Nitric oxide inhibits gastric acid secretion by increasing intraparietal cell levels of cGMP in isolated human gastric glands

Anna Berg,1 Stefan Redéen,2 Magnus Grenegård,3 Ann-Charlott Ericson,1 and Sven Erik Sjöstrand3

1Division of Cell Biology, Department of Biomedicine and Surgery, Linköping University; 2Surgery Department, University Hospital; and 3Division of Pharmacology, Department of Medicine and Care, Linköping University, Linköping, Sweden

Submitted 19 May 2005; accepted in final form 5 August 2005

Berg, Anna, Stefan Redéen, Magnus Grenegård, Ann-Charlott Ericson, and Sven Erik Sjöstrand. Nitric oxide inhibits gastric acid secretion by increasing intraparietal cell levels of cGMP in isolated human gastric glands. Am J Physiol Gastrointest Liver Physiol 289: G1061–G1066, 2005. First published August 11, 2005; doi:10.1152/ajpgi.00230.2005.—We have previously identified cells containing the enzyme nitric oxide (NO) synthase (NOS) in the human gastric mucosa. Moreover, we have demonstrated that endogenous and exogenous NO has been shown to decrease histamine-stimulated acid secretion in isolated human gastric glands. The present investigation aimed to further determine whether this action of NO was mediated by the activation of guanylyl cyclase (GC) and subsequent production of cGMP. Isolated gastric glands were obtained after enzymatic digestion of biopsies taken from the oxyntic mucosa of healthy volunteers. Acid secretion was assessed by measuring [14C]aminopyrine accumulation, and the concentration of cGMP was determined by radioimmunoassay. In addition, immunohistochemistry was used to examine the localization of cGMP in mucosal preparations after stimulation with the NO donor S-nitroso-N-acetylpenicillamine (SNAP). SNAP (0.1 mM) was shown to decrease acid secretion stimulated by histamine (50 μM); this effect was accompanied by an increase in cGMP production, which was histologically localized to parietal cells. The membrane-permeable cGMP analog dibutylrylcAMP (db-cGMP; 0.1–1 mM) dose dependently inhibited acid secretion. Addition of the effect of SNAP was prevented by preincubating the glands with the GC inhibitor 4H-8-bromo-1,2,4-oxadiazolo[3,4-d]benz[b][1,4]oxazin-1-one (10 μM). We therefore suggest that NO in the human gastric mucosa is of physiological importance in regulating acid secretion. Furthermore, the results show that NO-induced inhibition of gastric acid secretion is a cGMP-dependent mechanism in the parietal cell involving the activation of GC.

THE RELEASE OF GASTRIC HYDROCHLORIC ACID FROM PARIELAL CELLS IS KNOWN TO BE UNDER THE INFLUENCE OF BOTH HORMONAL AND NEUROLOGICAL FACTORS INVOLVING THE RELEASE OF HISTAMINE FROM ADJACENT ENTEROCHROMAFFIN-LIKE (ECL) CELLS AND OF GASTRIN FROM ANTRAL G CELLS AS WELL AS VAGAL STIMULATION VIA ACETYLCHOLINE. DOWNREGULATION OF GASTRIC ACID SECRETION HAS SO FAR BEEN CONSIDERED TO BE DUE TO WITHDRAWAL OF STIMULUS, MAINLY VIA SOMATOSTATIN, WHICH INHIBITS ECL CELLS AND G CELLS, RESULTING IN A REDUCTION OF HISTAMINE AND GASTRIN PRODUCTION (16). EVEN THOUGH SOMATOSTATIN HAS BEEN SHOWN TO INHIBIT ACID SECRETION VIA SOMATOSTATIN RECEPTORS ON PARIELAL CELLS (24), ITS REGULATION IS GENERALLY THOUGHT TO OCCUR THROUGH OTHER INDIRECT PATHWAYS.

