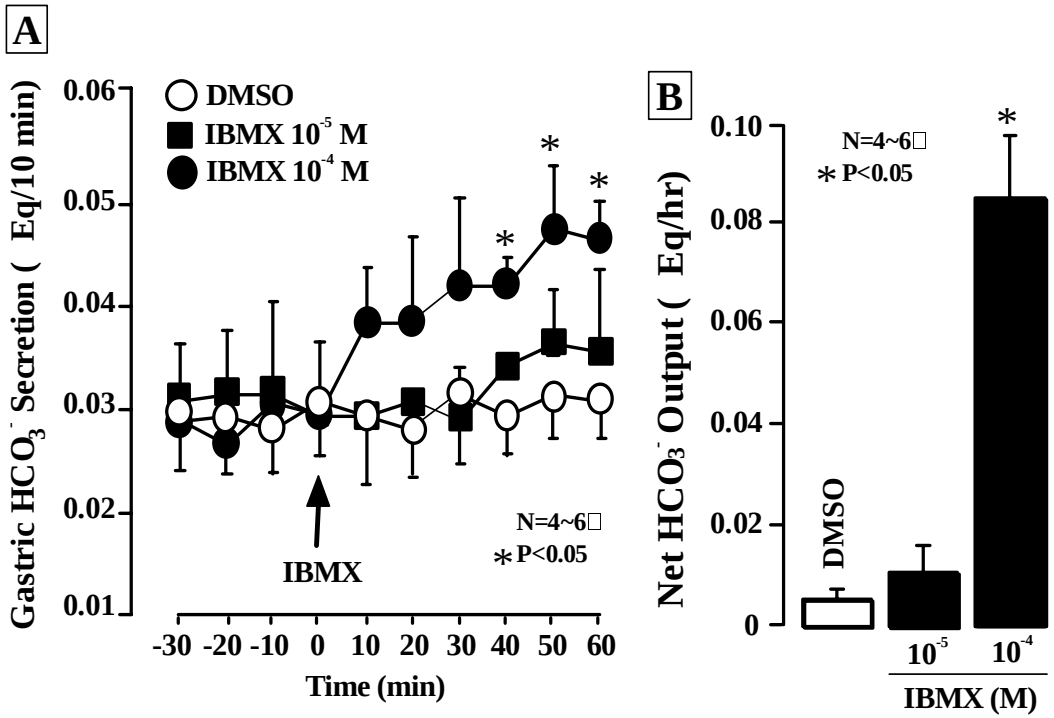

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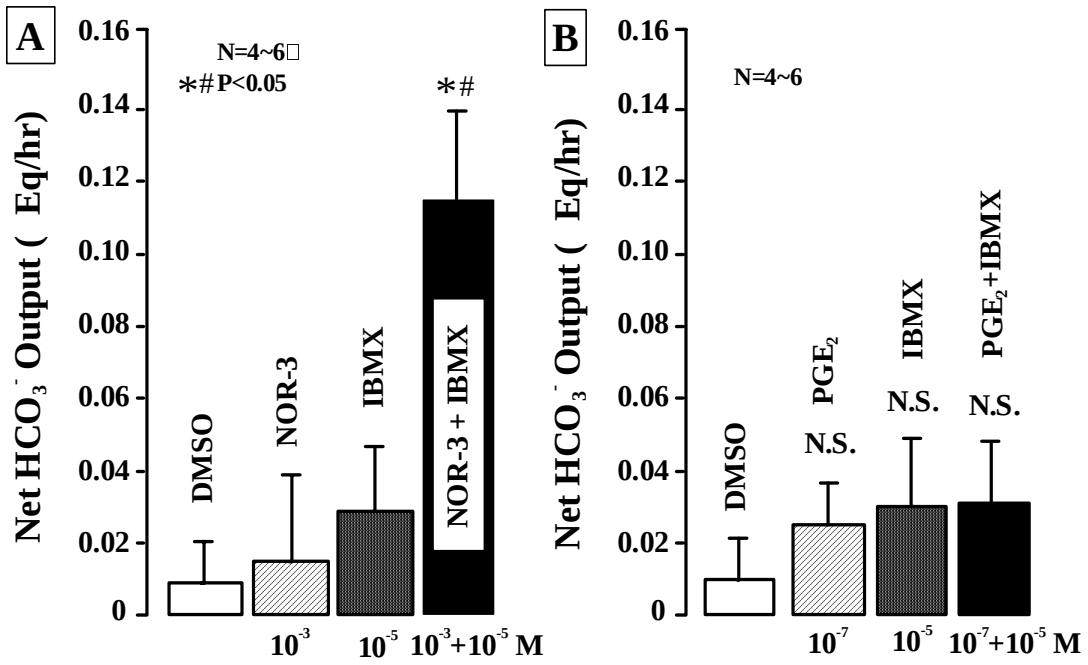

