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GASTRIC ACID SECRETION AND GUT PEPTIDES: MECHANISMS INVOLVED IN INFLAMMATORY RESPONSE

Tobias Rudholm Feldreich

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From DEPARTMENT OF MEDICINE
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ABSTRACT

The regulation of gastric acid secretion is complex and involves endocrine, paracrine, and neurocrine mechanisms. Among these, the interconnecting cross-talk between different gut peptides is an important part in the control of acid secretion.
The aims of this thesis were (1) to develop a new method of measuring intragastric pH for prolonged periods of time, and (2) apply developed and in-use methods using different substances and their impact on gastric acid secretion *in vivo* experiments on rats. (3) To study the changes in acid output and the migrating motor complex (MMC) when subjected to different substances, and (4) to further study the alterations in expression of different gut peptides in tissue samples *in vitro*, and the impact of inflammation in the gut.

The novel Bravo model developed gave reliable recordings compared to the chronic fistula model. The pH rose during treatment with esomeprazole and the acid output in the fistula model decreased accordingly. Gut peptides ghrelin and somatostatin increased in plasma when subjected to esomeprazole treatment, while gastrin remained unchanged. Ghrelin administered in bolus doses increased the intragastric pH in accordance with previous experiments.

The gut peptides somatostatin, neurotensin, and vasoactive intestinal peptide increased during pentagastrin-stimulated infusion and challenge with hydrochloric acid and polyethylene glycol both in plasma and intestinal perfusate, though the most pronounced elevation was seen in perfusate and with somatostatin. Gastrazole gave the most extensive inhibitory effect on acid secretion compared to ranitidine and esomeprazole.

The CCK_2-receptor antagonist YF476 inhibited acid secretion long-term and increased concentrations of ghrelin and somatostatin in plasma, but gastrin remained low. Tissue mRNA content of the peptides and their receptors were unchanged except for the ghrelin receptor. When subdued to NSAID gastrin, CCK_2-receptor and iNOS increased in mRNA expression while other peptides and receptors were unchanged.

Administration of NPS evoked a response in the MMC pattern with irregular spiking and prolonged cycle length of the activity fronts, and the mRNA expression of iNOS, TNF, and IL-1β increased in the tissue.

In conclusion, The Bravo model can be used as a complement to the chronic fistula model for measurements of pH. The regulation of gastric acid secretion is not only limited to the stomach, but also present in the smaller intestine where release of somatostatin seems to be most important. Different mechanisms are involved in the blockage of acid secretion when subjected to YF476, but under NSAID treatment the expression of gastrin and its CCK_2-receptor increase and COX-2 is activated which demonstrates a novel pathway for the study of gastric ulcerations. NPS, a novel neuropeptide influences the gastric motility and could have a role in inflammatory responses seen in the changes in the migrating motor complex and inflammatory markers iNOS, TNF, and IL-1β.

LIST OF PUBLICATIONS

I. **Rudholm T**, Hellstrom PM, Theodorsson E, Campbell CA, McLean PG, Naslund E. Bravo capsule system optimizes intragastric pH monitoring over prolonged time: effects of ghrelin on gastric acid and hormone secretion in the rat. World J Gastroenterol. 2008;14:6180-7.

II. **Rudholm T**, Wallin B, Theodorsson E, Näslund E, Hellström PM. Release of regulatory gut peptides somatostatin, neurotensin and vasoactive intestinal peptide by acid and hyperosmolal solutions in the intestine in conscious rats. Regul Pept. 2009;152:8-12.

III. **Rudholm T**, Gillberg L, Theodorsson E, Sanger G, Campbell CA, Boyce M, Näslund E, Hellström PM. CCK_2-receptor antagonist YF476 prevents NSAID-induced gastric ulceration through acid inhibition mediated by regulatory peptides. (Manuscript)

IV. **Rudholm T**, Gillberg L, Näslund E, Hellström PM. Neuropeptide S and the gastrointestinal tract: effects on the inflammatory markers TNF, IL-1β, subsequent expression of iNOS and motility in the rat. (Manuscript)

``Everything can change at any moment,
suddenly and forever.``

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LIST OF ABBREVIATIONS

ACh	Acetylcholine
ANP	Atrial natriuretic peptide
cAMP	3´-5´-cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CCK_2R	Cholecystokinin-2 receptor
CNS	Central nervous system
COX-2	Cyclooxygenase-2
D cell	Somatostatin-producing cell
EC cell	Enterochromaffin cell
ECL cell	Enterochromaffin-like cell
EIA	Enzyme immunoassay
ENS	Enteric nervous system
G cell	Gastrin-producing cell
GERD	Gastroesophageal reflux disease
GHS-R1a	Growth hormone secretagogue receptor 1a
Gr (X/A) cell	Ghrelin-producing cell
GRP	Gastrin-releasing peptide
H^+,K^+-ATPase	Hydrogen potassium adenosine triphosphate
HCl	Hydrochloric acid
HDC	Histidine decarboxylase
HP	*Helicobacter pylori*
HPLC	High-performance liquid chromatography
Hprt1	Hypoxantine-guanine phosphoribosyl transferase
H_2R	Histamine 2 receptor
IL-1β	Interleukin-1β
iNOS	Inducible nitric oxide synthase
MMC	Migrating motor complex
mRNA	Messenger ribonucleic acid
M_3R	Muscarinic 3 receptor
NaCl	Sodium chloride
NO	Nitric oxide
NPS	Neuropeptide S
NPSR(1)	Neuropeptide S receptor (1)
NT	Neurotensin
NSAID	Nonsteroidal anti-inflammatory drug
PACAP	Pituitary adenylate-cyclase activating polypeptide
PBS	Phosphate buffer solution
PEG	Polyethylene glycol
PPI	Proton pump inhibitor
qPCR	Real-time quantitative polymerase chain reaction
RIA	Radioimmunoassay
RTL	Recombinant T-cell ligand
SD	Sprague-Dawley
SEM	Standard error of the mean
$SSTR_2$	Somatostatin receptor-2
VIP	Vasoactive intestinal polypeptide

1 Introduction

1.1 Gastric acid

Up to the present day the study of gastric acid and its secretion and regulation, and our understanding of its relation to physiological and pathophysiological conditions are considerable. It ranges from Hippocrates (460-377 B.C.)[1] to modern times with the discovery of the so called conditioned reflexes by Ivan P. Pavlov during the 19th century[2], gastrin as the main stimulant of gastric acid secretion by John Edkins 1905[3], and James W Blacks discovery of the H_2-receptor in 1972 just to mention a few[4].
Gastric acid enables the digestion of protein and absorption of iron, calcium and vitamin B-12. It also prevents bacterial overgrowth and infections in the intestines[5]. The secretion must be regulated precisely not to cause ulceration[6] and maldigestion[7]. This regulation is highly complex and involves hormonal, neuronal, and paracrine mechanisms.

1.1.1 Functional anatomy of the stomach

The stomach consists of three distinct anatomical parts, fundus, corpus (or the body) and antrum, and two functional parts, the pyloric and oxyntic glands (figure 1).

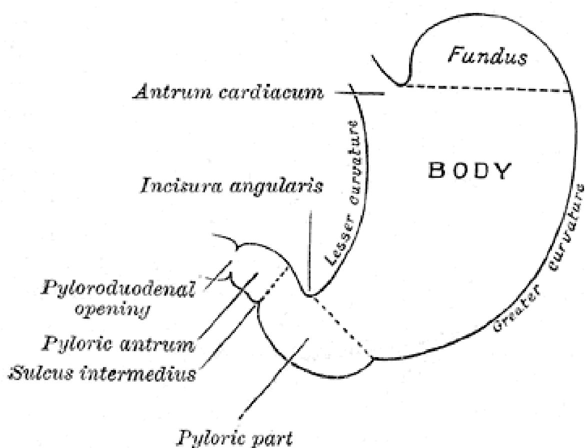

Figure 1. Schematic illustration of the stomach (lithography originally published in 1918 in Grey´s anatomy).

Fundus and corpus comprises 80% of the total organ and consists of the oxyntic or parietal cells, and the rest is composed of the antrum part in which the G-cells, or gastrin producing cells resides. It is estimated that the human stomach consists of $1x10^9$ parietal and $9x10^9$ gastrin cells[8](figure 1).

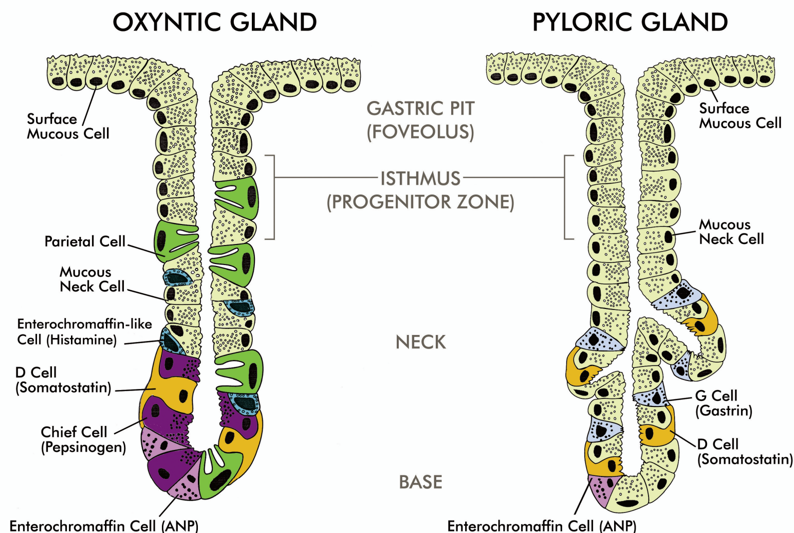

Figure 2. Anatomy of the gastric (oxyntic and pyloric) gland region in the mucosal lining of the stomach (reprinted with permission from the publisher, Mitchell L. Schubert).

One of the most prominent cell type in the oxyntic glands is the acid-secreting parietal cells[9] and the, at the base predominant zymogenic (chief) cells which secretes pepsinogen[10, 11] and leptin[12]. In addition to these cells the glands also contain neuroendocrine cells hosting a wide range of potential paracrine and hormonal signaling agents. A few of these have been identified as having physiological functions: (1) D cells containing somatostatin and amylin[13, 14]; enterochromaffin-like (ECL) cells containing histamine[15]; X/A-like or Gr cells containing ghrelin and obestatin[16, 17]; and enterochromaffin (EC) cells containing serotonin, adrenomedullin, and atrial natriuretic peptide (ANP)[18-20]. The pyloric glands contain D cells, X/A-like or Gr cells, and EC cells as do the oxyntic glands. In addition they also contain endocrine cells harboring orexin[21], and the gastrin-secreting G cells (figure 2).

1.1.2 Neuronal anatomy of the stomach

The stomach and the intestines are richly innervated with nerves and neuronal messengers in a system called the enteric nervous system (ENS). The ENS is made up of intrinsic and extrinsic afferent and efferent neurons distributed in two major complexes – the myenteric and submucosal plexuses[22]. The nerves and messengers serve such functions as gastrointestinal motility, acid secretion, hormone release, blood flow and mucosal defense mechanisms[23]. The ENS is part of the autonomic nervous system, which also includes the sympathetic and parasympathetic system, and is often referred to as the *little brain*[24]. This is because of its relative independence from the central nervous system (CNS).
The efferent nerve fibers are preganglionic and are connected to postganglionic neurons in the ENS. Unlike the preganglionic fibers the postganglionic directly innervate the target cells, i.e. the neuroendocrine and parietal cells. The postganglionic neurons contain a number of neurotransmitters, including acetylcholine (ACh), vasoactive intestinal polypeptide (VIP), and nitric oxide (NO) among others. These neurons regulate gastric acid secretion directly, via ACh

or indirectly through the action of gastrin from G cells, somatostatin from D cells, histamine from ECL cells, and ANP from EC cells[25, 26].

1.2 Regulation of gastric acid secretion

The hydrochloric acid is secreted by the parietal cells in the oxyntic glands (figure 2) and is assumed to reach the lumen of the stomach through the mucus layer by the relatively high intraglandular pressure generated during secretion. The parietal cells possess three different acid stimulatory receptors: CCK_2-receptor (CCK_2R), histamine 2 receptor (H_2R), and muscarinic 3 receptor (M_3R). There appears to be two main cellular mechanisms or pathways of acid secretion in the parietal cells: (1) activation of H_2R by histamine and subsequent increase in 3´-5´-cyclic adenosine monophosphate (cAMP) content in the parietal cells, and (2) activation of CCK_2R by gastrin and M_3R by acetylcholine (released from the parasympathetic nerve terminals) which stimulate increase in calcium concentration[27, 28] (figure 4). The secreted acid has a concentration of about 160 mmol L^{-1} or pH 0.8[29]. This is implemented through a series of complex interactions that are activated either directly through stimuli originating in the brain or through stimuli originating in the stomach, such as acid, distension, or protein[28] (figure 3).

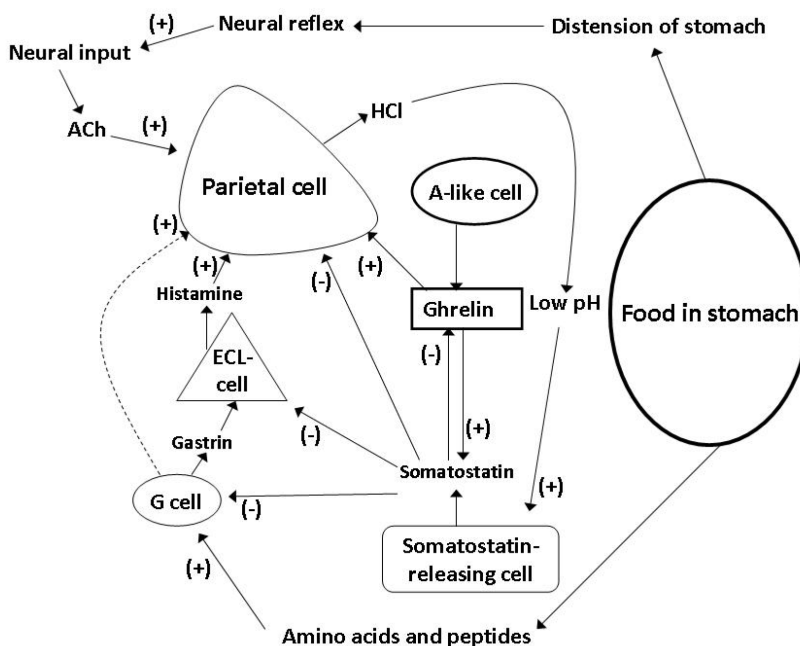

Figure 3. Schematic illustration of control of gastric acid secretion.

1.2.1 Gastrin

The peptide hormone gastrin is the main acid stimulatory agent during ingestion of a meal[30]. Gastrin is released from G cells in the antrum in an endocrine fashion and stimulates the parietal cells directly to produce gastric acid or indirectly through the release of histamine from the ECL cells. Gastrin binds to the CCK_2R located both on the parietal as well as the ECL cells

and activates a signaling cascade involving phospholipase C and release of intracellular calcium[31-33]. Although it is still not entirely clear if the direct effect of gastrin on the parietal cells is true[34, 35], it is believed that intracellular concentrations of cAMP have to reach a particular threshold for gastrin to be able to directly stimulate the parietal cells[36]. The primary action of gastrin on the parietal cells seems to involve sensitizing them to other secretagogues through synergistic interactions between signaling pathways. Presently the main mechanism by which gastrin is thought to stimulate gastric acid secretion is through the activation of CCK_2Rs on the ECL cells and following stimulation of histamine release[33, 37]. Gastrin also has trophic effects on the oxyntic mucosa and stimulates migration of gastric epithelial cells through their action on CCK_2R[31, 38, 39].

1.2.2 Histamine

Histamine is like gastrin one of the principal stimulants of gastric acid secretion. Released from the ECL cells they exert their action in a paracrine manner, stimulating acid secretion through H_2-receptors on the parietal cells[40]. Histamine also binds to H_3-receptors and indirectly stimulates release of acid by inhibiting somatostatin[41].

1.2.3 Somatostatin

Somatostatin is a powerful physiological inhibitor of gastric acid secretion[5]. Somatostatin has a widespread distribution throughout the central nervous system (CNS) and different peripheral tissues and organs[42-45]. In the stomach somatostatin is synthesized and released from cytoplasmic processes of the D cells of the fundic and antral mucosa[46]. These processes are in close contact with their target cells (e.g. gastrin, ECL, and parietal cells), and although somatostatin also is secreted via the local circulation and indirectly acts on its target, somatostatins main mode of action seems to be paracrine rather than endocrine[47-50]. In the stomach the action of somatostatin is mediated by somatostatin subtype 2 receptor ($SSTR_2$)[51, 52] by inhibiting acid secretion from the parietal cells and reducing the concentrations of circulating histamine and gastrin[44, 53-55].

1.2.4 Ghrelin

The stomach is the predominant source of circulating ghrelin[56, 57]. Production of ghrelin has been localized in the X/A or Gr cells of the oxyntic mucosa[58-60] but is also found in various other tissues and organs[57, 61]. Ghrelin binds and exert its actions via the orphan growth hormone secretagogue receptor type 1a (GHS-R1a)[62] that is highly expressed in hypothalamus, the pituitary and stomach, consistent with its influence on energy balance, food intake and appetite control[63-68]. Several other tissues and organs also house the GHS-R1a indicating broader functions of ghrelin other than food intake and growth hormone release[69-72]. Ghrelin levels are decreased by ingestion of food and increased by restriction of energy suggesting that ghrelin secretion is controlled by metabolic signals[73, 74]. Ghrelin has been reported to stimulate acid secretion[75, 76] which seems to involve the vagus nerve[77] and histamine release[78], although one study showed no effect on basal secretion and a decrease in pentagastrin-stimulated acid secretion in conscious rats with gastric fistula[79]. Furthermore, ghrelin secretion has shown to be inhibited by activation of somatostatin receptors. When native somatostatin or synthetic analogues such as octreotide were given in pharmacological doses there was a pronounced

reduction in circulating ghrelin. On the other hand ghrelin increases circulating somatostatin implicating a feedback mechanism between these peptides[76, 80, 81].

1.2.5 Neuropeptide S

Neuropeptide S (NPS), a novel neuropeptide first described in the patent literature[82], binds to a G-protein coupled receptor – neuropeptide S receptor (NPSR1) – and induces elevation of intracellular Ca^{2+} and cAMP[83-85]. NPS is involved in anxiety, food intake, sleep, pain, and modulation of stress responses[83, 86-91]. NPS and its receptor is primarily expressed in different regions of the brain[92], but recent data also indicate a strong expression pattern of NPSR1 and its ligand in the enteroendocrine cells of ileum, duodenum, colon and rectum, and in the submucosal neurons[93] suggesting a role for NPS-NSPR1 in gastrointestinal motor and sensory functions. One other peripheral cell type reported to be sensitive to NPS are the macrophages, which responds to NPS with reduced adhesion, increased phagocytosis, and chemotaxis[94]. Immunological responses involving NPS and macrophages have been suggested in skin and in the intestine[95], indicating that the NPS–NPSR system may have a role in modulating innate immunity and chronic inflammatory diseases of epithelial barrier organs[96, 97].

1.3 Pharmacology and acid secretion

A large body of work has been done during several decades to develop pharmacological treatments for the prevention of acid related disorders[4, 98-100]. Our comprehension of acid-peptic disorders was revolutionized with the discovery of the proton pump in the parietal cells (hydrogen-potassium adenosine triphosphate; H^+K^+ATPase) and *Helicobacter pylori* (HP) infections as the dominant cause of gastric and duodenal ulcers[101-104]. Now the development of the proton pump inhibitors (PPIs) became the focus of acid-related disease treatment, although better therapies are needed to eradicate for example night-time reflux, symptoms related to endoscopic-negative reflux disease, and extraesophageal manifestations of GERD[105].

1.3.1 Proton pump inhibitors

Since the discovery of omeprazole in 1987[105] proton pump inhibitors (PPIs) have been the gold standard for treatment of acid-related diseases[106]. The PPIs belongs to a group of so-called substituted benzimidazole derivatives and are weak bases accumulating in the acid compartments of the parietal cells. There, they undergo conversion from inactive to active form driven by the low pH in the cells. When in an active form the PPIs, such as omeprazole (Losec) or esomeprazole (Nexium), irreversibly and selectively bind to the gastric proton pump (H^+, K^+-ATPase) that drive the final step in acid secretion[107] (figure 4). PPIs are the treatment of choice in disorders such as peptic ulcer, gastrointestinal (GI) lesions, gastrooesophageal reflux disease (GERD), nonsteroidal anti-inflammatory drug (NSAID)-induced, dyspepsia, Zollinger-Ellison syndrome, and eradication of HP[106, 108-110].