Nitric oxide (NO) is a physiological signaling molecule that has been found to influence a wide variety of processes, such as blood flow, neutrophil and platelet adhesion, and neurotransmission (19). NO is produced from l-arginine by the enzyme NO synthase (NOS) (22) and has the potential to bind to iron heme-containing structures such as NOS, thereby regulating its own production (27). The most important physiological target of NO is soluble guanylyl cyclase (GC). NO binding to the heme moiety of GC induces a conformational change, which activates the enzyme, subsequently leading to the conversion of GTP into cGMP (21). Signaling via cGMP may be considered to be the main mechanism in which NO exerts its effect, as previously demonstrated in smooth muscle relaxation, neurotransmission, platelet inhibition, and intestinal ion secretion (17).

Investigations have shown that NO protects against mucosal damage and facilitates ulcer healing, mainly by affecting gastrointestinal functions such as mucous secretion, the microcirculation (8), and secretion of bicarbonate (29).

Evidence suggesting a connection between NO and acid secretion is beginning to grow. The effects of NO on gastric acid secretion have been described both in vitro and in vivo. In fact, several investigations using different animal models have shown that NO donor drugs influence stimulated gastric acid secretion (7, 11, 14, 15). Furthermore, NO has been shown to inhibit induced acid secretion in isolated glands and parietal cells (6, 7, 12, 15). Together with an earlier observation, where endocrine cells in close contact with parietal cells of the human gastric mucosa were found to express NOS (5), these findings suggest a physiological role for NO in the regulation of gastric acid secretion in humans.

The aim of the present study was to investigate the physiological mechanism underlying NO-induced inhibition of gastric acid secretion in humans with an emphasis on the role of NO-sensitive GC and cGMP.

MATERIALS AND METHODS

Subjects and Ethical Approval

Eighteen healthy men ranging in age from 20 to 32 yr were recruited as paid volunteers. The selection criteria stipulated that the subjects had to be free from disease and should not have taken any medication or imbibed alcohol for at least 1 wk before examination. The subjects fasted for at least 6 h before the examination.

Pharyngeal anesthesia was induced with lidocaine spray (Xylocain, AstraZeneca; Södertälje, Sweden), after which a routine gastroscope was performed using an Olympus GIF-100 endoscope. Pinch biopsy forceps (Olympus FB 24K-1) were used to take tissue specimens from the greater curvature immediately distal to the fundus. In all subjects, the costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Address for reprint requests and other correspondence: A.-C. Ericson, Div. of Cell Biology, Dept. of Biomedicine and Surgery, Linköping Univ., Linköping S-581 85, Sweden (e-mail: loter@ibk.liu.se).

http://www.ajpgi.org 0193-1857/05 $8.00 Copyright © 2005 the American Physiological Society

G1061
the gastric mucosa appeared to be normal, both macroscopically and histologically. All subjects tested negative for Helicobacter pylori infection in the urease breath test (Diabact UBT 50 mg [13C]urea, Diabact; Uppsala, Sweden).

The experimental procedures were approved by the Regional Ethics Committee for Human Research at University Hospital, Linköping, Sweden (file no. 02-039), and all subjects gave informed consent.

Materials

Collagenase type IA, dibutyryl-cGMP (db-cGMP), 3-isobutyl-1-methylxanthine (IBMX), and 4H-8-bromo-1,2,4-oxadiazolo[3,4-d]-benz[b][1,4]oxazin-1-one (NS-2028) were all purchased from Sigma-Aldrich (St. Louis, MO). 5-nitroso-N-acetylpenicillamine (SNAP) was obtained from Alexis (San Diego, CA), and [14C]-aminopyrine was obtained from Amersham Biosciences (Uppsala, Sweden). Antibodies for immunohistochemical analysis, rabbit anti-cGMP, and mouse anti-H'-K'-ATPase were purchased from Chemicon (Hamshire, UK) and Calbiochem-Merck Biosciences (Darmstadt, Germany), respectively. For radioimmunoassay, a polyclonal antiserum against cGMP produced at the Division of Pharmacology of Linköping University was used. All other chemicals and reagents were of analytic standard and from trusted producers.