Figure 4

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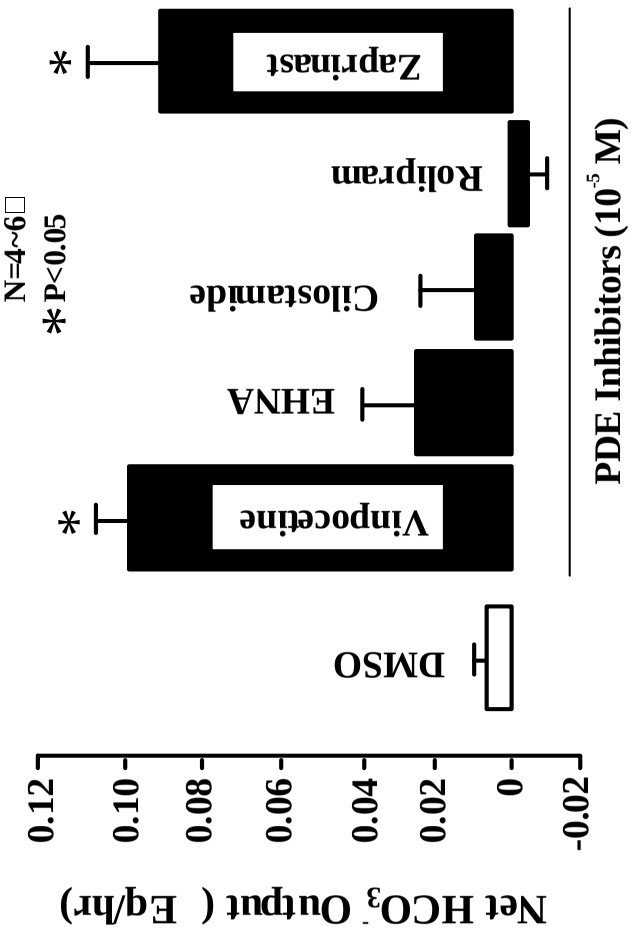

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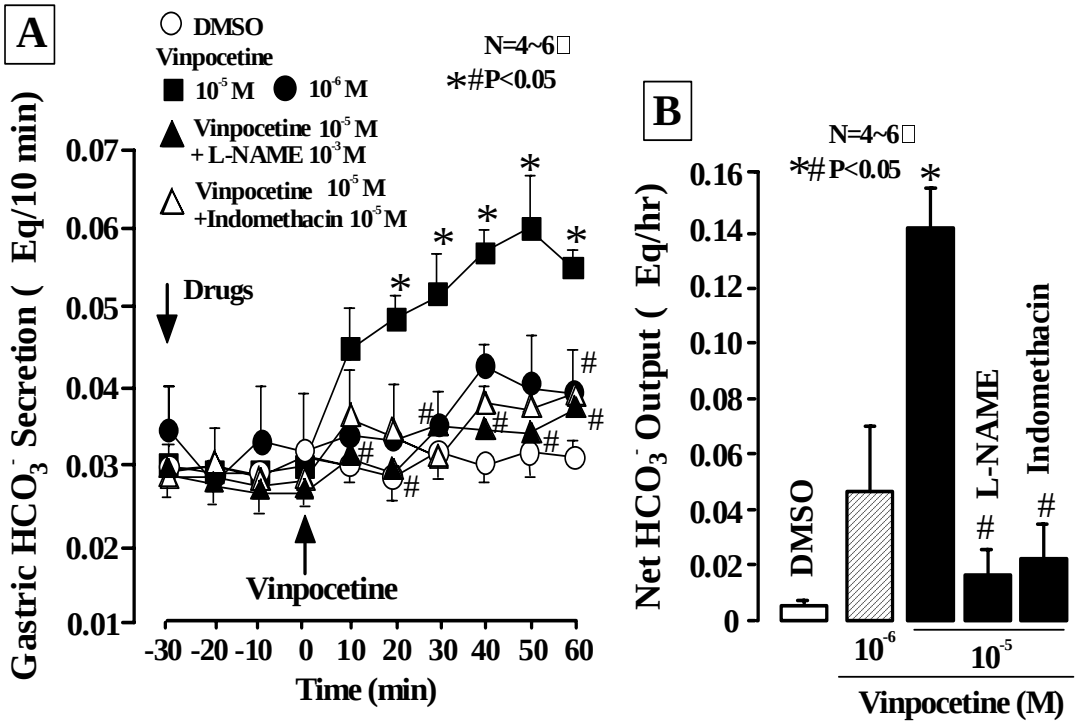