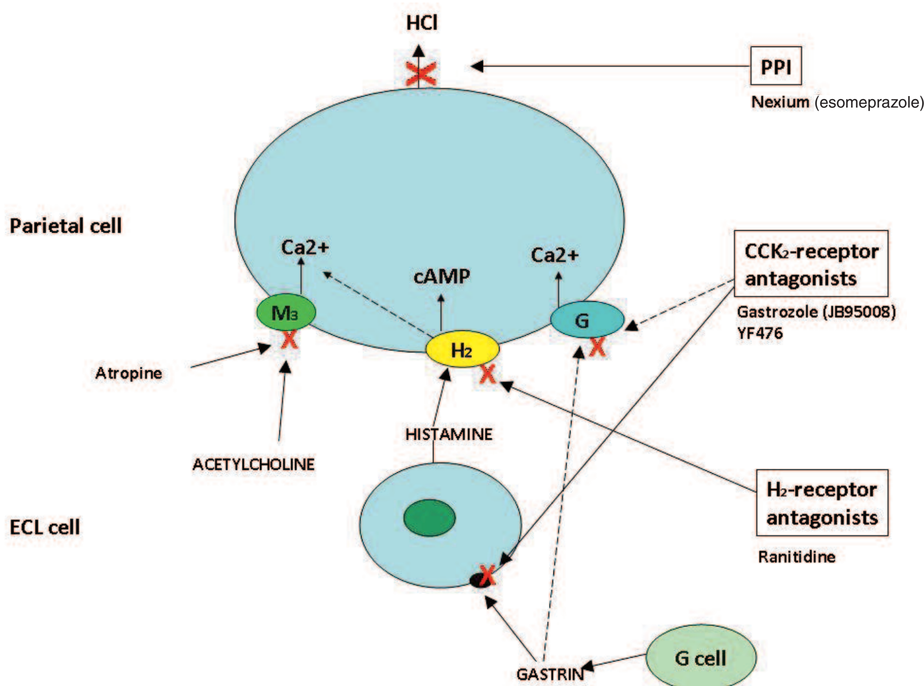

Figure 4. Illustration describing various receptor antagonists on the parietal and ECL cell.

1.3.2 H_2-receptor antagonists

The parietal cell possesses histamine H_2Rs. Stimulated by histamine, in response to release of gastrin from G cells these receptors then signals the subsequent secretion of gastric acid[28, 111]. Since the groundbreaking discovery of H_2-receptors and receptor antagonism[4], several histamine-like antagonists have emerged on the market. These have been used primarily in the treatment of peptic ulcers, but are also common as a combinatory treatment regime together with PPIs[112-115]. The H_2R antagonists block the histamine-driven stimulation of adenylate cyclase and generation of cAMP in the parietal cells. Acetylcholine and gastrin are capable to release histamine from ECL-cells and interacts in synergy with histamine to stimulate acid secretion; therefore the H_2R antagonists, such as ranitidine, inhibit all modes of stimulation by eliminating the direct and synergistic actions of histamine[112, 116] (figure 4).

1.3.3 CCK_2-receptor antagonists

The ECL cells in the oxyntic mucosa are rich in CCK_2R[117-119] and operate under control of gastrin, establishing the gastrin-ECL cell axis[120, 121]. As mentioned above, gastrin, histamine, and ACh are recognized as physiological stimulants of gastric acid secretion. There is some debate which specific pathways that control acid secretion from the stomach, but it seems accepted that gastrin stimulate acid secretion by mobilizing histamine from ECL cells which in turn stimulate parietal cells to produce gastric acid[122-126]. The CCK_2R antagonists have proven to inhibit the enzyme responsible for producing histamine, histidine decarboxylase (HDC), which is stimulated by means of gastrin on the CCK_2R[127-129]. Understandably, the inhibition of

gastric acid secretion with CCK_2R antagonists, such as YF476 or gastrazole, is a result of inhibition of histamine release from the ECL cells (figure 4).

2 Aims

The overall aim of this thesis was to study the effect of acid inhibition on gut peptide regulatory mechanisms in physiological and inflammatory reactions *in vivo* and *in vitro*. The more specific aims were:

- To develop a new model to study intragastric pH over prolonged periods of time using an implanted pH sensor.
- Apply developed and in-use methods using different substances and their impact on gastric acid secretion *in vivo* experiments on rats
- Study the changes in acid output and the migrating motor complex (MMC) when subjected to different substance
- To further study the alterations in expression of different gut peptides in tissue samples *in vitro*, and the impact of inflammation in the gut

3 Material and Methods

3.1 Ethics approvals

For all projects included in this thesis, ethics approval was granted by the Animal Ethics Committee in northern Stockholm (Stockholm, Sweden). Paper I (D no: 157/05, and 100/07), Paper II (D no: 297/90, 157/05, and 100/07), Paper III (D no: 157/05, 100/07, 191/08, and 353/09), and Paper IV (D no: 226/09, and 348/09).

3.2 Animals

For Paper I-IV, Sprague-Dawley (SD) male rats (300 g – 350 g) were purchased from Scanbur B&K AB (Sollentuna, Sweden) or Charles River (Kisslegg, BW, Germany).. The animals were fed *ad libitum* with a commercial rat diet (LABFOR, Lactamin R36, Kimstad, Sweden) and tap water.

3.3 Bravo capsule method (paper I and III)

In paper I we developed a new method for measuring intragastric pH based on the Bravo technique used in clinical setting. BravoTM module is a catheter-free intragastric pH registration system which utilizes telemetric transference (figure 5).

Figure 5. Picture of a Bravo pH registration module in a closed and opened mode.

A pre-calibrated (buffers pH 1.07 and 7.1) Bravo capsule (an electronic sensor encapsulated in PVC-plastic, 25x5x5 mm; Synmed Medicinteknik AB, Spånga, Sweden) is placed inside the stomach of the rat (figure 6).

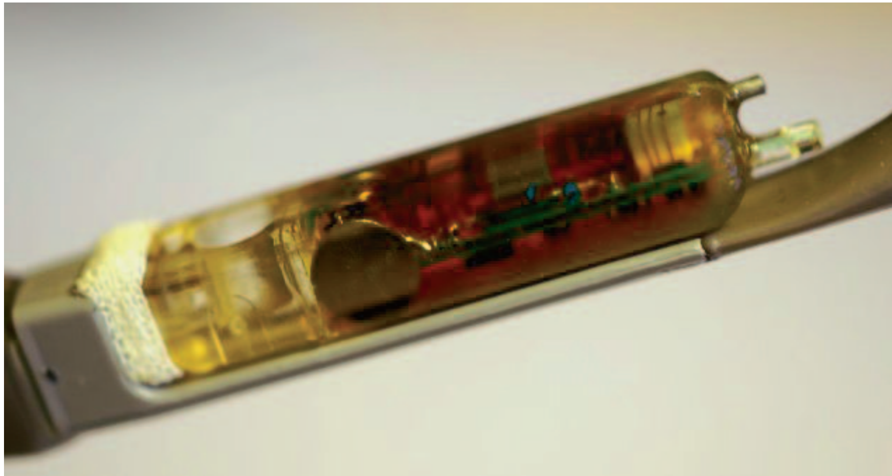

Figure 6. Picture of a Bravo capsule (measurements 25x5x5 mm).

The pH recorded by the Bravo capsule is transmitted to the Bravo receiver placed directly outside the rat's cage. The sampling frequency is 6 Hz and set for a 48-h registration period after which the data has to be downloaded, batteries replaced recording continued. This procedure can then be repeated in two more 48-h periods before the battery of the capsule finally runs out and has to be replaced by a new one.
For the Bravo system studies in papers I and III a midline incision was done, and a small opening created in the proximal greater curvature and the Bravo capsule was placed inside the stomach with the pH sensor pointing distally and anchored with a suture (figure 7).

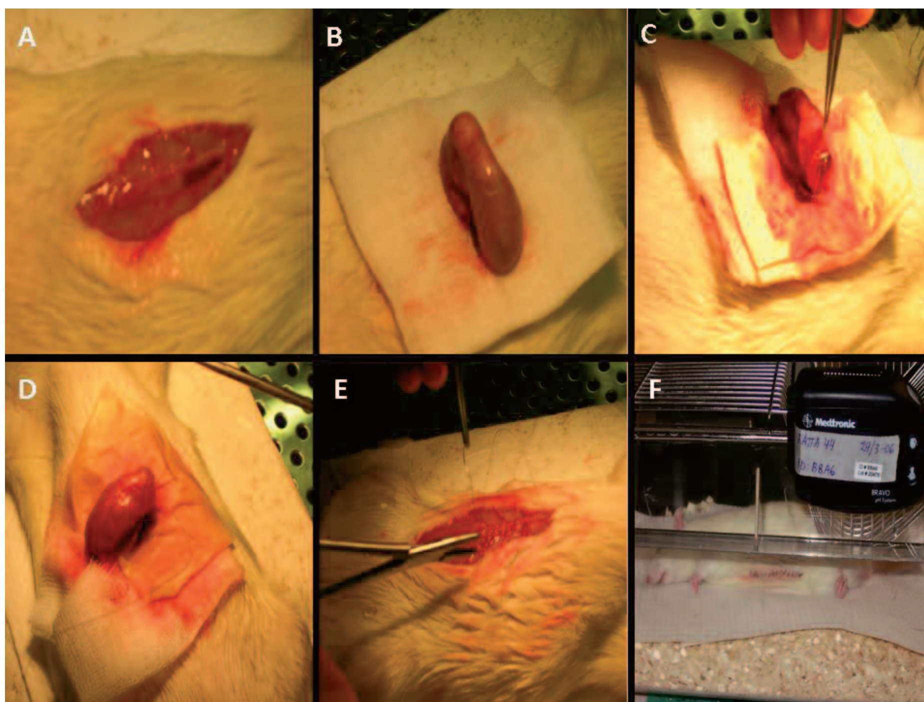

Figure 7. Pictures of the surgical procedures involving the Bravo capsule in the rat. (A) A midline incision was done and the stomach uncovered (B). An opening of the stomach was created, the gastric content removed and the capsule placed inside attached with a suture (C). The stomach was then sutured together as the midline incision and skin (D and E). The pH measurement was started (F) and the animal was placed in its cage to recover before the commencement of the experiment.

An indwelling catheter (Dow Corning Co., Midland, MI, USA) was inserted into the external jugular vein for injection of drugs or saline. Studies of intragastric pH began in the morning 2 days after surgery, and were carried out in conscious rats under normal conditions, or after a 16-h fasting period with free access to water. The animals gained weight and behaved in a normal fashion throughout all the experiments. At post-mortem examination, no mucosal lesions, obstruction of the pylorus or gastric distension were seen.

3.4 Chronic gastric fistula (paper I and II)

For the gastric fistula experiments animals were provided with a plastic cannula (a gastric fistula) placed proximal to the oxyntic gland area, and an IV catheter (Dow Corning Co.) implanted into the external jugular vein for drugs and infusion of saline and pentagastrin. After surgery and in between experimental periods the cannula was closed to prevent leakage. The animals recovered for at least 7 days after surgery before the commencements of the experiments. Prior to each experiment food was withheld for 18 h, but with free access to water. The stomach was rinsed with 10-15 ml luke-warm tap water to evacuate remaining food followed by a 30-min period before experiments were started. During the experiments, the conscious rats were placed in Bollman cages, and the gastric juice was collected at 30-min intervals. Volumes were measured to the nearest 0.1 ml and hydrogen ion concentration (pH) calculated by back-titration using 0.1 M sodium hydroxide. Acid output was calculated by

multiplying the secretion volumes with hydrogen ion concentrations and expressed as μmol per 30-min period.

3.5 Thiry-Vella loop (paper II)

After an overnight fast (18 h), the rats were anesthetized with pentobarbital (50 mg kg^{-1} intraperitoneally (IP), Apoteksbolaget, Umeå, Sweden) and a 10 cm long Thiry-Vella loop of the jejunum was prepared in each rat by dividing the duodenum at the pylorus and 6 cm farther distally, at the ligament of Treitz. A 10 cm long part of jejunum was removed immediately distal to the ligament of Treitz and a blind loop was constructed. To restore gastrointestinal continuity the continuous proximal and distal parts of the jejunum were anastomosed end-to-end. The oral end of the Thiry-Vella loop was closed, intubated with a catheter (PE 90, Clay Adams & Co., Becton Dickinson, Parsippany, NJ, USA), and attached to the abdominal wall. The distal end was brought through the abdominal wall and tunnelled subcutaneously to the back of the animal´s neck. Then, a mid-segment of the duodenal loop, 5-7 mm long, with the orifices of the bile and pancreatic ducts, was implanted end-to-side to the jejunum as a pouch 10 cm distal to the entero-anastomosis of the duodenum (figure 8).

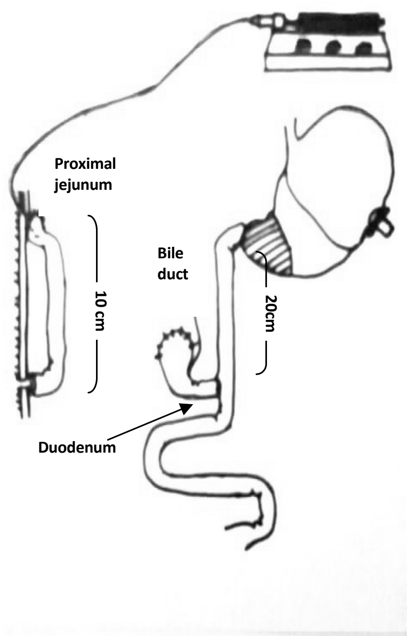

Figure 8. Schematic drawing of a Thiry-Vella loop of the proximal jejunum with duodenum, bile and pancreatic ducts anastomosed as a blind loop to the jejunum.

The rats had free access to water 24 h after surgery and access to food on the second postoperative day. A siliastic catheter (Dow Corning Co.), pretreated with heparin was inserted into the jugular vein for blood sampling and administration of saline and pentagastrin. Throughout the experiments saline was initially administrated via the jugular vein catheter for 120 min by an infusion pump (Sage Instruments, Cambridge, MA, USA). Pentagastrin (90 pmol $kg^{-1}min^{-1}$) was then added to the IV saline infusion for the remaining 90 min. In control experiments, the intestinal loops were perfused with 154 mmol L^{-1} NaCl at a rate of 2 ml h^{-1}.

In test experiments, either hydrochloric acid, HCl (200 mmol L^{-1}; pH 1.1; 380 mOsm kg^{-1}) or polyethylene glycol, PEG (1200 mOsm kg^{-1}; pH 7.2), was given during the pentagastrin infusion period (60 min). The concentrations of HCl and PEG were chosen as they caused maximal inhibition of pentagastrin-stimulated acid secretion[130]. The luminal content was collected during the experiments and kept on ice before analysis of gut peptides SOM, neurotensin (NT), and VIP.

3.6 Gastrointestinal motility *in vivo* in rats (paper IV)

Surgery was performed in animals under anesthesia with a mixture of midazolam (5 mg ml^{-1}, Aktavis AB, Stockholm, Sweden) and Hypnorm (fentanylcitrate, 0.315 mg kg^{-1} plus fluanisone 10 mg kg^{-1}; Janssen-Cilag, Oxford, CT, USA.) given subcutaneously (SC) at a dose of 1.5-2 ml kg^{-1} body weight. Temgesic® (Schering-Plough, Stockholm, Sweden) 0.05 mg kg^{-1} body weight was given SC after surgery to avoid post-operative pain. The abdomen was opened via a midline incision and three bipolar insulated stainless steel electrodes (SS-5T; Clark Electromedical Instr., Reading, UK) were implanted into the muscular wall of the small intestine, 5 (J1), 15 (J2) and 25 (J3) cm distal to the pylorus. The electrodes were tunneled to the back of the animal's neck. All animals were supplied with an indwelling silastic catheter (Dow Corning Co.) inserted into the external jugular vein for intravenous (IV) administration of Neuropeptide S (NPS) (NeoMPS, Strasbourg, France), or saline solution (sodium chloride 9 g L^{-1}; 300 mOsm kg^{-1} H_2O, Fresenius Kabi, Halden, Norway). After surgery, the animals were housed individually and allowed to recover for at least 7 days before experiments were undertaken. Experiments were carried out in conscious animals after an overnight fasting period (18 h) in wire-bottomed cages with free access to water. During the experiments the rats were placed in Bollman cages, and the electrodes were connected to electroencephalography preamplifiers (7P5B) operating a Grass Polygraph 7 B (Grass Instr., Quincy, MA, USA). The activity front (phase III) of the MMC, was identified as a period of distinguishable intense spiking with an amplitude at least twice that of the preceding baseline and a frequency of at least 40 spikes min^{-1}, propagating through the whole recording segment and followed by an inactive period, phase I of MMC. The cycle length, duration and propagation velocity of the activity fronts were calculated as a mean of the study period. The MMC cycle length, reflecting the interval between the propagated activity fronts was calculated at the J2 recording site. All experiments started with a control recording of baseline myoelectric activity with four regular MMCs propagating over all three recording sites. As the fifth activity front had vanished at the first electrode site, an IV infusion of either NPS, or saline solution was started using a microinjection pump (CMA 100; Carnegie Medicine, Stockholm, Sweden) and continued for 60 min, until the basal MMC pattern returned.

3.7 Gastric ulcer induction (paper III)

The animals were deprived of food but with free access to water 20 hours before the ulcer induction. The experiments were carried out in conscious animals given either a single dose of isotonic saline solution (sodium chloride 9 g L^{-1}; 300 mOsm kg^{-1} H_2O, Fresenius Kabi, Halden, Norway), esomeprazole (140 mg kg^{-1}; AstraZeneca, Stockholm, Sweden) or YF476 (100 mg kg^{-1} dissolved in 0.5 % methyl cellulose) one hour prior to the administration of diclofenac, 30 mg kg^{-1}. All substances were given via gavage and the animals left in their cages and at the end-point, 4 hours after the final dose of diclofenac, the animals were sedated with CO_2 and

euthanized by cervical dislocation. The abdomen was opened, the stomach removed and gently washed in phosphate buffer and spread open for photography.
The ulcer index[131] (expressed as mm) reflected the total length of gastric lesions per stomach as judged by three independent researchers blinded to the protocol. Tissue from the stomach was removed and placed in RNAlater (Ambion, Applied Biosystems, Carlsbad, CA, USA) for qPCR analysis.

3.8 Blood sample collection

Blood samples were withdrawn either from the jugular vein catheter during the experiments or full blood sampling by cardio puncture after sacrifice. Blood samples were centrifuged at 4° C, pipetted off and plasma collected. All samples were stored at -80°C until extraction procedures and radioimmunoassay (RIA).

3.9 Radioimmunoassay (paper I-III)

Paper I
The ghrelin (active) radioimmunoassay kit (Linco Research, St. Charles, MI, USA) was used, which utilizes ^{125}I-labeled ghrelin and ghrelin antiserum to determine active ghrelin in plasma. Intra- and interassay coefficients of variation were 7% and 14%.
Gastrin was analyzed using C-terminal-directed CCK/gastrin antiserum 2609/10[132]. Chloramine-T-labeled and HPLC-purified gastrin-17 (NeoMPS) was used as ligand and gastrin-17 as calibrator/standard. Intra- and interassay coefficients of variation were 6% and 8%.
An EIA kit from Phoenix Pharmaceuticals, Burlingame, CA, USA) was used to analyze somatostatin, which reacts 100% to somatostatin-14 and somatostatin-28. Intra- and interassay coefficients of variation were 5% and 14%.

Paper II
SOM-like immunoreactivity (LI) was analyzed with a detection limit of 2 pmol L^{-1}; intra- and interassay coefficients of variation were 7% and 11%[133].
NT-LI was analyzed using antiserum H which reacts with NT, NT (4–13) (118%), NT(8–13) (167%) and NT(9–13) (15%), but not with N-terminal fragments of NT. The detection limit of the assay was 8 pmol L^{-1}. Intra- and interassay coefficients of variation were 8% and 13%[134].
VIP-LI was analyzed in plasma and perfusates using antiserum VIP2 raised against natural porcine VIP. Detection limit of the assay was 3 pmol L^{-1}. Intra- and interassay coefficient of variation were 9% and 13%.

Paper III
The rat ghrelin (active) EIA kit (Millipore, Billerica, MA, USA) was used, utilizing ^{125}I-labeled ghrelin and antiserum which reacts 100% to active ghrelin in plasma, but not inactive des-octanoyl ghrelin. Intra-assay coefficient of variation was 7%.
Gastrin was analyzed using antiserum the C-terminally-directed CCK/gastrin antiserum 2609/10[135]. Chloramine-T labeled and HPLC-purified gastrin-17 (NeoMPS) was used as ligand and gastrin-17 as standard. The assay reacts 100% with gastrin-17 in plasma but not pentagastrin. The detection limit of the assay was 3 pmol L^{-1} and the intra-assay coefficient of variation 6%.

Somatostatin was analyzed using an EIA kit (Phoenix Pharmaceuticals), which reacts 100% to somatostatin-14, -25 and -28 in plasma. The intra-assay coefficient of variation was 5%.

3.10 PCR and RNA preparation

Tissue segments (20-30 mg) of the corpus were collected from the animals, quickly placed in RNA stabilizing reagent (RNA-later, Ambion) and stored at 4° C for 24 h before finally placed in -20° C freezer before PCR. The tissue samples were placed in RTL cell lysis buffer (Qiagen, Hilden, Germany), homogenized, and total RNA was then extracted using the RNeasy Mini Kit (Qiagen). A DNase digestion step (DNase I; Promega, Madison, WI, USA; incubation at 37° C for 30 min) was included to remove traces of chromosomal DNA. Samples with A260/ A280 ratio ≥1.8 were used for PCR. Complementary DNA (cDNA) synthesis was performed using oligo dT primers and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) on 1-5 µg of total RNA.