Isolation of Gastric Glands

The use of isolated gastric glands to measure secretory activity is a well-established and thoroughly evaluated method (9). The procedure, as previously described (6), is based on enzymatic treatment of biopsy specimens to yield gastric glands in solution. Briefly, biopsies were treated with a collagen solution to isolate complete free glands. The glands were kept at 37°C in a respiratory solution containing (in mM) 132.4 NaCl, 1.0 NaH2PO4, 1.2 MgSO4, 5.4 KCl, 5.0 Na2HPO4, 1.0 CaCl2, 0.01 indomethacin, and 10 glucose with 2 mg/ml human serum albumin.

To prevent degradation of cyclic nucleotides, 0.1 mM of the phosphodiesterase (PDE) inhibitor IBMX was added to all preparations.

Analysis of [14C]Aminopyrine Accumulation

Accumulation of radioactive aminopyrine was used as an indicator of acid secretory activity in isolated glands. The detailed experimental procedures have been described earlier (6). For the analysis of the influence of the cGMP analog db-cGMP on [14C]aminopyrine accumulation, isolated glands were incubated in respiratory medium supplemented with 0.1, 0.3, and 1 mM db-cGMP for 30 min at 37°C. The effect of NO on gastric acid secretion was evaluated by incubating the glands for 30 min with the NO donor SNAP (0.1 mM). To determine the involvement of GC activity, the influence of SNAP was analyzed in the absence or presence of the soluble GC-specific inhibitor NS-2028 (10 μM) (23). NS-2028 was added 30 min before SNAP, and glands were incubated at 37°C. In all series of experiments, the production of acid was provoked by adding histamine to yield a final concentration of 50 μM followed by incubating the samples at 37°C for 40 min, after which [14C]aminopyrine accumulation (AP ratio) was analyzed.

Each subject served as its own control, because we determined each individual’s AP ratio after histamine stimulation and expressed this value as 100%. The AP ratio after treatment with SNAP, cGMP, or NS 2028 was then recalculated and expressed as a percentage of the histamine-stimulated AP ratio. Presented data are based on single analyses.

Analysis of cGMP

Radioimmunoassay. Isolated glands were incubated in respiratory solution with or without SNAP (0.1 mM) for 30 min at 37°C and stimulated with histamine (50 μM) at 37°C. Glandular samples were taken at different times (0, 5, 30, 70, and 160 min) after stimulation, and the accumulation of cGMP was terminated by adding 10% ice-cold trichloroacetic acid (TCA). Thereafter, suspensions were centrifuged for 15 min at 4,000 g, and supernatants were extracted with 4 × 2 ml water-saturated diethyl ether. The aqueous phase was freeze dried in a vacuum freezer for 18 h and reconstituted in Na-acetate buffer (50 mM, pH 6.2). cGMP levels were determined by radioimmunoassay according to Axelssoon et al. (2). All measurements were performed in quadruplicate. For cGMP analysis after the inhibition of GC, glands were incubated in respiratory solution with or without NS-2028 (10 μM) for 30 min at 37°C. SNAP (0.1 mM) was added, and glands were preincubated for 30 min at 37°C and stimulated with histamine (50 μM). Incubation was terminated after 40 min, and 10% ice-cold TCA was added. The samples were then treated as described above.

Protein assay. The glandular sample (400 μl) or BSA protein calibrator (0–25 μg/ml) was mixed with 400 μl Coomassie blue reagent (Pierce; Rockford, IL). Appropriate volumes were placed in wells of a flat-bottomed 96-well microtest plate (Sarstedt; Nümbrecht, Germany). After 10 min, the absorbance was measured at 595 nm using a SPECTRAMax 340 microplate reader (Molecular Devices; Sunnyvale, CA). A water blank was subtracted from the standard and glandular samples; protein concentrations were determined using a standard curve plotted from the BSA standard. All measurements were performed in quadruplicate.