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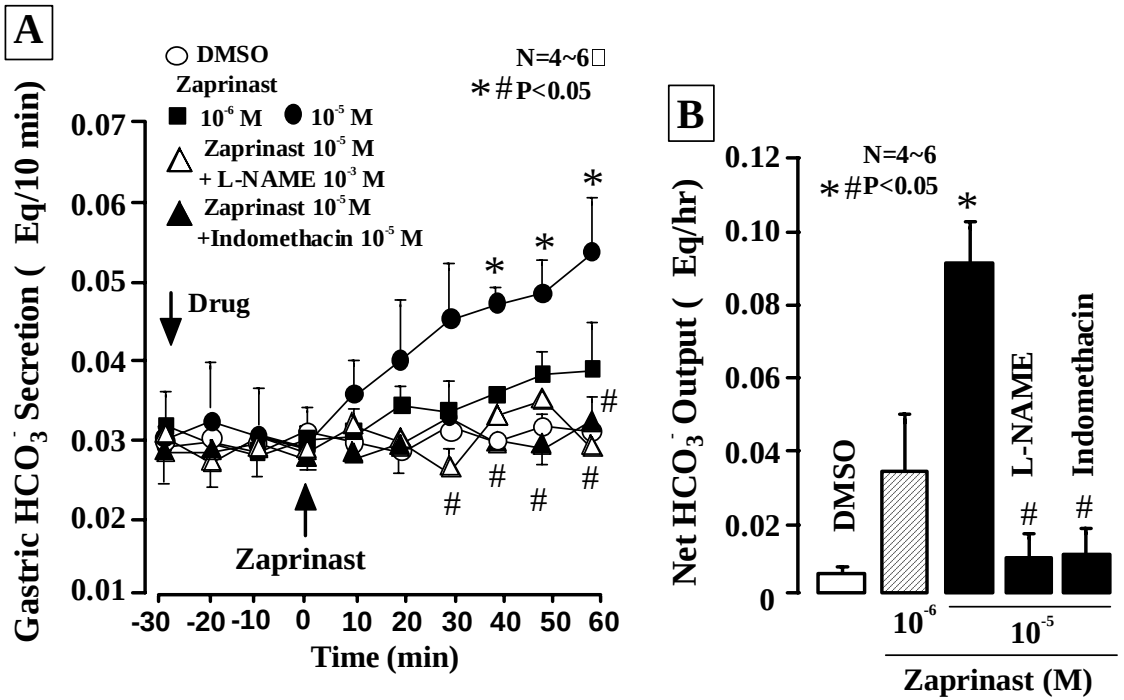