3.11 Quantitative real-time PCR (paper III and IV)

In *paper III* real-time qPCR was performed on an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using TaqMan gene expression Mastermix (TaqMan®, Applied Biosystems Europe BV, Stockholm, Sweden) for ghrelin, gastrin, somatostatin, and iNOS, and for the ghrelin receptor, CCK_2R and $SSTR_2$. Primers were purchased optimized for TaqMan gene expression assay (TaqMan®, Applied Biosystems Europe BV). The expression in each reaction was normalized by the expression of hypoxanthine phosphoribosyl transferase 1 (Hprt1). cDNA exposed to real-time qPCR was performed in 25 µL reaction volumes consisting of 20 x TaqMan universal PCR master mix. Amplification was carried out at 50° C for 2 min, 95° C for 10 min, 50 cycles of 95° C for 15 sec, and 60° C for 1 min. The absolute amounts of transcripts were determined by using several concentrations of standard cDNA (1000, 100, 10, and 1 ng) from Wt male SD rats, and qPCR analyses performed in triplicate of each sample and standards included in each experiment.
In *paper IV* the qPCR was performed on an iCycler iQ real-time detection system (Bio-Rad Labs, Hercules, CA, USA) analysing the mRNA expression of TNF, IL-1β and iNOS, and each gene normalized to 18S mRNA content. Primers were designed according to previous experimentations[136, 137] and manufactured by CyberGene® (Stockholm, Sweden). QuantiTect SYBR Green PCR Master Mix (Qiagen) was used for the PCR reaction mixture, each sample analyzed in triplicate. Melting curves were generated for each reaction to verify the identity of amplification product. Thermal cycling conditions: 13 min at 95° C to activate HotStarTaq DNA polymerase, followed by 40 cycles at 95° C for 15 s, 60° C for 30 s, and 72° C for 20 s.

3.12 Statistics

Values are expressed as mean ±SEM or SD; $P<0.05$ was considered statistically significant. The Prism software package 4.0 (GraphPad Software Inc., San Diego, CA, USA) was used for the statistical comparisons.

Paper I
Data obtained with the Bravo capsule was analysed using POLYGRAM NET™ pH software (Synmed Medicinteknik, Stockholm, Sweden). Studies with esomeprazole were analyzed as changes in pH from baseline at various time points (defined as 0.5 h prior to onset). For fistula

studies, the second 30-min collection was used as baseline for comparison with esomeprazole and pentagastrin. A Mann-Whitney test followed by a Kruskal-Wallis test was used for changes in baseline at the different time-points measured. Bland-Altman analysis was used for comparison of variability between the fistula and the Bravo system[138].

Paper II
The Kruskal–Wallis test with Dunn's multiple comparisons post-test was used to evaluate differences in acid secretion and peptide concentrations and differences in acid secretion and antagonist treatment between the groups. Friedman's test for paired observations with Dunnett's post-test was used to analyse variations in gut peptide concentrations.

Paper III
Data obtained with the Bravo capsule was analysed using the POLYGRAM NET ™ pH software. Results from YF476 and pentagastrin were analyzed as changes in pH at various time points from baseline (defined as 0.5 h prior to onset). The 2(-DeltaDelta C(T)) method was used for the qPCR measurements. To compare the data a one-way analysis of variance followed by a Dunnett´s multiple comparison test or Mann-Whitney test was used.

Paper IV
When comparing the MMC cycle length, duration and velocity of activity fronts, and in the fold expression changes in the qPCR analysis, the Mann-Whitney test was used.

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4 Results

4.1 Paper I

4.1.1 Comparison between Bravo and fistula methods

The Bravo system corresponded well with the chronic gastric fistula model as shown by Bland-Altman analysis. When treated with esomeprazole a rise in pH was seen in the Bravo system (pH 2.0 ±0.2 to 3.7 ±0.5) and a decrease in acid output in the fistula model (105 ±21 to 31 ±7 mmol L^{-1}; $P<0.05$). Pentagastrin stimulated acid secretion in the fistula model but a corresponding change in pH was not seen with the Bravo system.

4.1.2 Basal pH and pentagastrin with the Bravo system

The mean basal pH levels did not change significantly during fed or fasted conditions over a 24-h period (2.3 ±0.1 and 2.5 ±0.3, respectively). Neither did infusion of pentagastrin for 6 and 24 h during fed or fasted states compared to basal pH during saline infusion (fig 9).

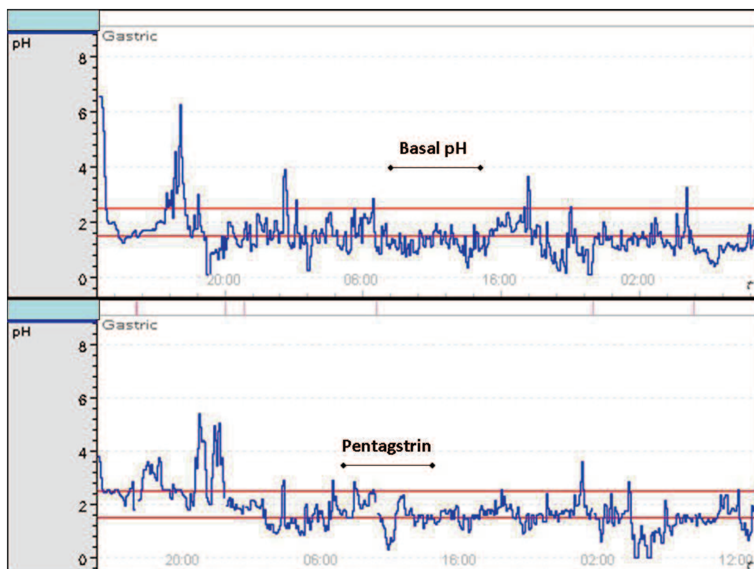

Figure 9. Standard recording with the Bravo system of intragastric pH in a rat during 48 h. The line in the top picture shows a basal period of intragastric pH registration for 6 h (8 am–2 pm) and the bottom one shows the effect of an infusion of pentagastrin during the same time period.

4.1.3 The effect of esomeprazole on pH and gut hormones

When given in bolus doses (1, 3, 5 mg kg^{-1}) esomeprazole increased intragastric pH in a dose-dependent manner (from pH 2.2 ±0.4 to 3.1 ±0.4 compared to baseline, 1.6±0.2) and was equally effective during both fed and fasted conditions compared to control (fig 10). Saline, as control did not change pH during fed nor fasted conditions.

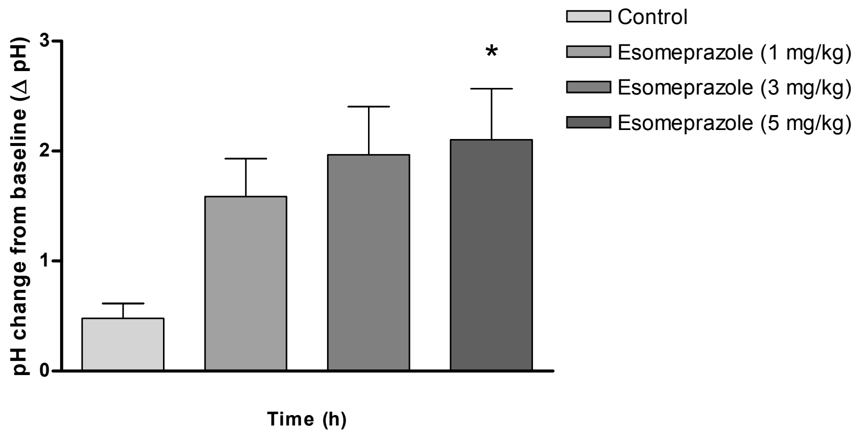

Figure 10. Change from baseline of intragastric pH ±SE after IV bolus of esomeprazole (1, 3, 5 mg kg^{-1}) and saline studied for 24 h during fed conditions. Mean for all doses *vs* control and for dose 1 mg kg^{-1} *vs* 5 mg kg^{-1} (*$P < 0.05$).

Esomeprazole (3 mg kg^{-1}) markedly elevated intragastric pH from 2.1 ±0.2 to 5.7 ±0.3 ($P<0.01$) during infusion of pentagastrin (90 pmol $kg^{-1}min^{-1}$) during 24 hours. When given as bolus dose esomeprazole 3 mg kg^{-1} increased the plasma concentrations of ghrelin and somatostatin, while gastrin remained stable over the period ($P<0.001$).

4.1.4 Ghrelin and pH

When administered IV ghrelin increased intragastric pH from day 1 to day 5 (2.5 ±0.6 to 2.8 ±0.5) compared to control (1.4 ±0.1 to 1.5 ±0.2) during the period ($P<0.01$).

4.2 Paper II

4.2.1 Perfusion of the Thiry-Vella loop with saline

Under control conditions perfusion of the intestinal loop with saline did not change basal gastric acid secretion. Pentagastrin stimulation (90 pmol $kg^{-1}min^{-1}$) increased acid secretion to half-maximum response ($P<0.01$), while the luminal peptide content (SOM, NT, and VIP) was unchanged. The only gut peptide that changed in plasma under control conditions was somatostatin which increased ($P<0.05$).

4.2.2 The effect of HCl and PEG

Perfusion of the loops with HCl inhibited pentagastrin-stimulated gastric acid secretion significantly more than PEG compared to saline ($P<0.01$; $P<0.06$ respectively) (fig 11). When challenged with acid the levels of SOM, NT, and VIP in the intestinal perfusate increased 1000- ($P<0.001$), 500- ($P<0.05$), and 260-fold compared to control. Perfusion with PEG gradually increased SOM reaching a maximum with a 3-fold increase over time ($P<0.01$) while NT and VIP did not change. In plasma, when challenged with HCl, only SOM increased ($P<0.01$) while NT and VIP were unchanged. PEG tended to increase SOM ($P<0.06$) as compared to NT and VIP.

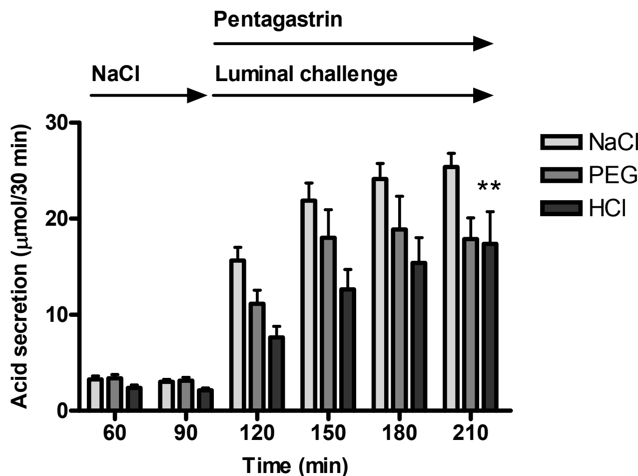

Figure 11. Luminal challenge of a jejunal Thiry–Vella loop with saline (NaCl, 154 mmol L^{-1}), hydrochloric acid (HCl, 50 mmol L^{-1}) and polyethylene glycol (PEG, 800 mOsm L^{-1}). ($P < 0.01$).

4.2.3 Effect of acid inhibitors on gastric acid secretion

In this study we examined the effect of the acid inhibitors esomeprazole, ranitidine, and gastrazole on acid output with different doses (0.15, 0.3, 1.5, 3, 9, 15 µmol kg^{-1}). At a dose of 0.3 µmol kg^{-1} gastrazole inhibited pentagastrin-stimulated acid secretion by 87 ±8% compared to esomeprazole 52 ±11%, and ranitidine 47 ±16%. Given at higher doses all acid inhibitors reached a similar maximum inhibition (see paper II).

4.3 Paper III

4.3.1 Intragastric pH and YF476

Administration of a bolus dose of YF476 (100 mg kg^{-1} intragastrically; 9 am and 3 pm) during continuous pentagastrin stimulation (90 pmol $kg^{-1}min^{-1}$; 6 h from 9 am to 3 pm and then from 4 pm to 8 am the following day; repeated for five consecutive days) increased intragastric pH compared to basal control conditions during the study period of five days. There were no changes of pH between night and day.

4.3.2 YF476, gut regulatory peptides and receptors

Given as bolus dose (100 mg kg^{-1} intragastrically) YF476 increased the plasma peptide ghrelin and somatostatin 12- and 6-fold, respectively, compared to control (P<0.001), while gastrin remain unchanged. There were no changes in gene expression of ghrelin, gastrin or SOM and their respective receptors in gastric tissue, except for the GHSR1a which decreased compared to the internal control, Hprt1 (p=0.026).

4.3.3 YF476 in gastric ulcer prevention

The gastroprotective ability of YF476 was in the same range as esomeprazole. This was reflected by the ulcer index which was reduced in the treatment groups receiving YF476 and esomeprazole (4.8 ±2.4 mm; P <0.001; 5.4 ±1.1 mm; P <0.01) compared to controls (70 ±14 mm).
The gene expression change of iNOS and gastrin with its corresponding receptor in gastric tissue increased 30- (P <0.01), more than 300- (P <0.01) and 18-fold (P <0.05) respectively. Ghrelin and somatostatin as well as their receptors did not change.

4.4 Paper IV

4.4.1 Small bowel motility

NPS administered IV at different doses (100-4000 pmol $kg^{-1}min^{-1}$) for 60 min induced irregular myoelectric spiking in the lower dose range (100, 300, 1000 pmol $kg^{-1}min^{-1}$). Higher doses (2000 and 4000 pmol $kg^{-1}min^{-1}$) besides inducing sporadic spiking, also prolonged the MMC cycle length and duration of the activity front in a dose-dependent manner. The propagation velocity was not changed (fig 12).

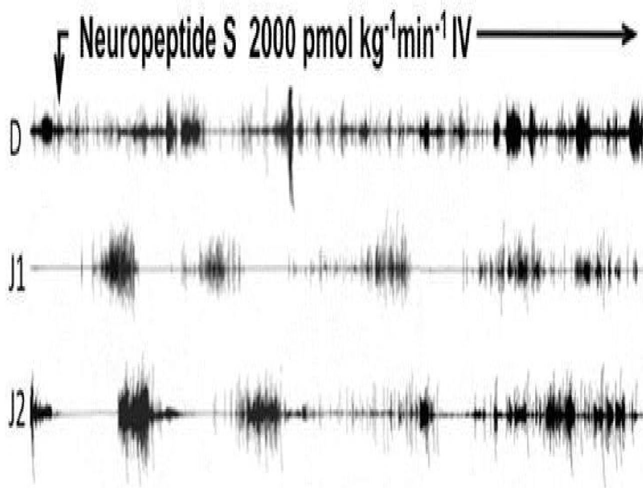

Figure 12. Electromyographic recording. Irregular spiking was induced during infusion of NPS at 2000 pmol $kg^{-1}min^{-1}$ intravenously (IV) followed by prolongation of the interval between activity fronts.

4.4.2 Expression of TNF, IL-1β, and iNOS

At 4000 pmol $kg^{-1}min^{-1}$ NPS induced a 1.8-, 2.9-fold increase in mRNA expression in tissue of TNF, IL-1β and iNOS compared to the internal control (18S).

5 General Discussion

We investigated the role of gut peptides on gastric acid secretion and their roles in inflammation.

In paper I we developed a novel method of measuring intragastric pH in the rat and compared it to a well known standard method, i.e. the chronic fistula. The purpose was to evaluate the Bravo system against the fistula method to see whether it was possible to make use of it as a supplement to the chronic fistula in an experimental model. We found that the Bravo model responded well in comparison to the fistula method and may be better from a physiological point of view. Animals restrained in small confinements, as in the chronic fistula, or restrained in any way should possibly have a negative influence on the results considering the stress imposed on them. The Bravo model allowed the animals to stay in their familiar environment during the experiments, and the capsule did not seem to hinder the digestion of food, taking into account the weight gained during the period of the trials. In the Bravo model rapid changes in pH after treatment of different substances could be registered instantaneously after administration which is not the case with the fistula model which relies on infrequent acid secretion volumes. On the other hand, the Bravo model is rather expensive, and because of the short battery time the experiments must start almost immediately after surgery, which could influence the results; even though we did not see any abnormalities in behavior or post mortal lesions. Also, the Bravo model does not show any true values of acid output because of the fact that it measures only pH intragastrically. This said, the Bravo capsule gave reliable recordings during all the experiments and should work as a complement to the chronic fistula model.
To be able to compare the two methods in experiments we gave pentagastrin which stimulated acid output in the fistula, but pH in the Bravo model was unchanged, this could be explained by the fact that pH as such is not affected by acid secretion changes. Esomeprazole, used as a comparable control of acid inhibition, showed an increased pH and decreased acid output accordingly when the two models were compared which gives us an indication of the functionality of the new model. When we measured the levels of the gut peptides gastrin, ghrelin, and SOM in plasma, gastrin proved to be unchanged in all the trials, which indicates that the capsule itself did not distend the stomach. Ghrelin and SOM increased during the treatment period. The mechanism behind this is not clear but a synergistic effect between these two peptides seems to be at hand [75, 139].

In paper II we examined the inhibitory impact of hydrochloric acid (HCl) and the hyperosmolal solution PEG on gastric acid secretion and release of gut peptides SOM, NT, and VIP, in addition to inhibitory drugs esomeprazole, ranitidine, and gastrozole. We found that HCl had the strongest inhibitory action compared to PEG, which also have been seen in other experiments [140, 141]. The subsequent release of peptides in the luminal perfusate, and in blood weighed in favor of SOM in the perfusate reaching 70 times higher concentrations than in blood. Generally, the luminal release dominated over the release to the blood. This implies a mechanism in which acid but also hyperosmolal solutions may reach the enteroendocrine cells, and even deeper into the mucosa to release peptides such as SOM, NT, and VIP. Considering the fact that these peptides influence both neuronal and endocrine pathways there could be a dual inhibitor mechanism involving either neuronal or endocrine responses to acid stimulation[5, 142, 143]. It is well known that low pH in the stomach affects the SOM-producing D cells to release SOM and subsequent inhibition of acid secretion[144, 145]. In our study we report that this phenomenon seems to be true not only for the stomach and duodenum, but also in the smaller

intestine. There could be a neuronal extrinsic mechanism involved in the small intestine, but it seems to be hidden by the stronger response of SOM to acid as shown in our work.
In this study we also evaluated the inhibitory effect of esomeprazole, ranitidine, and gastrazole on gastric acid secretion. Gastrazole proved to have the strongest inhibitory ability and this verifies the importance of gastrin as a key player in regulation of acid secretion from the parietal cell, and offers a novel pharmacological tool to study the mechanisms of gastric acid secretion.

In paper III we looked into the acid inhibitory effects of the CCK_2R antagonist YF476 mediated through regulatory peptides. The effects were studied both in animals receiving only YF476 under pentagastrin stimulation and animals with induced ulcers. Our study showed that YF476 administered twice daily in male rats during pentagastrin stimulation elevated pH significantly without any signs of desensitization of the drug. Further we found that YF476 increased pH to a level so as to prevent gastric ulceration by diclofenac in the same range as esomeprazole. Therefore YF476 could be expected to enter into treatments of different disorders such as peptic ulcer disease and upper gastrointestinal disorders[146]. In plasma the peptides ghrelin and SOM increased significantly compared to gastrin which was unchanged. The fact that somatostatin was increased during YF476 administration speaks of a further reduction in gastrin levels from the G cells[147, 148]. The reason for the corresponding increase of ghrelin is not fully explained but earlier research has shown this to be true[79] and may represent another aspect of regulatory mechanisms involved in gastric acid secretion[149].

During long-term administration of YF476 the most markedly change in mRNA expression was seen for the CCK_2R. This could reflect a compensatory effect considering that YF476 have their effect on the same receptor. The other receptors did also change but to a lesser extent and all to a considerable variability. This variation may be a natural condition in these individuals regarding their sensitivity to acid stimulation and regulatory peptides.
During exposure to NSAID-induced ulceration, an up-regulation of both gastrin and CCK_2R was seen, alongside the increase in iNOS expression, this may be important in the NSAID-induced ulcers seen. Reports have shown a subset of COX-2 dependent genes to directly influence gastric physiology. During treatment with NSAIDs there is an expression of this subset of genes implying a regulation under COX-2 expression[150]. In addition, gastrin enhances the stability and binding of the COX-2 mRNA, and there has been suggested that gastrin also activates COX-2 genes in CCK_2R containing cells that could further increase COX-2 expression[142, 151]. This is in line with our results and as the same genes are present in humans there is compelling new link between COX-2 and inflammation.

In paper IV we examined the impact of NPS on small bowel motility and inflammation. NPS noticeably provoked a response in the small intestine when administered peripherally with irregular spiking and prolonged MMC cycle length. It has been reported that NPS and its receptor NPSR1 is involved in different GI functions such as food intake, primarily gastric emptying[86] and susceptibility to inflammatory bowel disease[97]. This is implemented in our results with irregular spiking and extended MMC cycle length. There is some inconclusive evidence of NPS ability to stimulate motility reported by Han *et al* when administered intraperitoneally in doses much higher then ours. He could see no evident results, while there was a distinct evocation in motility when administered intracerebroventricularly (IVC)[88]. This pleads for a neurocrine or paracrine effect rather than an endocrine, considering the general concept of NPS as a neuropeptide. We also attempted to inquire whether NPS was able to elicit

an inflammatory response when administered IV, since it has been linked to inflammatory bowel disease. Our investigation showed an increase of TNF and IL-1β, while iNOS decreased. Recent discoveries of NPSR antagonists having no effects on basal motility but inhibiting exogenous NPS fits with the idea of NPS being associated with inflammation[152]. Though our results could not entirely abide this in terms of cytokines released, the ascertained increase in iNOS expression could be of importance since an uninterrupted up-regulation of NO in irritable bowel disease is beneficial[153, 154].

The inflammatory response of iNOS, TNF and IL-1β when subdued to NPS in our study increased in expression in the tissue which implies a clinical significance of NPS in mucosal healing abilities for conditions such as gastritis[155]. It is known though that TNF and IL-1β are strong inhibitors of gastric acid secretion under a pathophysiological response[156], and that during the same conditions the inflammatory response of iNOS seems to be an induction of NO to maintain the integrity of the gastric epithelium[157]. Secretion of NPS may possibly play a role in this process. The actions of ghrelin, gastrin and somatostatin, and related gut peptides are involved in the regulation of the normal physiology, but some also play a part in inflammation and control of inflammatory responses, such as ghrelin that seems to have a modulatory impact on the course of events during an inflammation[158-160]. This shows an intricate connection between different factors influencing acid secretion and inflammation.