Immunolocalization of cGMP

Biopsies were placed in respiratory solution supplemented with 0.1 mM SNAP and 0.1 mM IBMX for 2 h at 37°C, washed in PBS, fixed in 4% paraformaldehyde, and cryoprotected with 30% sucrose in PBS. Thin (5 μm) sections were cut using a Leica CM3050 cryostat (Leica Instruments). Sections were mounted on charged Super Frost* Plus glass slides (Menzel-Gläser), rinsed in PBS, and preincubated with 10% normal goat serum (DAKO) for 20 min. The slides were then rinsed in PBS and incubated overnight at room temperature with antibodies against cGMP (1:1,500) and H'-K'-ATPase (1:1,500) in PBS supplemented with 3% normal goat serum and 0.3% Triton X-100. Thereafter, the slides were washed in PBS for 5 × 10 min and exposed to 5 μg/ml biotinylated goat anti-rabbit IgG as well as FITC-conjugated goat anti-mouse IgG for 1–2 h. After slide were thoroughly washed in PBS, the antibody against rabbit IgG was detected using 20 μg/ml Texas red-Avidin (Vector). The slides were then rinsed, and coverslips were mounted using Vectashield Mounting Medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector). A Nikon Eclipse E800 fluorescence microscope equipped with a VFM EPI fluorescence attachment was used to study and evaluate the slides. A Bandpass filter with a wavelength band of 520–560 nm and a long-pass filter with a cut-on wavelength at 590 nm (for emitted light) were employed to visualize FITC and Texas red molecules. Colocalization of the two probes yields a yellow- to orange-colored staining.

Statistical Analysis

Data were tested for normal distribution (p plot) and analyzed by Wilcoxon’s signed-rank test using MINITAB Statistical Software. P values <0.05 were considered significant. Each individual served as its own control.

RESULTS

In the present study, we assessed the influence of NO on the content of cGMP in isolated human gastric glands. With the use of the weak base aminopyrine as an indicator of acid secretion, the effect of cGMP on histamine-stimulated acid...
secretion was also assessed. Histological, biochemical, and functional studies were carried out. The distribution of data was tested and could not be considered normally distributed. Nonparametric tests were used to determine statistical significances, and, hence, the presented results and calculations are based on median values. Because of interindividual variations in the secretory response, the AP ratio for each individual after histamine stimulation was recalculated and expressed as 100%. Hence, whenever the histamine-stimulated AP ratio was used as a control, any induced change in the AP ratio is indicated as the percentage of the histamine-stimulated AP ratio.

Effects of SNAP on cGMP Content in Isolated Gastric Glands

Isolated gastric glands taken from human gastric biopsies are known to secrete gastric acid after the administration of histamine. With the use of a combined immunoassay/protein analysis technique, it was found that cGMP content in the glandular preparation increased with incubation time and leveled off after about 70 min (n = 7; Fig. 1). In control experiments, without SNAP administration, only low amounts of cGMP were observed. The differences in cGMP concentration between treated and untreated glands were found to be statistically significant (P < 0.05) at 5, 30, and 70 min after histamine stimulation.

Immunolocalization of cGMP in Human Parietal Cells

With the use of a double immunohistochemical technique, the cellular distribution of SNAP-induced increase in cGMP was studied. Several kinds of mucosal cells showed fluorescence, indicating the presence of cGMP predominantly in the lamina propria. Immunoreactivity against cGMP was observed in a large number of parietal cells after SNAP treatment (0.1 mM; Fig. 2A). In control preparations, without SNAP treatment, staining could not be observed in parietal cells (Fig. 2B).

Effect of db-cGMP on Histamine-Stimulated Gastric Acid Secretion

After the administration of histamine to isolated human gastric glands, a pronounced secretion of acid was recorded. To clarify the functional role of parietal cell cGMP in acid secretion, various doses (0.1, 0.3, and 1 mM) of the membrane-permeable cGMP analog db-cGMP were added to the glands. A dose-dependent decrease of acid output due to db-cGMP was observed (Fig. 3). All tested specimens showed the same result, and changes were found to be statistically significant at all concentrations (P < 0.05, n = 6).

Effect of cGMP Suppression on SNAP-Induced Inhibition of Histamine-Stimulated Acid Secretion

Because the administration of db-cGMP was observed to lead to a reduced acid secretion, it was of interest to investigate whether SNAP-induced inhibition of acid secretion was mediated by GC and the endogenous production of cGMP. We administered NS-2028 (10 μM), a soluble GC inhibitor, to the glandular preparation before the addition of SNAP. Inhibition of GC in isolated human glands induced a decrease in cGMP concentration compared with control (Fig. 4). Furthermore, the inhibition of histamine-stimulated acid secretion induced by SNAP was reversed by pretreating the samples with NS-2028 (10 μM; Fig. 5). NS-2028 itself, up to 10 μM, did not have a significant effect (data not shown).