Figure 7

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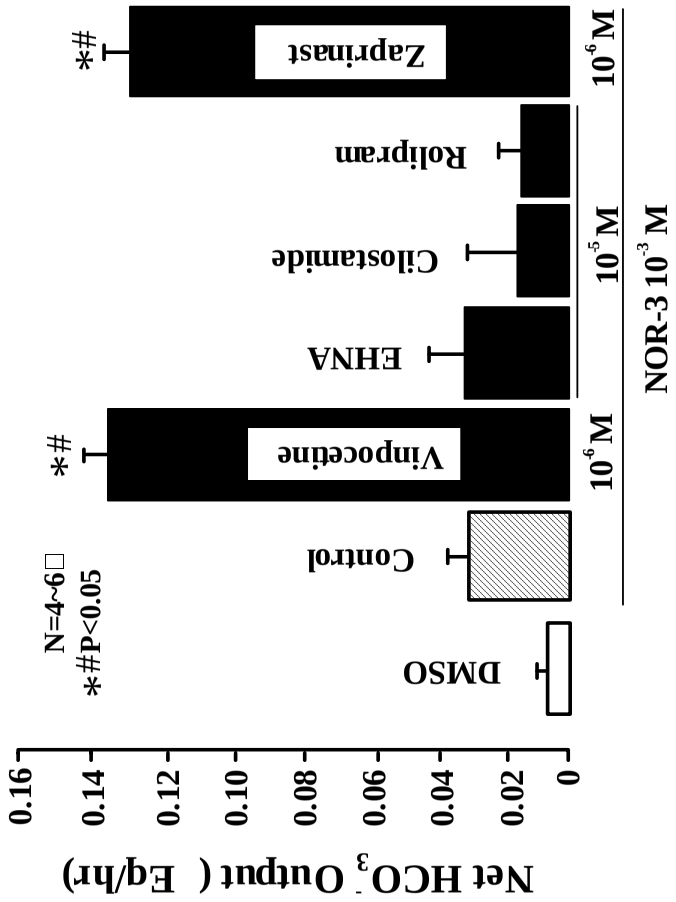

Figure 8

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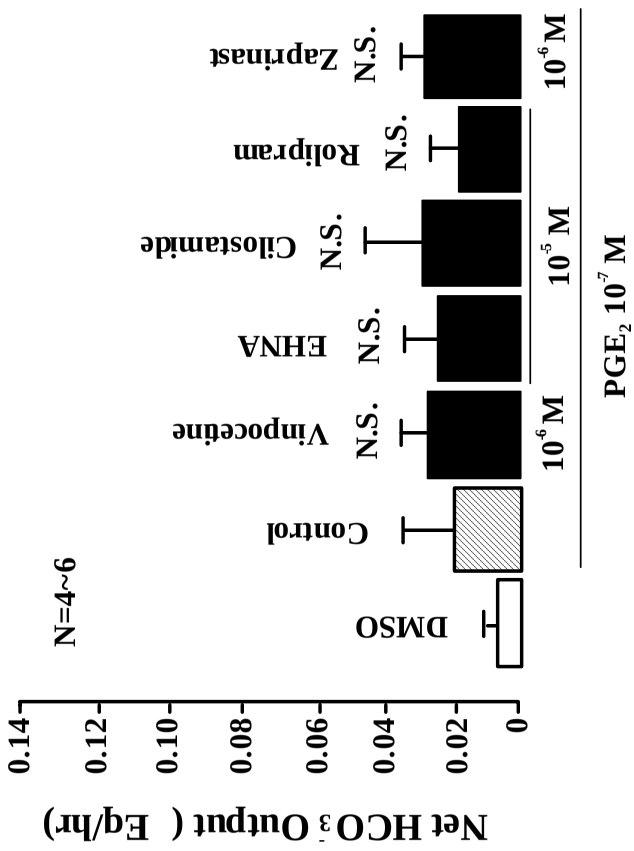