6 Summery and Conclusions

We have shown that gastric acid secretion is regulated by a number of different mechanisms, including neurocrine, paracrine, and endocrine pathways. This has been studied in different systems and in a newly developed model – The Bravo capsule system – we could conclude that prolonged studies of intragastric pH when challenged with acid stimulation (pentagastrin) or inhibition (esomeprazole) gave reliable results compared to the standard model of chronic fistula in rats, as seen by acid and gut peptide secretion measurements. Furthermore, we could support the idea that ghrelin has a stimulatory effect on gastric acid secretion when administered IV in rats.

We have also illustrated the effect of luminal exposure to hydrochloric acid and hyperosmolal solutions (PEG) on the release of peptides SOM, NT and VIP . Not only in the duodenum but also in the small intestine the acid, when released into the lumen triggers a negative feedback mechanism which secretes these intestinal peptides. Also PEG stimulates the peptides mentioned but to a much lesser extent. It is obvious that acid is the more powerful inhibitor of acid secretion in the smaller intestine because of the potential harm it may cause compared to the effects of osmolality.
There was a dominance of SOM release compared to the other peptides, and it was more pronounced in the luminal perfusate than in the bloodstream.
The inhibitory effect of the CCK_2R antagonist gastrazole was most potent compared to the actions of esomeprazole and ranitidine, which verifies the importance of gastrin for an acid response.

The CCK_2R antagonist YF476 inhibits gastric acid secretion and promotes mucosal healing. It increases gastrin, ghrelin, and SOM levels in plasma, and the mRNA expression of the ghrelin receptor in gastric tissue. Treatment with NSAID up-regulates the mRNA expression of gastrin and the CCK_2R, indicative of a connection between gastrin, CCK_2R, and COX-2 in the pathogenesis of NSAID-induced gastropathy.

We also studied the effect of NPS administration on intestinal motility and inflammatory response. We could conclude that a motility response similar to that in inflammatory reactions was seen. Though an early response to NPS did not without exception cause an inflammatory reaction, extended activation of the NPS/NPSR system may be involved in inflammatory responses of the gut.

7 Summary in Swedish

Utsöndringen av syra i magsäcken är en komplex process som involverar en mängd olika mekanismer. Bland annat är ”samtalet” mellan olika peptider en viktig beståndsdel i denna process.
Syftet med avhandlingen var att utveckla en ny metod för att mäta pH i råtta under en längre tid och att sedan jämföra denna nya metod med en etablerad. Vi ville studera eventuella förändringar i utsöndringen av syra och i MMC-mönstret på råtta vid behandling med olika substanser. Till yttermera visso ville vi undersöka eventuella förändringar i genuttryck av olika mag-tarmpeptider och inflammationsmarkörer i normal och inflammerad vävnad.

Våra resultat visade att Bravometoden stod sig väl i jämförelse med den etablerade fistelmetoden. Vid behandling med esomeprazol (nexium) steg pH-värdet i Bravometoden, medan utsöndringen av syra sjönk proportionellt i fistelmetoden. Mag-tarmpeptiderna ghrelin och somatostatin ökade i plasma medan gastrin förblev oförändrad vid behandling med esomeprazol. Vid behandling med ghrelin ökade pH-värdet liksom också tidigare forskning visat.

Peptiderna SOM, NT och VIP ökade både i plasma och tarmperfusatet vid behandling med HCl och PEG. Av de tre peptiderna var det SOM som ökade mest i framför allt tarmperfusatet, men också i plasma. Gastrazol påvisade den starkast hämmande effekten på utsöndringen av magsyra i jämförelse med de två andra syrahämmande preparaten ranitidin och esomeprazol.

YF476, en CCK_2-receptorhämmare, blockerade utsöndringen av syra under en längre tid och ökade koncentrationerna av ghrelin och somatostatin i plasma. Nivåerna av gastrin förändrades emellertid inte under samma tidsperiod. Mängden gastrin, ghrelin och somatostatin i magvävnaden varken ökade eller minskade när enbart YF476 gavs och av deras respektive receptorer var det bara GHSR1a som förändrades. Vid behandling med NSAID-preparat ökade dock uttrycket av gastrin, CCK_2-receptorn och iNOS, men ingen av de andra peptiderna och deras receptorer.

Behandling med NPS stimulerade till en förändring i MMC-mönstret med oregelbundet spikmönster och förlängt avstånd mellan aktivitetsfronterna. Mängden iNOS, TNF, and IL-1β ökade också i vävnaden.

Slutsatsen man kan dra är att Bravometoden är tillförlitlig vid mätningar av pH och kan användas som ett komplement till fistelmetoden. Regleringen av syrasekretionen tycks inte endast begränsad till magen utan verkar också kunna kontrolleras från tunntarmen av framför allt somatostatin som frigörs från magsäcken. Olika mekanismer ligger bakom YF476 syrahämmande förmåga och vid behandling med NSAID-preparat ökar uttrycket av gastrin, CCK_2-receptorn och iNOS.
NPS är en tämligen nyupptäckt neuropeptid som tycks påverkar motiliteten i mag-tarmkanalen och som kan ha en påverkan på inflammatoriska reaktioner. Detta såg vi en indikation på genom att NPS förändrade MMC-mönstret och ökade uttrycket av de inflammatoriska markörerna iNOS, TNF, and IL-1β.

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10 Appendix (paper I-IV)

I

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BASIC RESEARCH

Bravo capsule system optimizes intragastric pH monitoring over prolonged time: Effects of ghrelin on gastric acid and hormone secretion in the rat

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Abstract

AIM: To evaluate measurements of intragastric pH with the Bravo capsule system over a prolonged time.
METHODS: A Bravo capsule was placed inside the rat gastric body and pH was studied for periods up to five consecutive days. For comparison, a gastric fistula model was used. Effects of ghrelin and esomeprazole, with or without pentagastrin, on gastric pH were studied. In addition, effects of esomeprazole on plasma ghrelin, gastrin and somatostatin were analyzed.
RESULTS: All rats recovered after surgery. The average 24-h pH during free feeding was 2.3 ± 0.1 (n = 20) with a variation of 18% ± 6% over 5 d. Ghrelin, 2400 pmol/kg, t.i.d. increased pH from 1.7 ± 0.1 to 3.1 ± 0.3 (P < 0.01) as recorded with the Bravo system. After esomeprazole (1 mg/kg, 3 mg/kg and 5 mg/kg) there was a dose-dependent pH increase of maximally 3.4 ± 0.1, with day-to-day variation over the entire period of 8% ± 3%. The fistula and pH studies generated similar results. Acid inhibition with esomeprazole increased plasma ghrelin from 10 ± 2 pmol/L to 65 ± 26 pmol/L (P < 0.001), and somatostatin from 10 ± 2 pmol/L to 67 ± 18 pmol/L (P < 0.001).
CONCLUSION: pH measurements with the Bravo capsule are reliable, and comparable to those of the gastric fistula model. The Bravo system optimizes accurate intragastric pH monitoring over prolonged periods and allows both short- and long-term evaluation of effects of drugs and hormones.

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Key words: Gastric acid; Bravo system; Intragastric pH; Ghrelin; Somatostatin

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INTRODUCTION

In the past, different techniques have been employed to study gastric acid secretion in rodents. The main principle for these methods has been collection of gastric juice, and in order to measure acid secretion, pH titration has been carried out. One of the earliest methods was the pylorus ligation technique[1]. The principle of this method is distension of the stomach as a potent stimulus of acid secretion. Later, this method was altered with an esophageal ligature[2,3], after which the stomach of the rat was removed and secretions analyzed. Esophageal ligation in the pylorus-ligated rat has been shown to significantly inhibit acid secretion by inhibition of central vagus function[2]. Since then,

the most reliable method has been the chronic fistula method[4-6] where a gastric fistula is implanted at the greater curvature of the stomach. This technique requires movement restriction of the animal which is in a conscious state during the study. The gastric contents are collected and acid output measured. This technique allows re-use of animals following a recovery period from the experimental procedure. Other methods used today are perfusion of the gastric lumen[7] and isolated perfused, as well as vascularly perfused rat stomach[8-10].

Most of the above studies have the drawback that they do not measure intragastric pH directly and are not very physiological, as the animal is either restrained or anesthetized. The main goal of this study was to test the feasibility of a capsule normally used in the clinical setting in humans to measure gastroesophageal reflux disease (Bravo system) for monitoring intragastric pH in the rat. The Bravo capsule system has primarily been used in humans[11-14], but also in animals[15] for diagnosis of gastroesophageal reflux disease.

The aim of the study was to evaluate the Bravo capsule for pH monitoring in the rat. To validate the method, we compared the data to those of the standard gastric fistula model.

MATERIALS AND METHODS

Animals

Sprague-Dawley male rats (300-350 g) were purchased from Scanbur B&K AB (Sollentuna, Sweden). The rats were housed in wire-meshed cages at 24℃ with constant humidity and 12:12 h light-dark cycle. The animals were fed *ad libitum* with a commercial rat diet consisting of pellet (LABFOR, Lactamin R36, Kimstad, Sweden) and tap water prior to the studies. The experiments were approved by the Animal Ethics Committee in northern Stockholm.

Surgery

Surgery was performed under anesthesia with pentobarbital sodium (50 mg/kg; Apoteket AB, Stockholm, Sweden) intraperitoneally, and Hypnorm (fentanyl citrate, 0.315 mg/kg and fluanisone 10 mg/kg; Janssen, Oxford, USA) intramuscularly. Marcain (bupivacaine hydrochloride, 2.5 mg/kg; AstraZeneca, Södertälje, Sweden) was given subcutaneously after surgery along the abdominal incision.

For the Bravo system studies, a midline incision was performed, and a small opening created in the proximal greater curvature, and gastric contents were evacuated. An externally pre-calibrated (buffers pH 1.07 and 7.1) Bravo capsule (an electronic sensor encapsulated in PVC-plastic, 25 mm × 5 mm × 5 mm; Synmed Medicinteknik AB, Spånga, Sweden) was placed inside the stomach with the pH sensor pointing distally and anchored with a suture. An indwelling silastic catheter (Dow Corning Co., Midland, MI, USA) was inserted into the external jugular vein.

For the gastric acid fistula studies, rats were provided with a plastic gastric fistula placed immediately proximal to the oxyntic gland area near the greater curvature. The fistula was closed between experimental periods. A silastic catheter was implanted into the external jugular vein for drug administration.

Studies of intragastric pH (Bravo system)

Studies of intragastric pH began in the morning 2 d after surgery. The studies were carried out in conscious rats, one experiment for each rat, under normal conditions, or after a 16-h fasting period in wire-bottom cages with free access to water. The animals gained weight (10 ± 3.4 g during 1 wk) and behaved in a normal fashion, with a normal feeding pattern throughout the experiments. At post-mortem examination, no mucosal lesions, obstruction of the pylorus or gastric distension were seen. Drugs were administered through the external jugular vein in all experiments.

The pH recorded by the Bravo capsule was transmitted to the Bravo receiver placed directly outside the cage. The sampling frequency was 6 Hz. The Bravo system was set for a 48-h registration period, after which the data were downloaded, batteries replaced and recording continued. This procedure was then repeated in two more 48-h periods.

All test compounds were dissolved and diluted in isotonic saline solution (sodium chloride, 9 g/L; 300 mosm/kg H_2O, Fresenius Kabi, Halden, Norway).

The effect of ghrelin on pH: The effect of ghrelin on intragastric pH was studied with ghrelin (2400 pmol/kg) given t.i.d (08:00, 12:00 and 16:00) for 5 d in a row (n = 7).

Evaluation of basal pH: Baseline pH was studied over 24 h under fed (n = 20) and fasting (n = 8) conditions.

Effect of esomeprazole on pH: The effect of increasing bolus doses of esomeprazole (AstraZeneca) (1 mg/kg, 3 mg/kg or 5 mg/kg iv, n = 10) or saline (iv, n = 8) was studied for 24 h in fed rats. Furthermore, the effect of esomeprazole (3 mg/kg iv, n = 10) or saline (iv, n = 8) was studied for 24 h in fasting rats.

The effect of pentagastrin and esomeprazole on pH: The effect of esomeprazole (3 mg/kg iv, n = 10) or saline (iv, n = 8) was studied under pentagastrin (NeoMPS, Strasbourg, France) infusion (90 pmol/kg per min, iv) over 6 h in both fed and fasting rats. In these experiments, the rats were restrained in Bollman cages to mimic the gastric fistula studies and for infusion of pentagastrin.

The effect of a 24-h infusion of pentagastrin (90 pmol/kg per min iv, n = 6), of esomeprazole (9 pmol/kg per min, n = 6), or saline (0.154 mol/L, n = 6) on pH was studied.

Plasma levels of gut hormones: The effect of esomeprazole (3 mg/kg iv) on plasma levels of ghrelin, gastrin and somatostatin was studied. A group of animals (n = 10) was divided into two treatment groups (each n = 5). All animals were treated with esomeprazole

daily during 1 wk. The first group of animals was then euthanized, while the other group was followed for another week without esomeprazole and then euthanaized. Blood was drawn and centrifuged, and plasma assayed for concentrations of ghrelin, gastrin and somatostatin.

For ghrelin measurements, the ghrelin (active) radioimmunoassay kit (Linco Research, St. Charles, MI, USA) was used, which utilizes ^{125}I-labeled ghrelin and ghrelin antiserum to determine the level of active ghrelin in plasma. For the analysis, a Gamma Master 1277 (LKB-Wallac, Perkin-Elmer Inc, Massachusetts, NH, USA) was used. The intra- and interassay coefficients of variation were 7% and 14%, respectively.

Somatostatin was analyzed using an EIA kit (EK-060-03) from Phoenix Pharmaceuticals, Burlingame, CA, USA), which reacts 100% to somatostatin-14 and somatostatin-28. The intra- and interassay coefficients of variation were 5% and 14%, respectively.

Gastrin was analyzed using C-terminal-directed CCK/gastrin antiserum 2609/10 (Rehfeld, 1978). Chloramine-T-labeled and HPLC-purified gastrin-17 (NeoMPS) was used as radioligand and gastrin-17 as calibrator/standard. The intra- and interassay coefficients of variation were 6% and 8%, respectively.

Studies of gastric acid secretion (fistula)

Studies of gastric acid secretion began 7 d after surgery. The animals gained weight (8 ± 2.6 g during 1 wk) and had normal behavior during the experimentation periods. Prior to each experiment, food was withheld for 18 h, but with free access to water. At the start of the experiments, the stomach was rinsed with 10-15 mL luke-warm tap water to evacuate remaining food, followed by a 30-min period before the experiments were started. During the experiments, the conscious rats were placed in Bollman cages. Gastric juice was collected at 30-min intervals, and volumes measured to the nearest 0.1 mL. pH was calculated by back-titration using 0.1 mmol/L sodium hydroxide. Acid output was calculated by multiplying the secretion volumes with hydrogen ion concentrations and expressed as μmol per 30-min period.

Baseline acid secretion was studied for 60 min followed by esomeprazole (3 mg/kg iv), after which acid secretion was studied for another 2 h. During the experiment, saline was administered in the same amount as collected from the gastric fistula to compensate for the volume loss during the experiment. Furthermore, baseline acid secretion was studied for 60 min, followed by an infusion of pentagastrin (90 pmol/kg per min) for 4 h. After 1 h of pentagastrin infusion, a bolus of esomeprazole (3 mg/kg iv) was administered and acid secretion studied for another 3 h.

Data and statistical analysis

The data obtained with the Bravo capsule analyzed using (POLYGRAM NET™ pH Testing Application software, Synmed Medicinteknik) in 48-h periods. Results of studies with esomeprazole were analyzed by calculating changes in pH at various timepoints from baseline (defined as 0.5 h prior to onset of studies). For analysis of the fistula studies, the first 30-min collection was discarded and the second collection used as baseline for comparison with esomeprazole and pentagastrin.

All data are mean ± SE. A Kruskal-Wallis test followed by Mann-Whitney *U* test was used for statistical comparisons using specific time points for pH. $P < 0.05$ was considered statistically significant. For comparison of the variability between the fistula and the Bravo system the Bland-Altman analysis was used[16,17]. The Prism software package 4.0 (GraphPad Software Inc., San Diego, CA, USA) was used for the statistical comparisons.

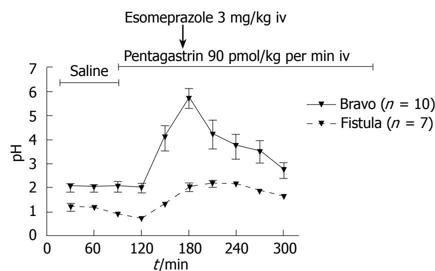

Figure 1 Change in pH ± SE in the Bravo system and gastric fistula model during fasting conditions after iv bolus of esomeprazole (3 mg/kg) and pentagastrin infusion (90 pmol/kg per min) for 2 h.

RESULTS

Comparison between the Bravo system and the fistula model

Pentagastrin resulted in a marked increase, 83 ± 9 mmol/L to 132 ± 8 mmol/L ($P < 0.05$) of acid output in the fistula model, which was not evident as a corresponding decrease in pH with the Bravo system. During esomeprazole treatment, there was a marked increase in pH from 2.0 ± 0.2 to 3.7 ± 0.5, as recorded with the Bravo system and correspondingly, a marked decrease in acid secretion from 105 ± 21 mmol/L to 31 ± 7 mmol/L in the fistula model ($P < 0.05$; Figure 1). Bland-Altman analysis of these conditions showed a high degree of agreement between the Bravo system and the fistula method as shown in Figure 2.

Evaluation of basal pH

A typical 120-h baseline registration including dose of esomeprazole (day 1, 3 and 5) with the Bravo system is shown in Figure 3. The feeding status did not alter the mean pH over 24 h, but increases in pH were observed during afternoon and night-time when animals were fed. The mean 24-h pH was 2.3 ± 0.1 during fed conditions and 2.5 ± 0.3 during fasted conditions, with 18% ± 6% variation during the next four 24-h periods. There was no difference in pH between daytime and night-time (1.4 ± 0.1 and 1.7 ± 0.2, respectively).

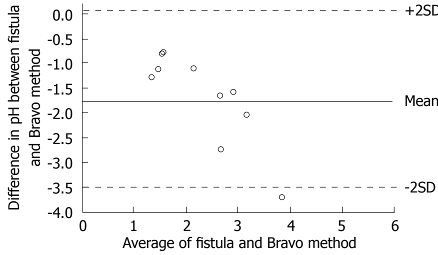

Figure 2 Bland-Altman analysis comparing the Bravo system with the fistula method. Mean value -1.7 with 2SD from -3.5 to 0.12.

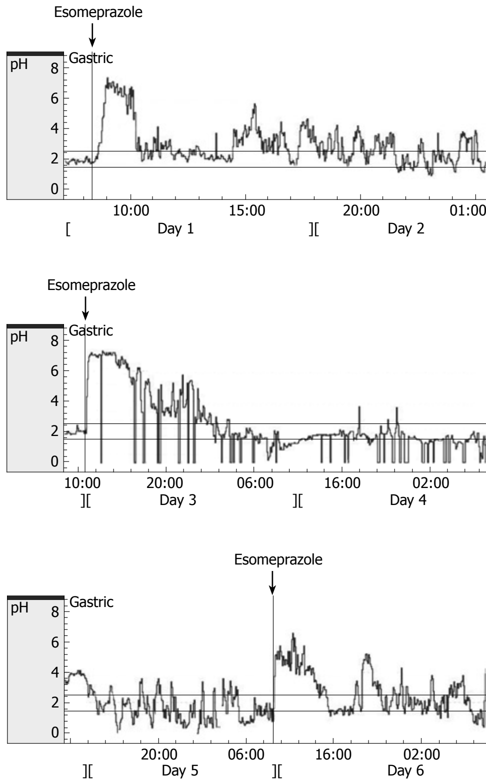

Figure 3 Standard recording with the Bravo system of intragastric pH in a rat during 6 d. The solid line indicates bolus doses of esomeprazole (3 mg/kg) given iv during 3 min under fed conditions.

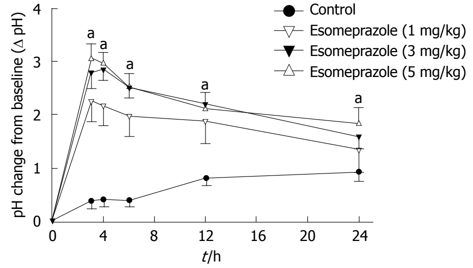

Figure 4 Change from baseline of intragastric pH ± SE after an iv bolus of esomeprazole in three different doses and saline studied for 24 h during fed conditions. Mean for all doses *vs* control and for dose 1 mg/kg vs 5 mg/kg ($^{a}P < 0.05$).