DISCUSSION

Previous work by our group has demonstrated that endogenous and exogenous NO inhibits histamine-stimulated gastric acid secretion in isolated human gastric glands (6). Additionally, we have shown that ECL cells, which do not stain for any known marker of gastric endocrine cells (e.g., somatostatin, serotonin, and histidine decarboxylase), express endothelial NOS (eNOS). These findings suggest that NO may act as a physiological inhibitor of gastric acid secretion and that NO is released in a paracrine fashion with a direct action on parietal cell function. The aim of the present investigation was to further clarify the role of NO by testing the hypothesis that NO stimulates cGMP production in parietal cells, leading to reduced acid secretion.

The NO donor SNAP is a pharmacological tool that has been extensively used to mimic and characterize the actions of NO in cells and tissues (13). We (6) have previously reported that inhibition of endogenously produced NO in isolated human gastric glands results in increased glandular aminopyrine uptake. This indicates that NO is produced within the glandular epithelium and may exert a local functional effect on acid secretion. Hence, we use SNAP as an exogenous source of NO at a concentration that has been evaluated and used in previous reports.

In many biological systems, the effect of NO is attributed to its binding to the heme-containing enzyme GC, causing an increase in intracellular cGMP concentration. Alternatively, NO-specific but cGMP-independent actions, including nitrilation and nitration reactions, have also been described (3). We found that SNAP caused a significant increase in cGMP in...
isolated human gastric glands. We (6) have previously shown, using the same technique, that SNAP induced an inhibition of histamine-stimulated gastric acid secretion 40 min after SNAP incubation. The present data thus indicate that an increase in glandular cGMP is present at the time of inhibition. The connection between NO-induced inhibition of gastric acid secretion and NO-mediated rises in cGMP was evaluated by incubating the glands with the membrane-permeable cGMP analog db-cGMP. Indeed, we found that db-cGMP dose dependently reduced histamine-stimulated acid secretion. Furthermore, when GC was blocked by NS-2028, SNAP-induced cGMP responses were decreased. This was accompanied by a reversal of the SNAP-mediated inhibition of acid secretion. These findings therefore show that GC and cGMP are involved in NO-induced inhibition of acid secretion. In comparison, other studies, using animal models, have observed that NO influences acid secretion both in vitro and in vivo. However, previous published results are contradictory; it has been suggested that NO inhibits acid secretion (7, 12, 14, 15), whereas others have indicated that NO activates secretion (11). Different regulatory mechanisms among species may explain these discrepancies. On the basis of our findings, we propose that NO reduces gastric acid secretion in humans and that this effect is mediated by cGMP.

Fig. 2. Double immunofluorescence staining of cGMP and H⁺-K⁺-ATPase in human oxyntic mucosa. cGMP was stained with a rabbit anti-cGMP antibody and biotinylated goat anti-rabbit IgG followed by Texas red-Avidin (red). Parietal cells were detected using a mouse anti-H⁺-K⁺-ATPase antibody and FITC-labeled goat anti-mouse IgG (green). A: tissue section from a biopsy treated with 0.1 mM SNAP, showing immunoreactivity against cGMP (arrows) in parietal cells. Colocalization of cGMP and H⁺-K⁺-ATPase appears as a yellow/orange color. In untreated tissue (B), no staining against cGMP in parietal cells was observed. Both control and SNAP-treated sections show immunoreactivity against cGMP in submucosal cells (arrowheads). Nuclei are stained with 4′,6-diamidino-2-phenylindole (DAPI; blue). Bar = 10 μm.

Fig. 3. Effect of different concentrations of dibutyl (db)-cGMP on [14C]aminopyrine accumulation (AP ratio) in isolated human gastric glands. Columns with the same pattern represent one individual. Although there is a large variance in the magnitude of the AP ratio between individual subjects, they all respond in a similar way with concentration-dependent inhibition of their AP ratio after treatment with 0.1, 0.3, and 1 mM db-cGMP. The differences were statistically significant at P < 0.05.