Figure 9

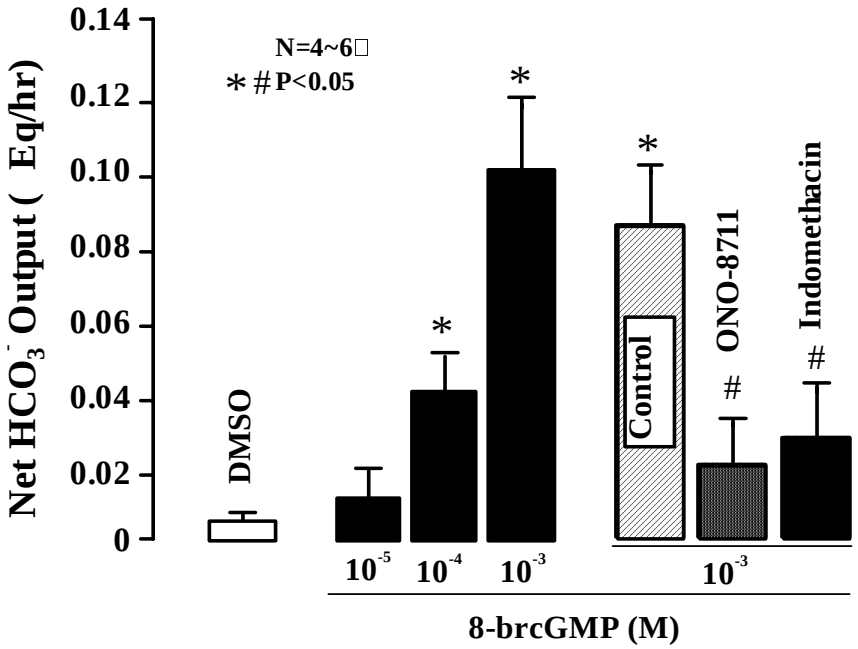

Figure 10

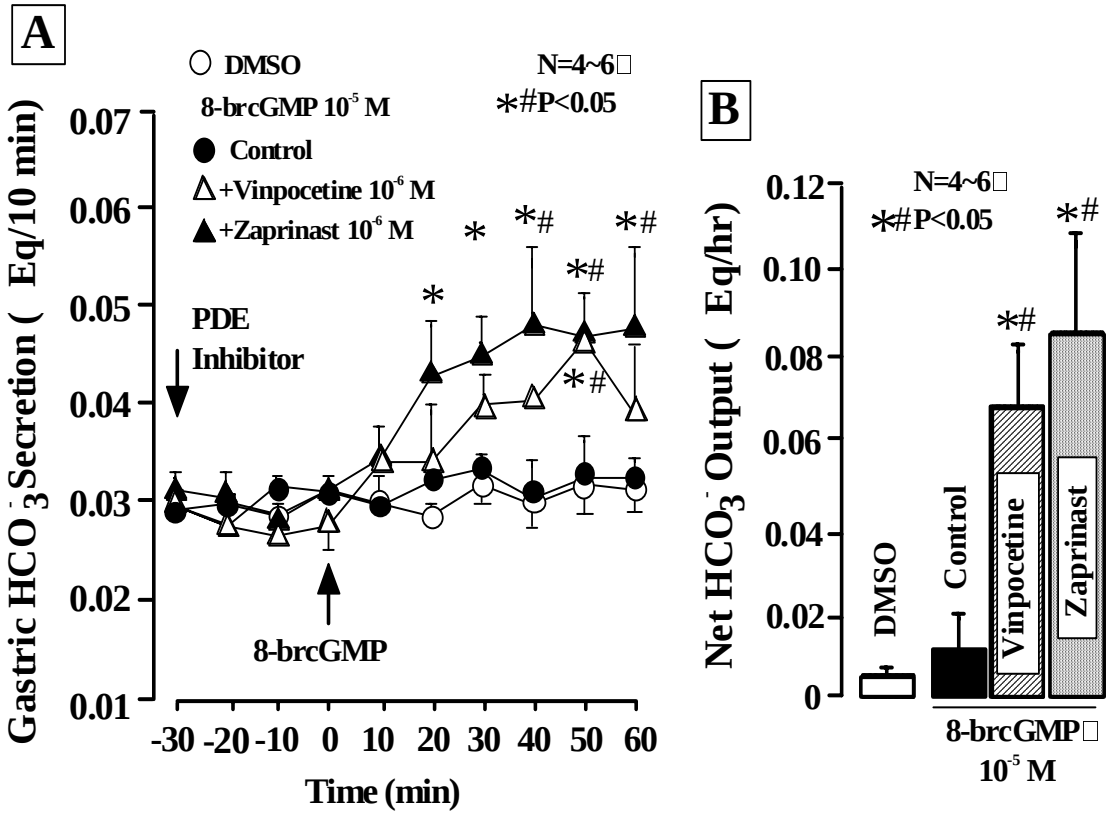

Figure 11