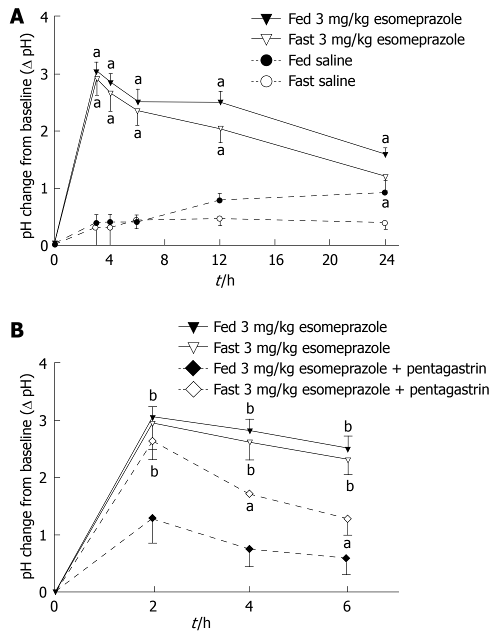

Figure 5 Changes of intragastric after an iv bolus of esomeprazole. A: Change from baseline of intragastric pH ± SE after an iv bolus of esomeprazole (3 mg/kg) or saline during fed or fasting conditions for 24 h ($^{a}P < 0.05$); B: Change form baseline of intragastric pH ± SE after an iv bolus of esomeprazole (3 mg/kg) or saline during pentagastrin (90 pmol/kg per min) infusion during fed or fasting conditions during 6 h. Mean esomeprazole *vs* esomeprazole and pentagastrin for fed ($^{b}P < 0.01$). Mean esomeprazole fed *vs* fasting esomeprazole and pentagastrin ($^{a}P < 0.05$). Mean esomeprazole fed *vs* fasting esomeprazole and pentagastrin ($^{b}P < 0.01$).

The effect of bolus esomeprazole on pH

As studied over 24 h, there was a dose-dependent increase of pH after esomeprazole, 1 mg/kg, 3 mg/kg, and 5 mg/kg, during free roaming conditions (Figure 4). Already 3 h after administration of esomeprazole, pH was significantly higher with 5 mg/kg, 3.1 ± 0.4, than with 1 mg/kg, 2.2 ± 0.4 ($P < 0.05$). Esomeprazole (3 mg/kg) increased intragastric pH during saline infusion over a 6-h period (2.5 ± 0.2) compared to baseline pH (1.6 ± 0.2), whereas saline did not ($P < 0.01$; Figure 5A).

Esomeprazole was equally effective during fed or fasting conditions (Figure 5A). As a control, saline did not change intragastric pH during either fed (baseline pH 1.4 ± 0.1) or fasting (baseline pH 1.6 ± 0.2) conditions (Figure 4, Figure 5A).

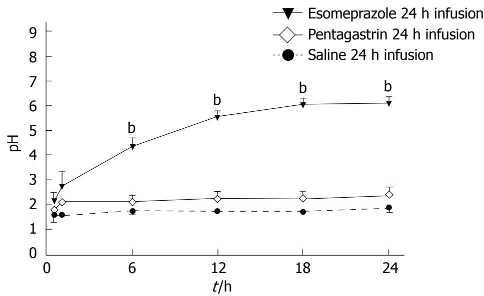

Figure 6 Change in mean pH ± SE over time. Mean esomeprazole (9 pmol/kg per min) 24 h infusion and pentagastrin (90 pmol/kg per min) infusion. (^bP < 0.01 *vs* control).

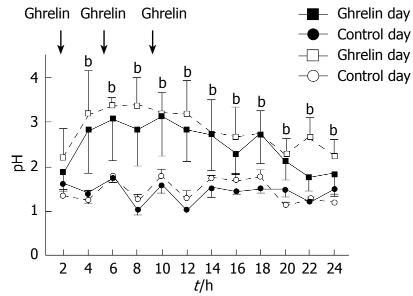

Figure 7 Change in mean pH ± SE during treatment with bolus dose ghrelin t.i.d. (2400 pmol/kg, ^bP < 0.01 ghrelin *vs* control). There was no day-to-day difference in variation of the ghrelin response during the 5 d.

The effect of pentagastrin on pH

Pentagastrin alone did not change pH over 6 h compared with fed (baseline pH 2.1 ± 0.2) or fasting (baseline pH 2.4 ± 0.2) conditions. After esomeprazole (3 mg/kg), pentagastrin infusion markedly decreased pH from 2.0 ± 0.3 to 1.0 ± 0.2 (P < 0.05, Figure 5B). This effect was most marked in fed animals.

The effect of 24-h infusion of esomeprazole on pH

After esomeprazole (9 pmol/kg per min) the average 24-h pH was substantially higher than in the controls, 5.7 ± 0.3 and 2.1 ± 0.2, respectively (P < 0.01). Pentagastrin alone did not change pH over the 24-h infusion period as compared to saline (Figure 6).

The effect of ghrelin on pH

Administration of ghrelin, t.i.d markedly increased gastric 24-h pH from day 1 (2.5 ± 0.6) to day 5 (2.8 ± 0.5) compared to control day 1 (1.4 ± 0.1) and day 5 (1.5 ± 0.2) (P < 0.01; n = 7). There was no significant day-to-day variation of the ghrelin effect during the five days (Figure 7).

Plasma levels of gut hormones

Esomeprazole (3 mg/kg) t.i.d resulted in a marked increase in plasma ghrelin and somatostatin concentrations as shown in Figure 8 (P < 0.001). Plasma gastrin, however, remained stable over the same time period (Figure 8).

DISCUSSION

This study demonstrates that the Bravo system can be used for studies of intragastric pH in rats and that the results are comparable to those of a standard fistula model. The system allows for long-term studies during unrestrained living conditions. There are several advantages with the use of the Bravo system. Previous models for studies of gastric acid secretion do not allow measurements of pH over a long time. Furthermore, during these studies, the animals are kept under stressful conditions, which to a certain degree, may influence the responsiveness of the animals to different stimuli. The Bravo system uses a telemetric system that records gastric pH during 24 h for up to five consecutive days. The day-to-day variation was within acceptable limits. The system allows for real-time recordings of intragastric pH with the ability to record from the start of a treatment until a detectable effect is seen. The system is suitable for long-term studies with continuous infusions that are difficult to perform using the fistula model, as the animals do not tolerate being restrained in cages during prolonged studies. The Bravo recording system is also a digital recording system, which means that primary data are logged, and permits detailed measurements as determined by the set sampling frequency.

The data are, however, limited to pH-values as no secretion volumes are obtained. With the gastric fistula model, recordings are made over no less than 15-min periods, which can be a limiting factor as regards rapid changes in pH, i.e. drug effects. However, in the fistula model, secretion volumes are recorded, which permit calculation of a true acid output. The Bravo system has a few drawbacks. It is expensive, the battery life of the capsule is short (5 d) and therefore, the animals can only be used in studies for about a week. This means that experiments must start immediately after the operation (in this case 2 d after the surgical procedure), and the recovery from surgery may influence the results and the comparison with the fistula model. Despite this, the Bravo system seems to be well tolerated, as the stomach of the rats did not show any abnormalities or mucosal lesions upon autopsy. The animals also gain weight and behaved in a normal fashion during the experiments.

From a physiological viewpoint, our results demonstrated expected results; intragastric pH in rodents was stable over time, with a slight increase during the night during fed conditions.

In addition, treatment with esomeprazole and pentagastrin gave expected results. The agreement between the Bravo system and the established fistula method was evaluated employing a Bland-Altman analysis. When the two methods were compared, the pH results obtained with the Bravo system were comparable to those obtained using the fistula model. The differences lie within acceptable limits of agreement approximately 95% of the time, and the variability

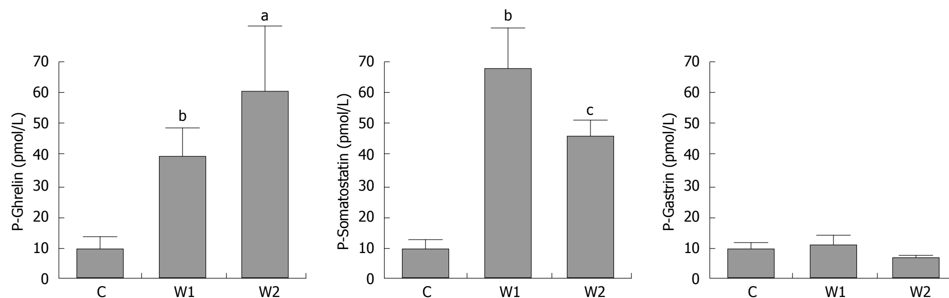

Figure 8 Gut peptide concentrations during treatment week 1 (W1) and week 2 (W2). The two groups of animals (n_{tot} = 10) were treated during one week with esomeprazole (3 mg/kg). After the first treatment week the first group (n = 5) were euthanaized and plasma were taken for peptide measurements. The other group (n = 5) went on for another week without any drug treatment and then euthanaized and plasma taken for analysis of peptides. $^aP < 0.05$; $^bP < 0.01$; $^cP < 0.001$ *vs* control.

was consistent across the graph; the scatter around the baseline (mean) did not increase with increasing means.

During comparative studies, the animals were restrained in Bollman cages for infusion of pentagastrin, so the experimental conditions were the same. During esomeprazole treatment, pH rose and gastric acid output decreased accordingly. There seemed to be a slight delay in response to esomeprazole when studied by the fistula method as compared to the Bravo system. The reason for this is probably related to the fact that the secretory response depends on the physical emptying of gastric contents from the fistula until measurements can be done. As judged from our experiments, this causes a delay of the recorded response of about 30 min. Pentagastrin increased acid output, but no change was seen in intragastric pH with the Bravo system. This is explained by the fact that a change in secretion volume does not affect the pH recorded, even though acid output is changed. The fact that pH does not change when introducing pentagastrin may be due to the constantly low basal pH level in the rat stomach.

The gut hormones assayed in this study, ghrelin, gastrin, and somatostatin, are all found in the mucosa of the stomach[18]. They operate in a coherent inhibitory/stimulatory fashion against one another, i.e. increasing levels of somatostatin stimulates ghrelin, while gastrin is inhibited[19,20]. Pentagastrin acts as an agonist on acid secretion and has a stimulatory effect on somatostatin, which in turn down-regulates the release of gastrin so that excessive amounts of acid are not produced[20]. The fact that basal plasma gastrin levels remained stable with the Bravo system indicates that the Bravo capsule by itself does not distend the stomach to such a degree that gastrin levels are affected[21].

Our results using the Bravo system, with an increase of intragastric pH during 1 wk after three times daily, administration of ghrelin, are in accordance with earlier studies[19,22], but at variance with another[23]. This may be explained by the fact that different methods for studying gastric acid secretion have been employed, some of which are dependent on gastric motility for the emptying of gastric secretions through the fistula. By using the Bravo system, we found no desensitization of the pH response to ghrelin. This is at variance with our previous studies on intestinal motility, in which a loss of the ghrelin response was shown[19,24]. This might be due to the fact that motility was stimulated by a continuous infusion of the hormone, whereas the pH effect was brought about by repeated injections of ghrelin, a form of administration that is considered less liable to desensitization effects. As ghrelin not only increases intragastric pH, but also stimulates gastric emptying in rodents[22,23,25,26]. This may be an erroneous factor in determining acid secretion using the fistula method.

With esomeprazole treatment, plasma concentrations of ghrelin and somatostatin were increased. This effect was maintained for 1 wk after esomeprazole treatment. The underlying mechanism for this increase in plasma ghrelin and somatostatin is not yet fully understood, but may be due to a direct effect of esomeprazole on ghrelin and somatostatin, but also by an indirect effect through changes in gastric pH. The counter-balancing effects between pentagastrin (low pH) and esomeprazole (high pH) as regards ghrelin levels point to a physiological role of ghrelin in the control of gastric acid secretion[27,28]. The rise in somatostatin concentration is likely due to a direct effect of the continuous doses of ghrelin, as pH was not affected. The lack of elevated levels of gastrin for the two groups are probably attributed to the increase in somatostatin[29] or, although less likely, low doses of esomeprazole[30,31].

To conclude, the Bravo capsule system is to be used for prolonged studies of gastric pH in free roaming conscious rats over days and is well tolerated, and could serve as a complement to the gastric fistula model, as shown by acid and gut hormone secretion measurements.

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COMMENTS

Background

The pharmacological treatment of gastrointestinal acid-related diseases aims at providing ulcer and mucosal healing, symptom relief and improved quality of life. Gastric acid inhibitory compounds are widely used in the clinical setting in order to treat not only benign gastric and duodenal ulcers, but also gastritis and reflux esophagitis. Over the past two decades, there has been a number of reports on the use of proton pump inhibitors (PPIs) such as omeprazole and the following competitors. The PPIs are activated in the acid environment in the stomach and inhibit the final step of gastric acid secretion. They bind in a non-competitive way to the H^+, K^+ -ATPase and inhibit secretion. Even though the PPIs have many good properties compared to other treatment regims, and are considered the treatment of choice in acid-related gastrointestinal diseases, there are drawbacks with PPI treatment. For instance, the onset of action is slow as compared to that of H2-receptor antagonists, which induce an immediate acid inhibition, and the duration of action may be too short giving room for night-time acid breakthrough. So far, treatments have got around this problem by recommending a two-dose regimen. Pharmaceutical development has been directed against finding a compound with profound acid inhibitory action over prolonged periods of time, not permitting night-time acid breakthrough to take place. The development of such drugs, however, require new methods of studying gastric acid secretion over prolonged periods, up to 120 h over or more.

Research frontiers

Research concerning acid-related diseases has been focused on PPIs targeted against the H^+, K^+ -ATPase of the stomach and H2-receptor antagonists. Recent studies have shown that the proton pump is the most likely candidate for a sustainable therapeutic application in the regulation of acid suppression. One of the hurdles in this field is the possibility to perform long-term measurements of acid secretion in the development of pharmacological treatment of acid diseases. Although PPIs are highly effective as a class, differences in their pharmacokinetics, such as bioavailability, metabolism, and elimination half-life, may translate into differences in clinical outcomes.

Innovations and breakthroughs

Over the latest years, new drugs have emerged on the market, such as being PPIs (3rd generation), new potassium channel blocking agents that inhibit gastric secretion (P-CAP), and even combinations of PPIs and H2-receptor antagonists. A second line to this further development is to be expected and with this new method, developed as a tool for evluation of such long-acting drugs, may become a feasible tool in the clinical setting for treatment of acid-dependent diseases.

Applications

Our research demonstrates stable recordings with the Bravo capsule system in the rat. The animals were given PPI and ghrelin and this resulted in an almost immediate response in pH, sustained during approximately 6 h. The capsule model was compared with the fistula model and showed agreement in compliance between the two methods. This indicates that the capsule model could eventually replace the fistula model. It seems better to use the former method because of less strain on the rats, and easier and more gentle handling and experimental procedures. Furthermore, the Bravo system set-up is easy to manage and the information recorded allows many different analysis variables. The system also records over five consecutive days, which previously has not been possible in this setting.

Terminology

Bravo capsule system: A catheter-free system used to measure esophageal pH (acidity) levels in patients who have or are suspected of having gastroesophageal reflux disease, but has now also been used for intragastric titration of pH.

Peer review

The measurement of intragastric pH with the Bravo capsule system is comparable to that of the gastric fistula model, and is useful for prolonged studies of gastric pH, even in free roaming conscious rats over days, as described. Although further studies are required, this study indicates the novel possibility for investigating the acid and gut hormone secretion under more physiological conditions.

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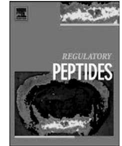

Release of regulatory gut peptides somatostatin, neurotensin and vasoactive intestinal peptide by acid and hyperosmolal solutions in the intestine in conscious rats

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ABSTRACT

The impact of exposure of the intestinal mucosa to acid and hyperosmolal solutions on the release of the inhibitory gut peptides somatostatin (SOM), neurotensin (NT) and vasoactive intestinal peptide (VIP) was studied in conscious rats during pentagastrin-stimulated gastric acid secretion. The animals were equipped with a chronic gastric fistula to measure acid secretion and a jejunal Thiry–Vella loop for intestinal challenge with saline, hydrochloric acid (HCl, 200 mmol L^{-1}) or hyperosmolal polyethylene glycol (PEG, 1200 mOsm kg^{-1}). Gut peptide concentrations were measured in intestinal perfusates, and in plasma samples collected during stimulated acid secretion, and at the end of experiments with luminal challenge of the loops.

After pentagastrin-stimulation acid secretion was dose-dependently inhibited by intravenous administration of the gastrin receptor antagonist gastrazole, as well as ranitidine and esomeprazole by maximally 73±10%; 95±3%; 90±10%, respectively.

Acid perfusion of the Thiry–Vella loop caused a prominent release of SOM both to the lumen (from 7.2±5.0 to 1279±580 pmol L^{-1}) and to the circulation (from 18±5.2 to 51±9.0 pmol L^{-1}) simultaneously with an inhibition of gastric acid secretion. The release of NT and VIP was not affected to the same extent. PEG perfusion of the loop caused a release of SOM as well as NT and VIP, but less. Simultaneously acid secretion was slightly decreased.

In conclusion, intestinal perfusion with acid or hyperosmolal solutions mainly releases SOM, which seems to exert a major inhibitory action in the gut, as shown by inhibition of acid secretion. The other peptides NT and VIP also participate in this action but to a much lesser degree. The operative pathways of these gut peptides hence involve both endocrine (SOM) and paracrine actions (SOM, NT, VIP) in order to exert inhibitory functions on the stomach. The inhibitory action of gastrazole, was in a similar range as that of SOM implying that physiological acid-induced inhibition of gastric acid may primarily be exerted through inhibition of gastrin endocrine secretion.

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1. Introduction

Luminal acid and hyperosmolal solutions in the proximal small intestine inhibit gastric acid secretion stimulated by pentagastrin [1–4]. It has been suggested that acid and hyperosmolal solutions act at different sites along the gut to inhibit gastric acid secretion. Acidification of the jejunum inhibits gastric acid secretion by endocrine, paracrine and neurocrine mechanisms [5–8]. The underlying mechanisms for the inhibitory properties of acid and hyperosmolal solutions on gastric acid secretion have not yet been fully elucidated. Somatostatin (SOM) is suggested to be a mediator of the acid-induced inhibitory processes [8,9] as SOM reduces both histamine release from enterochromaffin-like (ECL) cells and exerts a direct inhibitory effect on parietal cell function [10]. Among other gut peptides, neurotensin (NT) in an endocrine fashion, and vasoactive intestinal peptide (VIP) acting as a neurotransmitter or in a paracrine fashion, are considered to contribute in the peptidergic interplay of inhibitory mechanisms for gastric acid secretion [11]. The mechanisms by which NT and VIP inhibit parietal cell function are unknown. Inhibition of the function of the isolated oxyntic cell mucosa has not been demonstrated and no receptors for these inhibitors are found on parietal cells.

The aim of the present study was to investigate the effects of luminal acid and hyperosmolal solutions on gastric acid secretion and the simultaneous release of SOM, NT and VIP to the circulation and to the intestinal lumen, acting in an endocrine or paracrine fashion to inhibit gastric acid secretion. Moreover, the effect of a novel gastrin receptor antagonist gastrazole, was studied and compared with ranitidine and esomeprazole.

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2. Materials and methods

All experiments were approved by the local Animal Care Committee and performed according to the guidelines of the National Institute of Health (USA).

2.1. Surgical procedures

The experiments were carried out on 51 male Sprague–Dawley rats (B&K Universal Ltd, Sollentuna, Sweden) kept under standardized conditions (room temperature 22 °C, humidity 60%, automatically regulated 12 h lighting cycles) and were fed *ad libitum* on a commercial diet (BK standard feeding, BK). After an overnight fast, the rats were anesthetized with pentobarbital (50 mg kg^{-1} intraperitoneally, Apoteksbolaget, Umeå, Sweden) and operated as described elsewhere [4]. In short, a 10 cm long Thiry–Vella loop of the jejunum was prepared in each rat by dividing the duodenum at the pylorus and 6 cm farther distally, at the ligament of Treitz. Immediately distal to the ligament of Treitz a 10 cm long part of jejunum was removed and a blind loop was constructed. The continuous proximal and distal parts of the jejunum were anastomosed end-to-end to restore gastrointestinal continuity. The oral end of the Thiry–Vella loop was closed, intubated with a PE 90 catheter (Clay Adams & Co., Becton Dickinson, Parsippany, NJ, USA), and attached to the abdominal wall. The distal end was brought through the abdominal wall as a mucocutaneous fistula. The catheter was tunnelled subcutaneously to exit at the back of the animal's neck. Then, a mid-segment of the duodenal loop, 5–7 mm long, with the orifices of the bile and pancreatic ducts, was implanted end-to-side to the jejunum as a pouch 10 cm distal to the entero-anastomosis of the duodenum. To quantify acid secretion the rats were provided with a plastic gastric cannula placed proximal to the oxyntic gland area near the greater curvature [12]. The rats had free access to water 24 h after surgery and access to food on the second postoperative day. A few days later a siliastic catheter (Dow Corning Co., MI, USA) was inserted into the jugular vein for administration saline and pentagastrin, as well as for blood sampling. The catheters were pretreated with heparin [13] to avoid clotting in the catheter lumen.