Fig. 4. Blockade of guanylyl cyclase (GC) and its effect on cGMP content in isolated human gastric glands. Columns with the same pattern represent one individual. The concentration of cGMP in isolated glands was measured with radioimmunoassay and expressed as picomoles per milligram of protein. When isolated glands are treated with SNAP (0.1 mM), the production of cGMP is induced, which is significantly (P < 0.05) decreased in all subjects when GC is blocked with NS-2028 (10 μM).
So far, little is known about the effects of cGMP in gastric parietal cells. Therefore, only speculations based on cGMP’s physiological role in other cell systems can be made. In smooth muscle cell relaxation, cGMP is known to activate PKG and decrease cytosolic Ca$^{2+}$. Rises in cytosolic Ca$^{2+}$ are a central component in the regulation of gastric acid secretion (18). By regulating transportation and docking of tubulovesicles to the apical membrane, Ca$^{2+}$ facilitates membrane recycling and becomes a vital element in acid secretion (30). Theoretically, it is possible that NO/cGMP acts on parietal cells by interfering with Ca$^{2+}$ signaling, thereby causing a decrease in acid secretion due to impaired translocation of H$^+$ - K$^+$-ATPase to the apical membrane. Another possibility is that NO/cGMP inhibits its gastric acid secretion by accelerating the breakdown of cAMP. This is because cGMP allosterically activates type II PDE, which catalyzes the degradation of cAMP (4). Indeed, other studies have shown that the addition of db-cGMP causes the suppression of cAMP-provoked gastric acid secretion in isolated rat parietal cells (12), but, in our experimental design, PDE activity was suppressed by the presence of IBMX in preparations. Consequently, cGMP must exert other mechanisms of action to reduce gastric acid secretion from isolated human glands.

The NO/cGMP signaling pathway has been shown to regulate other secreting cells, for instance, the release of amylase from the parotid gland (28), secretion of HCO$_3^-$ in the duodenum (10), and release of glucagon from pancreatic islets (20). Moreover, in enterocytes, cGMP has been known to affect secretion via the cystic fibrosis transmembrane conductance regulator. This Cl$^-$ channel is phosphorylated by PKG and is responsible for an enhanced secretion of Cl$^-$ and water as a result of increases in cGMP intracellular content (31). The fact that cGMP can affect ion channels in other cells in the gastrointestinal tract and adjacent glands suggests that Cl$^-$ or K$^+$ channels coupled to proton exchange in parietal cells may be potential targets for the NO/cGMP signaling system. Studies have demonstrated that NO is able to inhibit the enzyme carbonic anhydrase (CA) both in vivo and in vitro (26). Because CA in parietal cells is Ca$^{2+}$ sensitive, NO may act either directly on the enzyme or indirectly via increases in cytosolic Ca$^{2+}$, thereby reducing the available hydrogen ions for the proton pump (25). Currently, we are elucidating the exact mechanism(s) underlying NO/cGMP-mediated inhibition of parietal cells from the human gastric mucosa.

In conclusion, the NO donor SNAP was found to cause inhibition of stimulated acid secretion in an isolated human gastric gland preparation. This downregulation of secretory function was accompanied by an increase in cGMP production in parietal cells. Similarly, the administration of db-cGMP to isolated glands caused a dose-dependent inhibition of gastric acid secretion. The SNAP-induced reduction of acid secretion was prevented by blocking cGMP production using a GC inhibitor. The present findings thus suggest a physiological role for NO in the regulation of gastric acid secretion in the human mucosa. However, additional studies with a focus on signal transduction in isolated cell systems should be carried out to elucidate the mechanism underlying the inhibition of gastric acid secretion by the NO/cGMP signaling pathway.
ACKNOWLEDGMENTS

We thank Marja Tjädermo for valuable technical assistance, Inga-Lill Andersson for help with the gastroscopic procedures, and Ulf Hannestad for carrying out the urease breath tests.

GRANTS

This study was supported by AstraZeneca Research and Development.

REFERENCES