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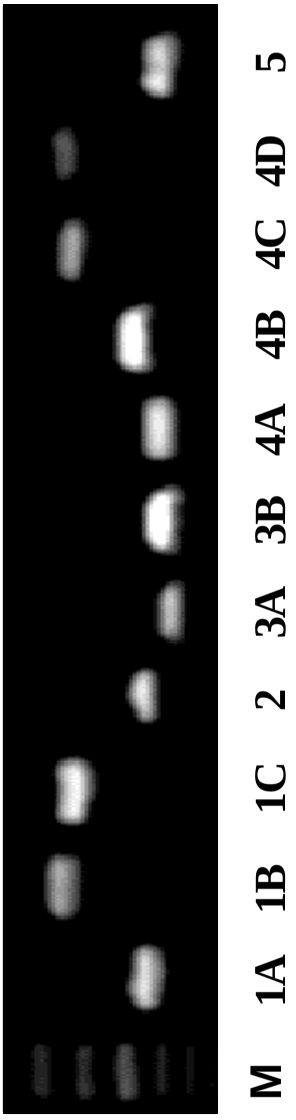

Figure 12

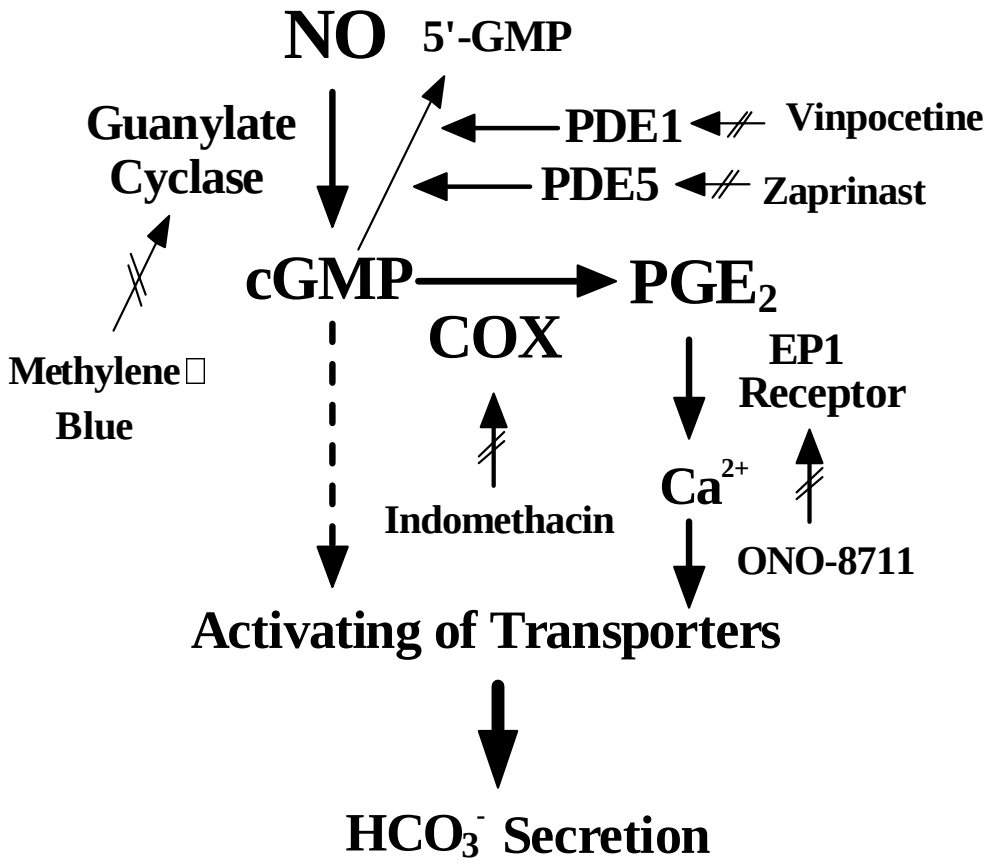

Figure 13