2.2. Experimental procedures

Studies of acid secretion and release of gastrointestinal peptides began 7–10 days after surgery. Food, but not water, was withheld for 18 h before the experiments were undertaken. Before the start of the experiments, the rats were placed in Bollman cages to which they were previously accustomed. Initially, the stomach was rinsed with tap water. Then, gastric juice was collected at 30-min intervals of which the first two were discarded, and back-titrated to pH 7.0 (Autoburet, Copenhagen, Denmark) as described earlier [3,4]. The acid output was expressed in μmol 30 min^{-1}. Throughout the experiments, which lasted 2.5 h, 0.15 mol L^{-1} NaCl was initially administrated via the jugular vein catheter for 120 min at a rate of 2 ml h^{-1} by an infusion pump (Sage Instruments, Cambridge, MA, USA). Pentagastrin (NeoMPS S.A., Strasbourg, France) at a dose of 90 pmol kg^{-1} min^{-1} was added to the intravenous saline infusion for the remaining 90 min. This dose has previously been shown to produce maximal acid response in chronic fistula rats [12]. In control experiments, the intestinal loops were perfused with 154 mmol L^{-1} NaCl at a rate of 2 ml h^{-1} throughout the experiments as described above. In test experiments, saline was exchanged either for HCl (200 mmol L^{-1}; pH 1.1; 380 mOsm kg^{-1}) or for PEG (1200 mOsm kg^{-1}; pH 7.2), which was given during 60 min starting from the fifth basal 30-min period and continuing during the whole pentagastrin infusion period. The concentrations of HCl and PEG were chosen as they caused maximal inhibition of pentagastrin-stimulated acid secretion [1,14].

Experiments on the effect of acid inhibitors were carried out in fasted rats with a gastric fistula. Gastrazole, ranitidine or esomeprazole were administered IV to conscious rats on top of 90 pmol kg^{-1} min^{-1} pentagastrin. The effects of gastrazole (0.15, 0.3, 3 μmol kg^{-1}), ranitidine (0.15, 0.3, 3 μmol kg^{-1}) and esomeprazole (0.15, 3, 9, 15 μmol kg^{-1}) administration were examined. Acid output was expressed as μmol [H^+] per 30-min period. Experiments were carried out using individual animals exposed to both pentagastrin and antagonist treatments.

2.3. Extraction of samples and radioimmunoassay

During the experiments, perfusates from the loops were collected on ice during each 30-min period for analysis of gut peptide concentrations. Blood samples withdrawn from the jugular vein catheter for the same purpose were obtained at the end of the fourth basal period as well as at the end of the experiments. However, in control experiments blood samples were collected only at the end of experiments. The blood samples were centrifuged at 4 °C, pipetted off and plasma collected. All samples were stored at −80 °C until extraction procedures and radioimmunoassay (RIA). The peptides were then absorbed onto and eluted from Sep Pak C18 cartridges (Waters, Millipore Co., Milford, MA, USA). Elute A consisted of 0.1% trifluoroacetic acid, 0.06 mol L^{-1} NaCl and 99.9% water. Elute B consisted of 0.1% trifluoroacetic acid, 19.9% water, 0.06 mol L^{-1} NaCl and 80% methanol. Samples were then evaporated to dryness at 45 °C under nitrogen gas before dissolution and RIA.

SOM-like immunoreactivity (LI) was analyzed as described earlier [15]. The detection limit was 2 pmol L^{-1}, and the intra- and interassay coefficients of variation were 7% and 11% respectively.

NT-LI was analyzed using antiserum H which reacts with NT, NT (4–13) (118%), NT(8–13) (167%) and NT(9–13) (15%), but not with N-terminal fragments of NT. The detection limit of the assay was 8 pmol L^{-1}, and the intra- and interassay coefficient of variation were 8% and 13% respectively [16].

VIP-LI was analyzed in plasma and perfusates using antiserum VIP2 raised against conjugated natural porcine VIP. The antiserum does not cross-react with gastrin, pancreatic polypeptide, glucagon, neuropeptide Y or NT. The detection limit of the assay was 3 pmol L^{-1}. Intra- and interassay coefficient of variation were 9% and 13% respectively.

2.4. Statistics

The results are presented as means ± SEM. Differences in acid secretion and peptide concentrations and differences in acid secretion and antagonist between the groups were evaluated using the Kruskal–Wallis test with Dunn's multiple comparisons post-test. Variations in gut peptide concentrations were evaluated using the Friedman test for paired observations with Dunnett's post-test. $P<0.05$ was considered significant.

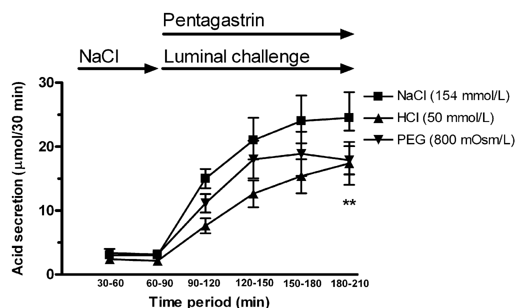

Fig. 1. Change of gastric acid output during basal and pentagastrin-stimulated gastric acid secretion during luminal challenge of a jejunal Thiry–Vella loop with saline (NaCl), hydrochloric acid (HCl) and polyethylene glycol (PEG). **$p<0.01$.

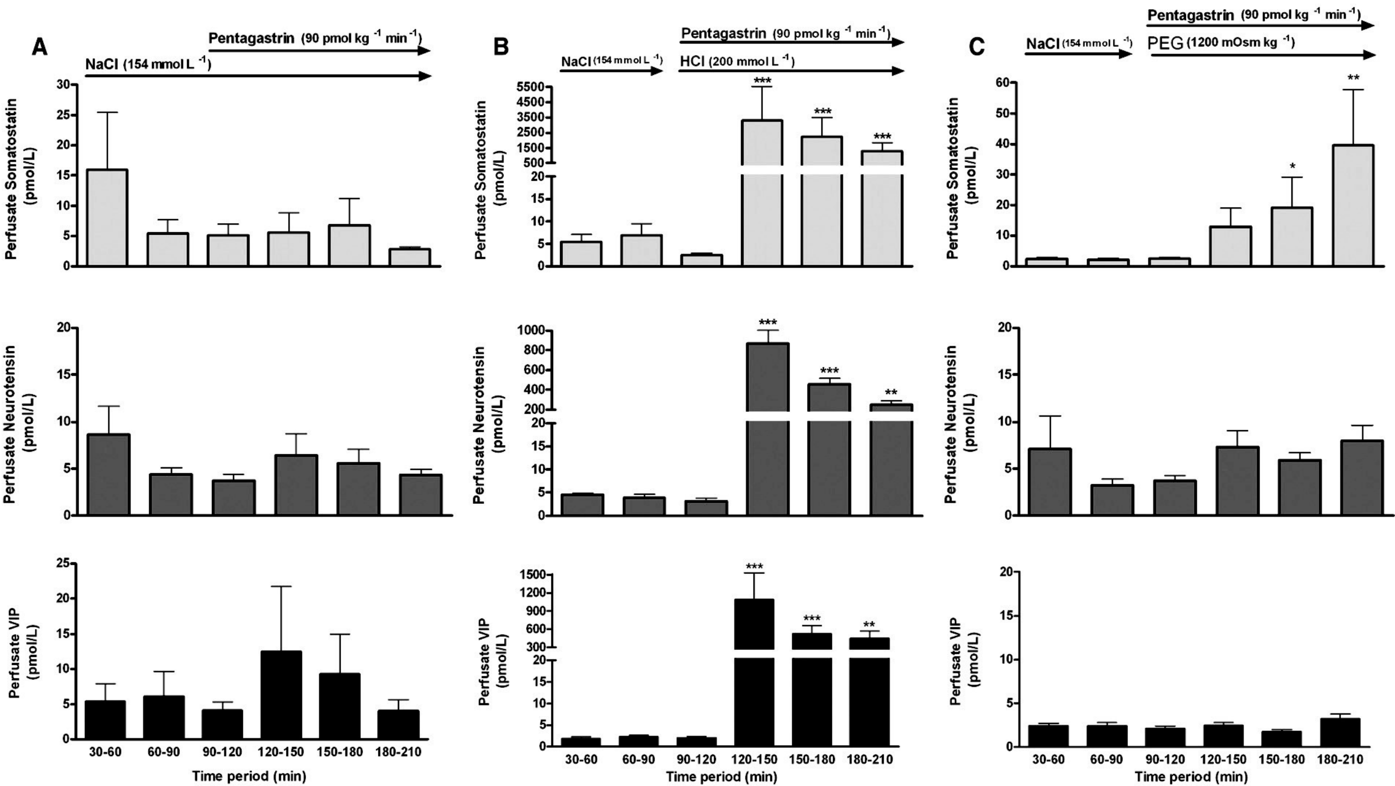

Fig. 2. A–C. Luminal concentrations of somatostatin, neurotensin and vasoactive intestinal peptide (VIP) the luminal perfusate during basal and pentagastrin-stimulated gastric acid secretion after challenge of a jejunal Thiry–Vella loop with saline, or hydrochloric acid (HCl) (**$p<0.01$, ***$p<0.001$), or polyethylene glycol (PEG) (*$p<0.05$, **$p<0.01$).

3. Results

3.1. Control conditions

Under control conditions with saline perfusion of the intestinal loop there was a stable basal acid secretion. Upon stimulation with pentagastrin 90 pmol kg^{-1} min^{-1} acid secretion increased to maximal levels ($p<0.01$), whereas the luminal concentrations of SOM, NT and VIP did not change (Fig. 2A). In plasma, the circulating levels of SOM increased ($p<0.05$) after introduction of pentagastrin, but the levels of VIP and NT did not change (Fig. 3).

3.2. Challenge with hydrochloric acid

Perfusion of the intestinal loop with HCl reduced the pentagastrin-stimulated gastric acid secretion compared to control ($p<0.01$) (Fig. 1). With a delay of 30 min we found a marked release of the levels of SOM in the intestinal perfusate increased 1000-fold ($p<0.001$), similar to NT and VIP which increased 500- ($p<0.05$) and 260-fold ($p<0.05$), respectively (Fig. 2B). The increase of gut peptide release displayed no significant differences between SOM, NT and VIP, neither as regards concentrations or time profile.

At the same time the circulating levels of SOM in plasma increased markedly ($p<0.01$) compared to VIP and NT, which were unchanged. In comparison with exposures of the Thiry–Vella loop to NaCl and PEG, the plasma increase of somatostatin in response to HCl was markedly increased ($p<0.001$) (Fig. 3).

3.3. Challenge with hyperosmolal polyethylene glycol

Perfusion of the intestinal loop with hyperosmolal PEG tended to lower the gastric acid output compared to control ($p<0.06$) (Fig. 1).

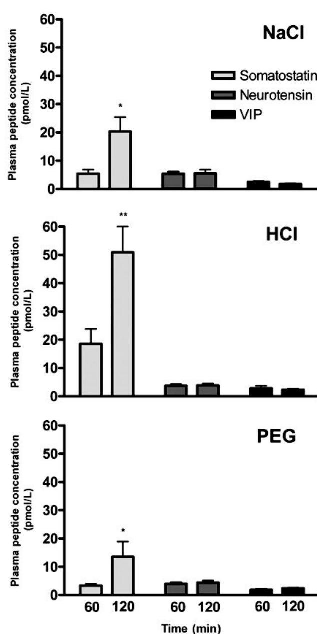

Fig. 3. Plasma concentrations of somatostatin, neurotensin and vasoactive intestinal peptide during basal (60 min) and after challenge of a jejunal Thiry–Vella loop with saline (NaCl), hydrochloric acid (HCl) and polyethylene glycol (PEG) (120 min). *$p<0.05$, **$p<0.01$.

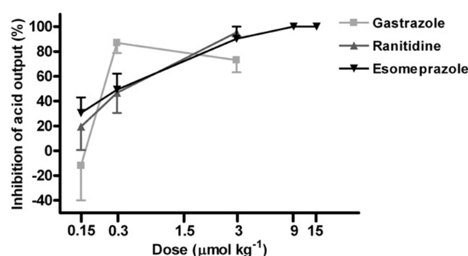

Fig. 4. Dose–response inhibition of acid secretion 30 min after intravenous administration of gastrozole, ranitidine or esomeprazole.

Concomitantly, the levels of SOM in the luminal perfusate gradually increased over time reaching maximum with a 3-fold increment ($p<0.01$). This was significantly more outspoken ($p<0.01$) than for NT and VIP, which did not change (Fig. 2C). The plasma levels of SOM in plasma tended to increase ($p<0.06$) as compared to VIP and NT (Fig. 3).

3.4. Pharmacological acid inhibitors

Intravenous administration of an acid inhibitor (gastrazole, ranitidine and esomeprazole; each $n=6$) produced dose-dependent inhibition of pentagastrin-stimulated gastric acid secretion (Fig. 4). Gastrazole was the most potent when given at a dose of 0.3 μmol kg^{-1} (87±8%) compared to ranitidine at the same dose (47±16%) and esomeprazole (52±11%). At higher dosages all three drugs reached a similar maximal inhibition of 73±10%, 95±3% and, 90±10% for gastrazole, ranitidine and esomeprazole, respectively.

4. Discussion

Peptidergic regulatory mechanisms play important roles in the inhibition of gastric acid secretion [17–22]. Long experience has shown that inhibition of acid secretion is mainly sensitive to low pH as well as high osmolality [1–4,23,24]. We have therefore chosen to study the inhibitory action of two different stimuli representative for physiological conditions, namely 200 mM hydrochloric acid and 1200 mOsm polyethylene glycol. We found that acid caused the most marked inhibition of acid secretion, while hyperosmolality not quite significantly decreased acid secretion during the 120-min period studied [14]. During this period, however, gross changes in gut peptide release were observed [23]. Two ways of output were analysed; secretion to the lumen and to the bloodstream. Broadly speaking the luminal release outweighed the release to the bloodstream. This was most evident for SOM where the luminal concentrations reached levels about 70 times higher than those measured in the circulation. Even for NT and VIP there was a pronounced increase to the lumen which was shown for the bloodstream [25]. Our data suggest that mainly acid, but also hyperosmolal solutions, may reach the enteroendocrine cells to stimulate the release of gut peptides, such as SOM and NT. In addition it seems that acid may penetrate even deeper into the mucosa in order to influence the release of neuropeptides from neuronal tissues. This opens the possibility that acid may stimulate dual inhibitory mechanisms mediated either through endocrine and neuronal mechanisms or depending on the circumstances that one pathway may suppress or dominate over the other [26].

It is well known that SOM-containing D-cells of the antrum are sensitive to low pH and upon stimulation release their peptide in order to reduce acid secretion as a balancing physiological feedback to counteract a deleterious effect of acid [20,26,11]. As shown in this study this also seems to go for the small intestine as an isolated Thiry–Vella loop of the jejunum was capable of inhibiting gastric acid secretion.

Thus, a SOM-mediated endocrinological acid inhibitory mechanism seems to be valid not only for the antrum and duodenum [27], but also for the greater part of the small intestine. Whether the origin of the SOM is intestinal or released from the gastric mucosa by another secretagogue from the small intestine is not yet confirmed. The possibility of a supplementary neuronal acid inhibitory mechanism is most likely to be due to direct stimulation of local nerve plexuses in the gut wall. Extrinsic pathways may be involved but to a lesser extent and may be masked by the seemingly stronger endocrine response to HCl in the small intestine with SOM and CCK as the main acid inhibitors [8].

Esomeprazole, ranitidine and gastrazole inhibits gastric acid secretion through different cellular mechanisms. Ranitidine is a potent H_2-receptor blocker [28], while gastrazole inhibits gastric acid secretion through the CCK-2 receptor [29,30]. According to our present results gastrazole seems to be more potent than ranitidine and esomeprazole to inhibit acid secretion. The temporally prompt effect of gastrazole verifies the importance of gastrin for acid secretion from the parietal cell, and offers a new principle for pharmacological studies of acid secretion, namely inhibition of endocrine gastrin stimulation of the parietal cell.

When acid is released into the lumen of the duodenum, and also further into the small intestine this signal seems to trigger a negative feedback mechanism which releases different intestinal peptides [8,2]. Acid seems to be more powerful in activating inhibitory mechanisms with release of intestinal peptides in the small intestine than hyperosmolal solutions. Primarily SOM contributes to inhibition of gastric acid secretion which seems valid for both acid and hyperosmolal solutions in the gut [1,2]. The reason for this is obvious, as acid is a far more powerful aggressor from which the intestinal mucosa has to be protected than the effects of osmolality.

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III

CCK_2-receptor antagonist YF476 prevents NSAID-induced gastric ulceration through acid inhibition mediated by regulatory peptides

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Short title: CCK_2-receptor antagonist prevents NSAID ulcers

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Abstract

Aim: Study effects of the CCK_2-receptor antagonist YF476 on gastric pH, plasma peptide levels, gene expression of peptides and receptors for prevention of NSAID ulcerations in rats. *Method:* A Bravo capsule inside the stomach measured pH for five consecutive days. Gastric and jugular vein catheters were implanted for blood sampling, and administration of drugs. Plasma and gastric tissue was analysed for gastrin, ghrelin, somatostatin, their receptors and inducible nitric oxide synthase (iNOS) using immunoassays and quantitative real-time PCR. *Results:* Gastric pH recorded over 24 hours was 2.3±0.6 in controls; variation 19±4 % over study days. With YF476, gastric pH rose to 3.5±0.3 ($P<0.001$). Ulcer index showed gastroprotective effects of YF476 and esomeprazole against diclofenac-induced gastric ulcers compared to control (4.8±2.4, $P<0.001$; 5.4±1.1, $P<0.01$; control 70±14 mm). After YF476 treatment, plasma ghrelin and somatostatin increased 12- and 6-fold, respectively ($P<0.001$), whereas gastrin was unchanged. Gene expression of gastrin, ghrelin, somatostatin and the receptors CCK_2 and $SSTR_2$ in stomach did not change after YF476 except ghrelin receptor, which decreased compared to internal control (p=0.026). Diclofenac induced 30-fold expression of gastric iNOS ($P<0.001$), 300-fold expression of gastrin ($P<0.01$), and nearly 20-fold of CCK_2-receptor ($P<0.05$). Ghrelin, somatostatin and their receptors were unchanged. *Conclusion:* The CCK_2-receptor antagonist YF476 increased gastric pH without concomitant hypergastrinemia. Other stomach peptides, ghrelin and somatostatin, increased in circulation while their tissue content and receptors were unaffected except ghrelin receptor which increased. Thus, YF476 may prevent NSAID ulcerations by inhibition of acid secretion involving somatostatin and over-expressed gastrin and CCK_2-receptor.

Key words: Diclofenac, gastric acid, gut hormones, nitric oxide synthase, pH.

Introduction

The acid-producing oxyntic part of the rat stomach is rich in endocrine and paracrine cells, including enterochromaffin-like (ECL)-cells containing histamine[1], G-cells localised to the antrum[2] A-like or Gr cells holding ghrelin and obestatin[3], and D-cells with somatostatin[4]. An important part of the acid secretion process is the stimulation and release of gastrin and the subsequent activation of its receptor, the cholecystokinin-2 (CCK_2) receptor, located on the histamine producing ECL-cells[5]. Among other important physiological regulators of gastric acid secretion somatostatin is a potent inhibitor of gastric acid secretion[6], and ghrelin which seems to stimulate acid secretion probably through dual actions mediated by the vagus nerve and histamine[7-9].
The standard treatment in management of gastroesophageal reflux (GERD), and peptic ulcer disease is proton pump inhibitors (PPIs) which cause a remarkable suppression of acid secretion by inhibiting the final common step in the proton pump[10, 11]. Although PPIs seem to be the most effective therapy one problem with this treatment is the hypergastrinemia with rebound acid hypersecretion resulting in acid-related symptoms once PPI therapy is withdrawn[12-15]. Another problem is the loss of durability of acid suppression during the night or early morning when massive acid secretion takes place[10]. This nocturnal acid breakthrough is defined as the presence of intragastric pH < 4 during the overnight period for at least 60 continuous minutes in patients taking PPIs. Nocturnal acid breakthrough occurs in a sizeable part of patients on PPI therapy and has clinical consequences in particular in patients with complicated GERD[16].
The search for more physiologically adapted compounds for optimal therapy is under continuous development. Considering gastrin as the main stimulant of food-induced acid secretion, different approaches have been made to deal with acid-related diseases by inhibition of this pathway. YF476, a CCK_2-receptor antagonist, structurally related to benzodiazepine derivatives, has recently received clinical interest[17, 18]. The rationale of using a CCK_2-receptor antagonist in preventing acid secretory disorders is based on the drugs ability to counteract the hypergastrinemia that occurs under PPI treatment. Furthermore, in patients with extended PPI treatment who still experience acid-related symptoms due to hypergastrinemia a CCK_2-receptor antagonist would be the drug of choice[19,20].
The primary aim of this study was to investigate the effect of YF476 on gastric long-term pH and its healing properties on non-steroidal anti-inflammatory drug (NSAID)-induced gastric ulcerations. In addition, as a secondary aim, the plasma levels of the regulatory peptides gastrin, ghrelin and somatostatin produced by endocrine cells in the stomach were studied. Furthermore, tissue gene expression of gastrin, ghrelin and somatostatin and their respective receptors; CCK_2-receptor, ghrelin receptor and somatostatin receptor-2 ($SSTR_2$) were investigated, using real-time quantitative PCR (qPCR) in normal and in NSAID-induced gastric ulcerations using the expression of inducible nitric oxide synthase (iNOS) as a marker of inflammatory activity.

Material and Methods

Chemicals

YF476, (R)-1-[2,3-dihydro-2-oxo-1-pivaloylmethyl-5-(2-pyri-dyl)-1H-1, 4-benzo-diazepin-3-yl]-3-(3-methylaminophenyl)urea, was generously supplied by Malcolm Boyce (Hammersmith Medicines Research, London, UK).

Animals

Sprague-Dawley male rats (300-350 g) were purchased from Scanbur B&K AB (Sollentuna, Sweden). The animals were fed *ad libitum* with a commercial rat pellet diet (LABFOR, Lactamin R36, Kimstad, Sweden) and tap water. At least 7 days prior to the experiments rats were allowed to adapt to the environment in housed in wire-meshed cages at 24° C with constant humidity and 12:12 hour light-dark cycle. During recovery after surgery, the rats were daily trained to accept experimental conditions. Experiments were then carried out in conscious animals after a fasting period, at most 15 hours, in wire-bottomed cages with free access to water. The experiments were approved by the Animal Ethics Committee in northern Stockholm.

Surgical procedures

Surgery was performed under anesthesia with a mixture of midazolam (5 mg mL^{-1}, Midazolam Aktavis AB, Stockholm, Sweden) and Hypnorm (fentanylcitrate, 0.315 mg kg^{-1} plus fluanisone 10 mg kg^{-1}; Janssen-Cilag, Sollentuna, Sweden) given subcutaneously (SC) at a dose of 1.5-2 mL kg^{-1} body weight. Buprenorfin (Temgesic® 0.3 mg mL^{-1}, Schering-Plough, Stockholm, Sweden) 0.05 mg kg^{-1} body weight was given SC after surgery to relieve post-operative pain.

A Bravo capsule (an electronic pH sensor encapsulated in PVC-plastic, 25x5x5 mm; Synmed Medicinteknik AB, Spånga, Sweden) was surgically placed inside the stomach as previously described[21]. In brief, a midline incision was done, creating a small opening in the proximal greater curvature and gastric contents evacuated. Then, the capsule was sutured to the mucus layer of the stomach with the pH sensor pointing distally. An indwelling silastic catheter (Dow Corning Co., Midland, MI, USA) pretreated with heparin[22] was inserted into the external jugular vein in all animals, except those given gavage, for administration of saline and pentagastrin, as well as for blood sampling. Finally, a catheter (Dow Corning Co.) provided with a small flange was anchored in the fundus part of the stomach near the lesser curvature. The catheters were then tunneled at the back of the animal’s neck and the opening soldered with heat to prevent leakage of gastric contents.

Experimental procedures

Studies of intragastric pH. Experiments were carried out in conscious rats (n=12) and began in the morning two days after surgery. The studies were carried out as one experiment in each rat. The animals gained weight (8.0 ±4.1 g) and behaved in a normal manner with normal feeding pattern throughout the experiments. At post-mortem examination, no mucosal lesions, obstruction of the pylorus or gastric distension were seen. YF476 was administered as a

suspended solution (100 mg kg^{-1} body weight dissolved in 0.5% methyl cellulose) through the gastric catheter in all experiments twice daily (8 am and 5 pm).
The pH recorded by the Bravo capsule was transmitted with sampling frequency 6 Hz to the Bravo receiver placed immediately outside the cage. The Bravo system was set for a 48-h recording period after which data was downloaded, batteries replaced and recording continued. This procedure was then repeated for two more periods up to 120 hours.

Studies on stomach regulatory peptides and receptors. The effect of YF476 on the plasma concentrations of regulatory peptides and gastric pH was studied with bolus dose of YF476 (100 mg kg^{-1} body weight) given intragastrically twice daily (9 am and 4 pm) during stimulation of acid secretion with pentagastrin 90 pmol $kg^{-1}min^{-1}$ IV for 6 h (9 am to 3 pm) and then for 16 h (4 pm to 8 am) repeated for five consecutive days. A sample of 200 µL blood was drawn twice during the experiment, 1.5 h after each administration of YF476, centrifuged at 3000 rpm for 10 min and 100 µL of plasma collected and stored as aliquots in -20° C freezer for subsequent analysis of regulatory peptides.

Studies on NSAID-induced gastric ulcers. The animals (n=24) were deprived of food but had free access to water 18-20 h before induction of gastric ulcerations with diclofenac. The rats were then given a single dose of either saline solution (sodium chloride 9 g L^{-1}, Fresenius Kabi, Halden, Norway), esomeprazole (140 mg kg^{-1}; esomeprazole (Nexium 40 mg, AstraZeneca, Södertälje, Sweden) or YF476 (100 mg kg^{-1} dissolved in 0.5% methyl cellulose) one hour prior to the administration of diclofenac (Voltaren® 25 mg mL^{-1}, Novartis, Basle, Switzerland) 30 mg kg^{-1}, via gavage and left in their cages for 4 hours after the final dose. The animals were then sedated with CO_2 and euthanized by cervical dislocation. The abdomen was opened, the stomach removed and gently washed in phosphate buffered saline and pinned open for photographic documentation (Fig. 1). The ulcer index[23] (expressed as mm) reflects the total length of gastric lesions per stomach as judged by three independent researchers blinded to the protocol.

Tissue sampling and RNA isolation

At the end of experiments the animals (n=12 for the pH study; n=24 for the diclofenac study) were euthanized with an overdose pentobarbital sodium (Apoteksbolaget, Solna, Sweden) or sedated with CO_2 and euthanized by means of cervical dislocation. Tissue segments (20-30 mg) of the corpus of the stomach were collected from each animal, quickly placed in RNA-stabilizing reagent (RNAlater, Qiagen, Hilden, Germany) and stored at 4° C for 24 h before finally placed in -20° C freezer before qPCR analysis.
For RNA extraction tissue samples were placed in RTL cell lysis buffer (Qiagen, Hilden, Germany) and homogenized by means of a rotor-stator knife (Ultra-Turrax T8, IKA®-Werke, Staufen, Germany). Total RNA was then extracted using the RNeasy Mini Kit according to the manufacturer's instruction (Qiagen). Finally, a DNase digestion step (DNase I; Promega, Madison, WI, USA) with incubation at 37° C for 30 min was included to remove traces of chromosomal DNA.

Samples with A260/ A280 ratio ≥1.8 were used for qPCR. Complementary DNA (cDNA) synthesis was performed on 1-5 µg of total RNA using oligo dT primers and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA).

Immunoassays

Gastrin was analyzed using antiserum the C-terminally-directed CCK/gastrin antiserum 2609/10 [24]. Chloramine-T labeled and HPLC-purified gastrin-17 (NeoMPS, Strasbourg, France) was used as radioligand and gastrin-17 as calibrator/standard. The assay reacts 100% with gastrin-17 in plasma but not with pentagastrin. The detection limit of the assay was 3 pmol L^{-1} and the intra-assay coefficient of variation 6%.
Somatostatin was analyzed using an enzyme immunoassay kit (EK-060-03, Phoenix Pharmaceuticals, Burlingame, CA, USA), which reacts 100% to somatostatin-14, -25 and -28 in plasma. The intra-assay coefficient of variation was 5%.
For ghrelin measurements the rat ghrelin (active) enzyme-linked immunosorbent assay kit (EZRGRA-90K, Millipore, Billerica, MA, USA) was used, that utilizes ^{125}I-labeled ghrelin and ghrelin antiserum which reacts 100% to active ghrelin in plasma, but not to inactive des-octanoyl ghrelin. The intra-assay coefficient of variation was 7%.

Real-time quantitative polymerase chain reaction

Real-time qPCR was performed on an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) using TaqMan gene expression Mastermix (TaqMan®, Applied Biosystems Europe BV, Stockholm, Sweden) for gastrin, somatostatin, ghrelin, iNOS, and for the receptors CCK_2, ghrelin receptor, and $SSTR_2$ (gene expression assays no. Rn01644838, Rn00561967, Rn00572319, Rn00561646, Rn00565867, Rn00571116, Rn00583419; amplicon lengths 60, 117, 82, 77, 109, 91, 78 respectively). Primers were purchased as primer set optimized for TaqMan gene expression assay (TaqMan®, Applied Biosystems Europe BV). The expression in each reaction was normalized by the expression of hypoxanthine phosphoribosyl transferase 1 (Hprt1) (gene expression assay no. Rn01527840, amplicon length 64) as internal control.
Complementary DNA subjected to real-time qPCR was performed in 25 µL reaction volumes consisting of 20 x TaqMan universal PCR master mix (Applied Biosystems Europe BV). Amplification was carried out at 50° C for 2 min, 95° C for 10 min, 50 cycles of 95° C for 15 sec, and 60° C for 1 min. The absolute amounts of transcripts were determined by using several concentrations of standard cDNA (1000, 100, 10, and 1 ng) that were reverse transcribed from the stomach of Wt naive males. qPCR analyses were performed in triplicate of each sample and standards included in each experiment.

Data analysis and statistical analysis

The data obtained with the Bravo capsule was analysed using the POLYGRAM NET ™ (pH Testing Application software, Synmed Medicinteknik) in 48-h periods. Results of studies with YF476 and pentagastrin were analyzed by calculating changes in pH at various timepoints from baseline (defined as 0.5 h prior to onset). The qPCR measurements were calculated using the 2(-DeltaDelta C(T)) method[25].
All data are expressed as mean±SEM. Data were compared using a one-way analysis of variance followed by a Dunnett´s multiple comparison test or Mann-Whitney test. *P* less than 5% was considered significant.

Results

Intragastric pH. The collective data shown in figure 1 describes the pH changes during a 24-h period. Under control conditions, the average 24-h pH was 2.3 ±0.6 with a variation of 19 ±4% over the 5-day study period. After administration of YF476 (100 mg kg^{-1}) gastric pH significantly increased from baseline 2.1 ±0.3 to 3.5 ±0.3 already after the first dose. This difference in pH was maintained throughout the study period ($P<0.001$). There were no significant differences between diurnal pH changes (9 am to 3 pm vs 3 pm to 9 am) when YF476 was given, except that pH was maintained at a higher level.

Gastroprotection. As seen in figures 2C and 2D the macroscopic appearance revealed gastroprotective properties of YF476 comparable to those of esomeprazole on diclofenac-induced gastric ulcers (Fig. 2A). This was reflected by the ulcer index (Fig. 3), which markedly diminished in the groups receiving YF476 (4.8 ±2.4 mm; $P<0.001$) or esomeprazole (5.4 ±1.1 mm; $P<0.01$), as compared to controls (70 ±14 mm).

Stomach regulatory peptides. Administration of YF476 (100 mg kg^{-1}) significantly increased ghrelin and, somatostatin, but not gastrin as compared to control. Ghrelin increased 12-fold compared to control from 17 ±7 to 211 ±12 pmol L^{-1}; $P<0.001$), while somatostatin increased 6-fold from 42 ±6 to 260 ±23 pmol L^{-1}; $P<0.001$) (Fig. 4).

Gene expression of stomach peptides and receptors. As shown in figure 5, when YF476 was given there was a great variability and no significant changes of the expression of gastrin, ghrelin, or somatostatin in gastric tissue, or the corresponding receptors, except for the expression of the ghrelin receptor which decreased (p=0.026) compared to internal control.

Gastroprotection and gene expression. After diclofenac the gene expression of iNOS increased 30-fold ($P<0.01$), the expression of gastrin and its receptor increased more than 300-fold ($P<0.01$), and 18-fold ($P<0.05$), respectively. The expression of ghrelin and somatostatin or their receptors did not change (Fig 6).

Discussion

YF476 is a member of a new class of drugs, the so-called CCK_2-receptor antagonists, which is believed to counteract the effects of gastrin on parietal cells and ECL-cells that are shown to express the CCK_2-receptor on their surface. Hence, it is expected that treatment with YF476 should result in a diminution in gastric acid secretion and reduction of certain symptoms and pathogenic mechanisms of acid peptic diseases or upper gastrointestinal disorders[26].
Our study shows that YF476 administered twice daily increased gastric pH up to about 3.5. This took place with a limited variability and no indication of decreased sensitivity to the drug over five days in a row. Studies of experimental NSAID-induced gastric ulcerations further showed a gastroprotective effect of YF476 comparable to that of esomeprazole. Long-term treatment with YF476 under challenge with pentagastrin increased the plasma concentrations of ghrelin and somatostatin, whereas plasma gastrin was only barely elevated not reaching statistical significance. At the same time mRNA expressions of gastrin, ghrelin and somatostatin did not change after exposure to YF476. Of the corresponding receptors the

ghrelin receptor decreased, whereas the CCK_2-receptor and $SSTR_2$ were unchanged. On the other hand, NSAID-induced ulcerations as characterized by increased gene expression of iNOS, revealed an up-regulation of both gastrin and the CCK_2-receptor, while neither ghrelin nor somatostatin or their receptors were changed.

The ECL-cell of the oxyntic mucosa is functionally related to acid secretion through a paracrine release of histamine which activates the parietal cell. The ability of the ECL-cell to undergo proliferation in response to the trophic stimulus of hypergastrinemia has important implications in pathology, being involved in the development of ECL-cell carcinoid tumors of rodents treated with powerful inhibitors of acid secretion[27]. In addition, most human gastric carcinoids are composed of ECL cells[28]. Hence, the ECL-cell response to gastrin in humans has contributed to the development of CCK_2-receptor antagonists, assumed to counteract effects of the hypergastrinemia caused by inhibition of acid secretion. CCK_2-receptor antagonists act by blocking the gastrin receptor leading to a reduction in histamine elaboration and release[29]. We found that YF476 inhibited acid secretion thereby increasing pH to a level high enough to prevent gastric damage of NSAID. This finding speaks in favour of a pharmacologically applicable use of the compound. At the same time there was a limited increase of circulating gastrin perhaps due to the acid inhibitory effect of the drug[30]. Our data also show that circulating somatostatin was increased during YF476 treatment which might further reduce local gastrin release from G-cells[31,32]. The biological significance of the parallel increase of ghrelin is unclear but is likely to represent another component of the local acid regulatory mechanisms[33]. Previous research by our group has also found ghrelin IV to increase somatostatin[34]. This opens the possibility that ghrelin apart from food intake, also affects secretory functions implying a multifacetted role in nutritional intake and digestion. Long-term administration of YF476 increased the mRNA expression of the CCK_2-receptor, the ghrelin receptor, and $SSTR_2$ most markedly of the CCK_2-receptor. Due to this, it can be argued that the changes in receptor expression may reflect a compensatory adjustment or mobilization of CCK_2-receptor because of inhibition of that same receptor by YF476. Considering the localization of CCK_2-receptor, on parietal cells as well as ECL cells[5], seem to be main targets for inhibition acid secretion in order to avoid compensatory endocrine hypersecretion or receptor expression[35]. The variability of gut peptides and receptor expression between different animals in this study is compelling, but may reflect a natural condition with a high variability between different individuals as regards their sensitivity to stimulation of gastric acid secretion and regulatory peptides.

In order to disentangle inflammatory gene expression patterns involved in the ulcer-inducing capacity of diclofenac we found that not only mRNA of iNOS was increased, but also expression of gastrin and CCK_2-receptor. These alterations speak in favour of an up-regulation of a local gastrin-CCK_2-receptor link, which might be of additional importance for the development of NSAID-induced ulcers. Thus, the CCK_2-receptor blocker YF476 would here find another mechanism of action. In agreement with this, Walduck *et al.* have found that there is a cyclooxygenase (COX)-2 dependent subset of genes, some of which directly influence gastric physiology (Gastrin, Galr1), epithelial barrier function (Tjp1, connexin45, Aqp5), inflammation (Icam1), apoptosis (Clu) and proliferation (Gdf3, Igf2). Under treatment with NSAID there is a differential expression of 140 genes, 97 of which seem unique, indicating that these genes are regulated only under COX-2 expression[36]. Moreover, gastrin increases the stability of COX-2 mRNA increasing its half-life increasing from 0.5 to 8 h and enhances COX-2 mRNA binding. In addition, data *in vitro* suggest that gastrin transactivates the COX-2 promoter in cells expressing the CCK_2-receptor and can further increase COX-2

expression[37]. In line with our results, there seems to be a panel of COX-2 dependent genes to which the gastrin gene[38] and the CCK_2-receptor gene[39] can be added. As the same gene profiles are present in humans[40], these data provide important new links between COX-2, the inflammatory processes, epithelial repair and integrity

In conclusion, gastric acid secretion was inhibited by the CCK_2-receptor antagonist YF476. This promotes mucosal healing with a numerical increase of plasma gastrin and further increases of ghrelin and somatostatin, while tissue mRNA expression of the ghrelin receptor increases. Under treatment with NSAID, the mRNA expression of the peptide gastrin as well as its receptor, CCK_2-receptor are up-regulated suggestive of a local coupling link between gastrin, the CCK_2-receptor and COX-2 in the pathogenesis of NSAID-induced gastropathy.

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Figure legends:

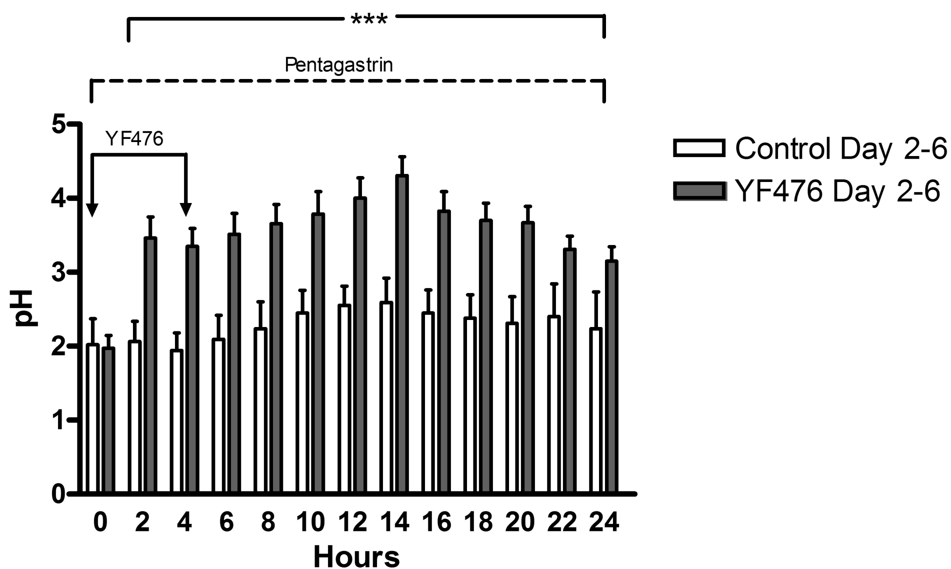

Fig. 1. Change in mean pH±SEM in the Bravo system during *ad libitum* conditions after bolus dose of YF476 (100 mg kg^{-1} intragastrically) under continuous pentagastrin stimulation (90 pmol $kg^{-1}min^{-1}$) for 6 h from 9 am to 3 pm and then from 4 pm to 8 am the following day; repeated for five consecutive days (*** P<0.001).

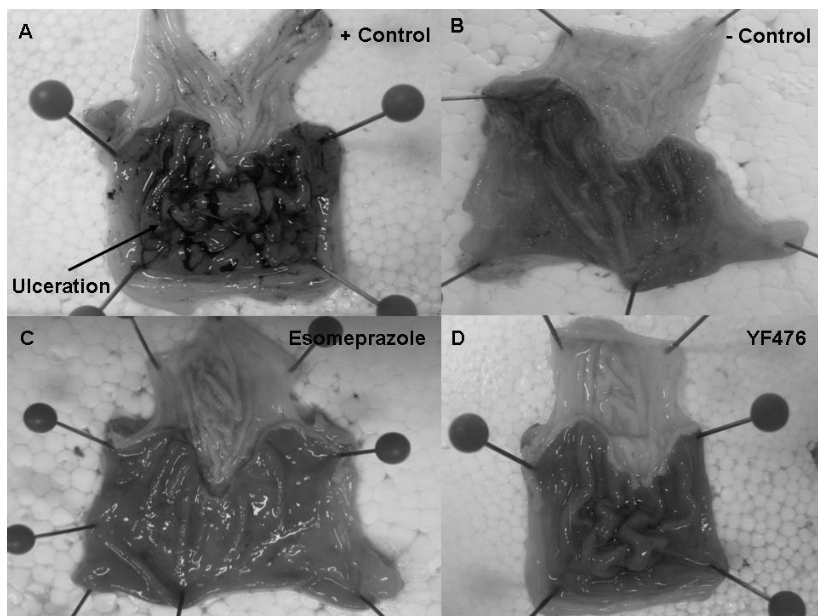

Fig. 2A-D. Representative photographs of excised rat stomachs 4 h after different challenges.. (A) shows positive control given saline 1 h prior to administration of diclofenac (30 mg kg^{-1}) with saline, (B) represents the negative control were animals received saline alone, (C) shows the preventive effect of esomeprazole (140 mg kg^{-1}) and (D) the comparative effect of YF476 (100 mg kg^{-1}) given in the same fashion as the saline control.(cf. A).

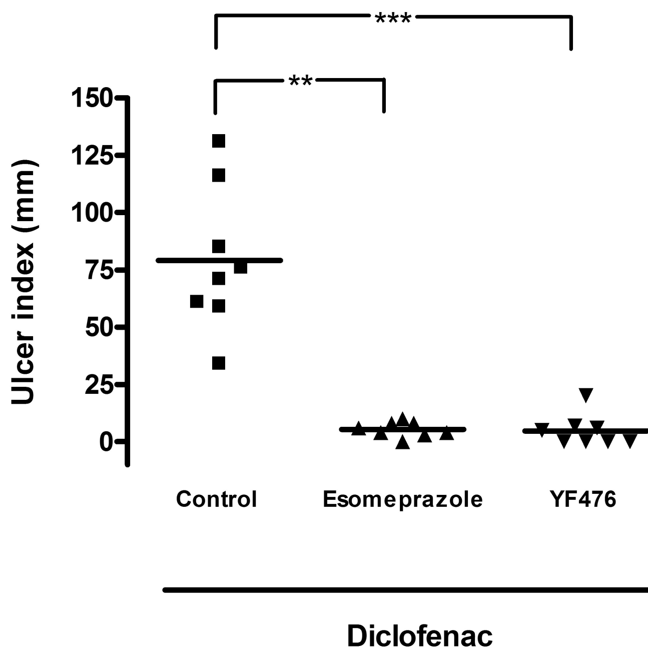

Fig. 3. Effect of esomeprazole and YF476 on gastric ulcer induced by diclofenac (30 mg kg^{-1}). Both esomeprazole (140 mg kg^{-1}) and YF476 (100 mg kg^{-1}) was given 1 h prior to the administration of diclofenac and compared to saline control, all by means of gavage. Ulcer index was determined 4 h after administration of diclofenac. Values are expressed as mean ±SEM (**P<0.01, ***P<0.001).

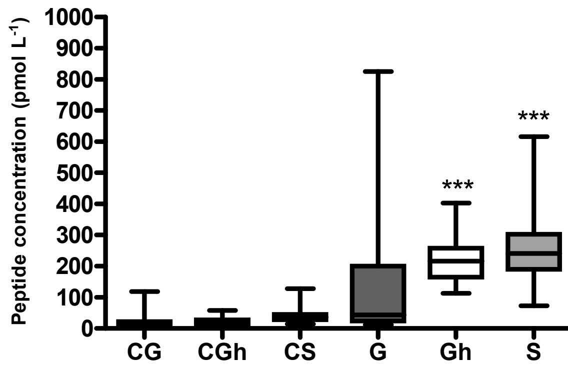

Fig. 4. Peptide concentrations in plasma expressed as mean ±SEM after administration of YF476 (100 mg kg^{-1} intragastrically) compared to controls. C (Control), G (gastrin), Gh (ghrelin), and S (somatostatin). (*** *P*<0.001).

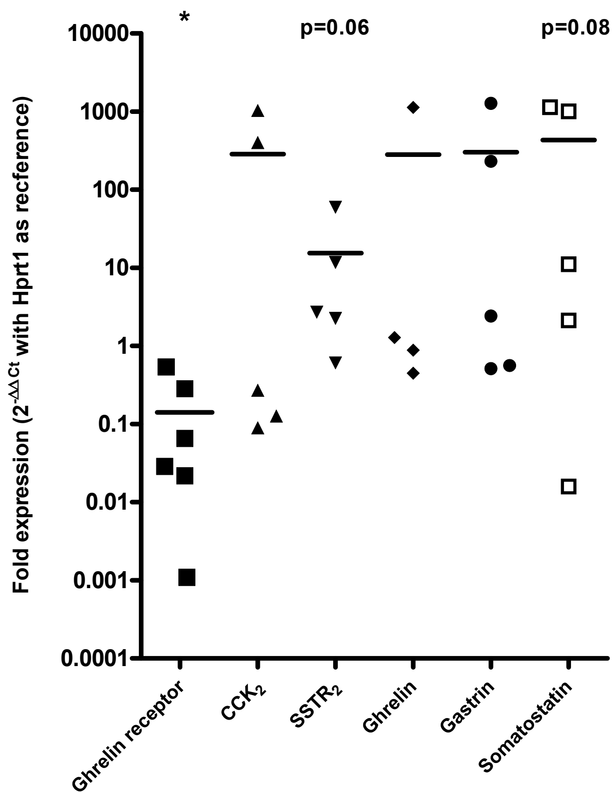

Fig. 5. Effects of bolus dose YF476 (100 mg kg^{-1} IV) and infusion of pentagastrin (90 pmol kg^{-1}min^{-1} IV) on the mRNA expression of different gut peptides and their receptors. Values are expressed as mean fold change in gene expression ±SEM (* *P*=0.0260). CCK_2 (cholecystokinin-2 receptor), $SSTR_2$ (somatostatin receptor-2).

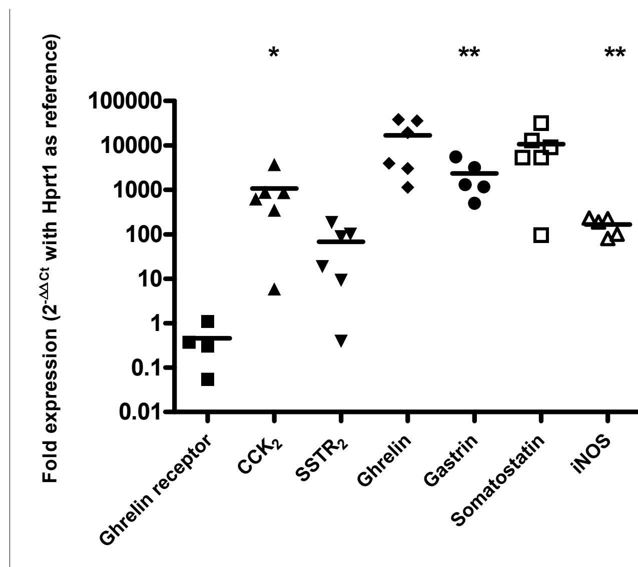

Fig. 6. Effects of bolus dose YF476 (100 mg kg^{-1} IV), esomeprazole (140 mg kg^{-1}) and control (saline) followed 1 h later by diclofenac (30 mg kg^{-1}) on the mRNA expression of gut peptides, their receptors, and iNOS. All drugs were given by gavage The values are expressed as mean fold change in gene expression ±SEM; * P=0.0260 (CCK_2-receptor) ** P=0.0079 (Gastrin), ** P=0.0043 (iNOS). CCK_2 (cholecystokinin-2 receptor), $SSTR_2$ (somatostatin receptor-2), iNOS (inducible nitric oxide synthase).

IV

Neuropeptide S in the gastrointestinal tract: effects on motility and inflammatory markers TNF, IL-1β, and iNOS in the rat

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Short title: Neuropeptide S and inflammation in the gut

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Abstract

Background: Neuropeptide S (NPS) is expressed by gastrointestinal (GI) enteroendocrine cells in rat and man. Effects of NPS are mediated through the NPSR1 receptor and associated with anxiety and nociception, as well as increased risk of inflammatory bowel disease. Polymorphisms of the NPSR1 receptor are linked to motor and sensory disturbances of the gut suggesting a role for NPS in functional GI disorders. We studied effects of NPS infusion on fasting small bowel motility, and expression of tumor necrosis factor (TNF), interleukin-1 beta (IL-1 beta) and inducible nitric oxide synthase (iNOS) as biomarkers of inflammation.
Methods: Studies of migrating myoelectric complexes (MMCs) were carried out in rats with electrodes implanted in the small bowel, and a jugular vein catheter. After baseline recording of 60 min with four activity fronts, NPS was infused IV for 60 min. Myoelectric activity was recorded using a Grass Polygraph with EEG preamplifiers. Tissue samples were obtained for evaluation of gene expression of TNF, IL-1β and iNOS.
Results: NPS at low dosage (1000 pmol $kg^{-1}min^{-1}$) increased irregular spiking, while high dosage (2000 and 4000 pmol $kg^{-1}min^{-1}$) significantly reduced spiking activity and increased the MMC cycle length (P=0.0152 and P=0.0022, respectively). NPS (4000 pmol $kg^{-1}min^{-1}$) induced 1.8 to 2.6-fold increase in mRNA expression of TNF and IL-1β, although less than diclofenac. iNOS expression was numerically increased by 1.4-fold.
Conclusion: NPS is involved in motility responses of the small intestine when administered peripherally and does so in a dose-response manner. NPS seems capable to increase cytokines as inflammatory markers.

Key words: Inflammation, migrating myoelectric complex, MMC, biomarkers, nitric oxide

Introduction

Neuropeptide S (NPS) is a recently discovered neuropeptide that is highly expressed in different regions of the central nervous system and gastrointestinal (GI) tract[1]. Its effects are mediated through the NPSR1 receptor, which expression parallels that of NPS in various tissues[1,2]. There is strong evidence that NPS promotes anxiolytic-like effects in rodents[1-5], increases wakefulness, suppresses sleep and induces hyperlocomotion[3,6]. NPS also either inhibits[7] or stimulates[8] food intake through orexin-containing neurons, and is considered to be involved in inflammatory reactions[9]. Effects of NPS are mediated through the receptor NPSR1 and increase the mRNA expression of cholecystokinin, vasoactive intestinal peptide, peptide YY and somatostatin[10]. Polymorphisms of the NPSR1 have been associated with motor and sensory disturbances, such as hastening of colonic transit, as well as pain, gas, and urgency sensations upon distension of the gut suggesting a role for NPS in functional GI disorders[11].

The aim of this study was to examine the effect of prolonged NPS infusions in a broad dose range on the fasting motility pattern, as well as on the gene expression of the cytokines tumour necrosis factor (TNF) and interleukin (IL)-1β and inducible nitric oxide synthase (iNOS) as biomarkers of an inflammatory response.

Material and Methods

Animals

Sprague-Dawley male rats (n = 42; 300-350 g) were purchased from Scanbur AB (Sollentuna, Sweden). The rats were housed in wire-meshed cages at 24° C with constant humidity and 12:12 h light-dark cycle. The animals were allowed to acclimatize to the new environment at the animal facility, and were fed *ad libitum* with a commercial rat diet consisting of pellets (LABFOR, Lactamin R36, Kimstad, Sweden) and tap water prior to the studies. The experiments were approved by the Animal Ethics Committee in northern Stockholm (diary no. 226/09:348/09; 20091116 – 20100315).

Surgical procedure

Surgery was performed in 30 rats under anesthesia with a mixture of midazolam (5 mg ml^{-1}, Aktavis AB, Stockholm, Sweden) and Hypnorm (fentanylcitrate, 0.315 mg kg^{-1} plus fluanisone 10 mg kg^{-1}; Janssen-Cilag, Oxford, CT, USA.) given subcutaneously (SC) at a dose of 1.5-2 ml kg^{-1} body weight. Temgesic® (Schering-Plough, Stockholm, Sweden) 0.05 mg kg^{-1} body weight was given SC after surgery to avoid post-operative pain.

The abdomen was opened via a midline incision. The animals were then supplied with three bipolar insulated stainless steel electrodes (SS-5T; Clark Electromedical Instr., Reading, UK) implanted into the muscular wall of the small intestine, 5 (J1), 15 (J2) and 25 (J3) cm distal to the pylorus. All animals were supplied with an indwelling silastic catheter (Dow Corning Co., Midland, MI, USA) inserted into the external jugular vein for intravenous (IV) administration of NPS. The electrodes were pierced through the abdominal muscle wall and together with the vein catheter tunneled to the back of the animal's neck. After surgery, the animals were housed individually and allowed to recover for at least 7 days before experiments were undertaken. All animals were monitored daily.

Experimental procedures

Small bowel motility. Experiments were carried out in conscious animals after an overnight fasting period in wire-bottomed cages with free access to water. The rats were placed in Bollman cages during the experiments, and the electrodes were connected to

electroencephalography preamplifiers (7P5B) operating a Grass Polygraph 7 B (Grass Instr., Quincy, MA, USA). The main characteristic feature of myoelectrical activity of the small intestine in the fasted state, the activity front (phase III) of the migrating motor complex (MMC), was identified as a period of clearly distinguishable intense spiking activity with an amplitude at least twice that of the preceding baseline and a frequency of at least 40 spikes min^{-1}, propagating aborally through the whole recording segment and followed by a period of quiescence, phase I of MMC. The MMC cycle length, duration and propagation velocity of the activity fronts were calculated as a mean of the study period. The MMC cycle length, reflecting the interval between the propagated activity fronts was calculated at the J2 recording site. All experiments started with a control recording of baseline myoelectric activity over a period of about 60 min with four regular MMCs propagating over all three recording sites. As the fifth activity front had vanished at the first electrode site, an IV infusion of either NPS(NeoMPS, Strasbourg, France) at doses of 100, 300, 1000, 2000 or 4000 pmol $kg^{-1}min^{-1}$ (each dose, n=6), or saline solution (sodium chloride 9 g L^{-1}; 300 mosm/kg H_2O, Fresenius Kabi, Halden, Norway) (n = 6) was started using a microinjection pump (CMA 100; Carnegie Medicine, Stockholm, Sweden) and continued for 60 min, after which the experiment continued until the basal MMC pattern returned (within a total experiment time of 6 h).

Tissue sampling and RNA isolation. Twelve rats were challenged with NPS at a dose of 4000 pmol kg^{-1} min^{-1} IV during 60 min. At the end of experiments animals were euthanized with pentobarbital (Apoteksbolaget, Solna, Sweden). Tissue segments (20-30 mg) were collected from the corpus of each animal, quickly placed in RNA stabilizing reagent (RNA-later, Ambion, Applied Biosystems, Austin, TX, USA) and stored at 4° C for 24 h before storage in -20° C freezer preceding real-time PCR analysis. The tissue samples were then placed in RTL cell lysis buffer (Qiagen, Hilden, Germany) and homogenized by means of a rotor-stator knife (Ultra-Turrax T8, IKA®-Werke, Staufen, Germany). Total RNA was extracted using the RNeasy Mini Kit according to the manufacturer's instruction (Qiagen). Finally, a DNase digestion step using DNase I (Promega, Madison, WI, USA) with incubation at 37° C for 30 min was included to remove traces of DNA.
Samples with A260/A280 ratio ≥1.8 were used for PCR. Complementary DNA (cDNA) synthesis was performed on 1-5 µg of total RNA using oligo dT primers and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) at temperatures 25° C for 10 min, 42° C for 50 min, 70° C for 15 min, respectively.

Real-time quantitative polymerase chain reaction. mRNA expression of TNF, IL-1β and iNOS was analyzed using real-time quantitative polymerase chain reaction (qPCR) performed on an iCycler iQ real-time detection system (Bio-Rad Labs, Hercules, CA, USA). Primers (TNF, IL-1β) were designed according to literature[12] or our previous experimentations (iNOS)[13] and manufactured by CyberGene® (Stockholm, Sweden) (Table 1). PCR reaction mixture was prepared using QuantiTect SYBR Green PCR Master Mix (Qiagen). Each sample was analyzed in triplicate according to the manufacturer's instructions (Qiagen). For each reaction, melting curves were generated to verify the identity of amplification product. Thermal cycling conditions were 13 min at 95° C to activate the HotStarTaq DNA polymerase, followed by 40 cycles of 95° C for 15 s, 60° C for 30 sec, and 72° C for 20 sec. The expression of each gene was normalized to 18S mRNA content and calculated relative to control using the $2^{-\Delta\Delta Ct}$ method[14].

Statistical analysis

All values are expressed as mean ±SEM. Mann-Whitney test were used when comparing the MMC cycle length, duration and velocity and in the fold expression changes in the qPCR analysis. $P < 0.05$ was considered statistically significant. The Prism software package 4.0 (GraphPad Software Inc., San Diego, CA, USA) was used for the statistical comparisons.

Results

Small bowel motility. Infusion of NPS (100-4000 pmol $kg^{-1}min^{-1}$) changed the appearance of the myoelectric activity and the MMC cycle length. In the lower dose range irregular and sporadic myoelectrical spiking was induced (Fig. 1), while higher dosage range also prolonged the MMC cycle length in a dose-dependent manner (Fig. 1, 2, Table 2). NPS at 2000 pmol $kg^{-1}min^{-1}$ signficantly prolonged the cycle length, which was even more marked at 4000 pmol $kg^{-1}min^{-1}$. At the same time the duration of the activity front was dose-dependently increased while the propagation velocity was not changed (Table 2).

Expression of inflammatory markers TNF, IL-1β and iNOS. As shown in table 3 NPS at 4000 pmol $kg^{-1}min^{-1}$ for 60 min, induced an 1.8 to 2.9-fold increased mRNA expression of TNF, IL-1β, and iNOS compared to internal control. All results showed a considerable variation.

Discussion

Our present investigation demonstrates that peripheral infusion of NPS evokes a motility response in the small intestine. Firstly, at low doses irregular spiking was induced, and secondly the MMC cycle length increased to at least a doubling of the interval between the activity fronts. To this end, our studies showed NPS to be capable of inducing a numerical increase of the recognized inflammatory biomarkers TNF, IL-1β, and iNOS.

In the rat, the NPS receptor is expressed in the autonomic nervous system and GI tract[2,15], which suggests a role for NPS and its receptor (NPSR) in the regulation of GI functions. Hence, NPS has been reported to have an effect on food intake[7,8], which is influenced on GI motility primarily gastric emptying[16-20]. Thus an effect on GI motility is at hand as evidenced by our present findings of increased spiking activity of the small bowel as well as prolonged MMC cycle length. In addition to effects on physiological conditions, the NPS receptor is associated with susceptibility for inflammatory bowel disease[21]. Irregular spiking is commonly seen in inflammatory conditions, be it direct[22,23] or indirect[24]. In agreement with this Han et al.[25] found that NPS administered intracerebroventricularly inhibits colonic transit implying a motility effect in the gut. For comparison, the same authors found no *in vivo* effects of NPS when given intraperitoneally even at high dosages up to about 100 times those used in our experiments, or in studies *in vitro*. These results are inconsistent with ours, even if we used a comparably high dosage of NPS as compared to other gut peptide hormones or neuropeptides to achieve a motility response[26-30]. This fact is in agreement with a paracrine or neurocrine effect of NPS, rather than an endocrine effect, and also seems to fit with the general concept of NPS as a neuropeptide.

In order to bring about a physiological motility effect of NPS its receptor has to be localized in the intestines. Recent data show that the receptor variant NPSR1-A mRNA is expressed by enteroendocrine cells in the gut. Challenge with NPS results in up-regulation of peptides such tachykinin 1, neurotensin and galanin which encode peptide hormones to be liberated by enteroendocrine cells. Moreover, challenge with pro-inflammatory cytokines, such as TNF and interferon-beta increased NPSR1 expression in monocytes[1]. These findings also seem to have a bearing on humans since NPS and NPSR are expressed in colon as shown by *in situ*

hybridization[31] and can stimulate cell proliferation and mitogenic signals in human colon cancer cells[32].
As the NPS/NPSR system previously has been associated with the risk of developing inflammatory bowel disease we sought to investigate whether challenge with NPS is able to induce an inflammatory response. In our study, NPS given at a dose and during a period that evokes a motility response did not invariably cause an increase of the cytokines TNF, IL-1b, and iNOS. This does not unambiguously speak in direction of NPS as an inflammatory mediator.
Recently, antagonists for the NPSR were identified[33,34]. Since the antagonist was found to have no effect on basal motility but inhibited the response to exogenous NPS, it seems that NPS is not constantly tonically active, but may be activated under certain circumstances to target a particular function. This data fits with the idea of NPS being associated with inflammatory reactions. However, even though our present study could not conclusively support this picture in terms of cytokine increase, the observed increase in iNOS may be of interest as inducible NO production seems detrimental during acute colitis, but sustained up-regulation of NO is beneficial in terms of IBD[35,36]. Comparative data using diclofenac to trigger gut injury clearly disclosed an inflammatory reaction with an immense increase of iNOS expression (III).
In conclusion, peripheral NPS brings about a motility response in the gut similar to that observed in endotoxinemia and inflammatory reactions, whereas the early response to NPS did not invariably cause an inflammatory response why prolonged activation of the NPS/NPSR system may be needed to incite a chronic inflammation in the gut.

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Figure legends:

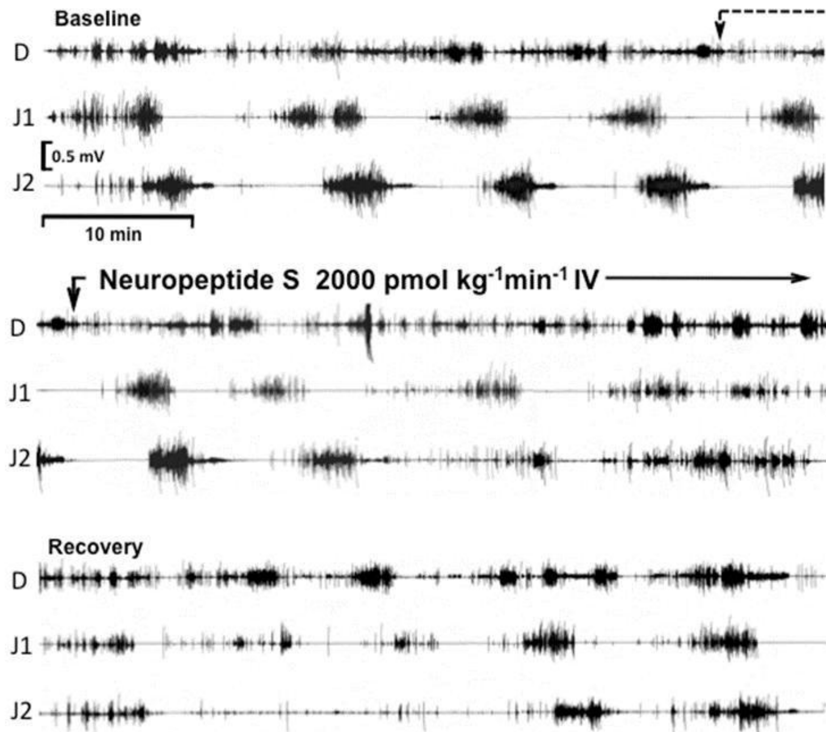

Fig. 1. Electromyographic recording showing the effect of Neuropeptide S on the myoelectrical activity of the small intestine. Upper panel: After four activity fronts of the migrating myoelectric complex under fasting conditions, infusion of neuropeptide S was started and continued during 60 min (arrow with broken/solid line). Middle panel: During infusion of Neuropeptide S at 2000 pmol $kg^{-1}min^{-1}$ intravenously (IV) irregular spiking was induced followed by prolongation of the interval between activity fronts. Lower panel: Upon termination of infusion there was a slow recovery of the intestine until recurrence of activity fronts.

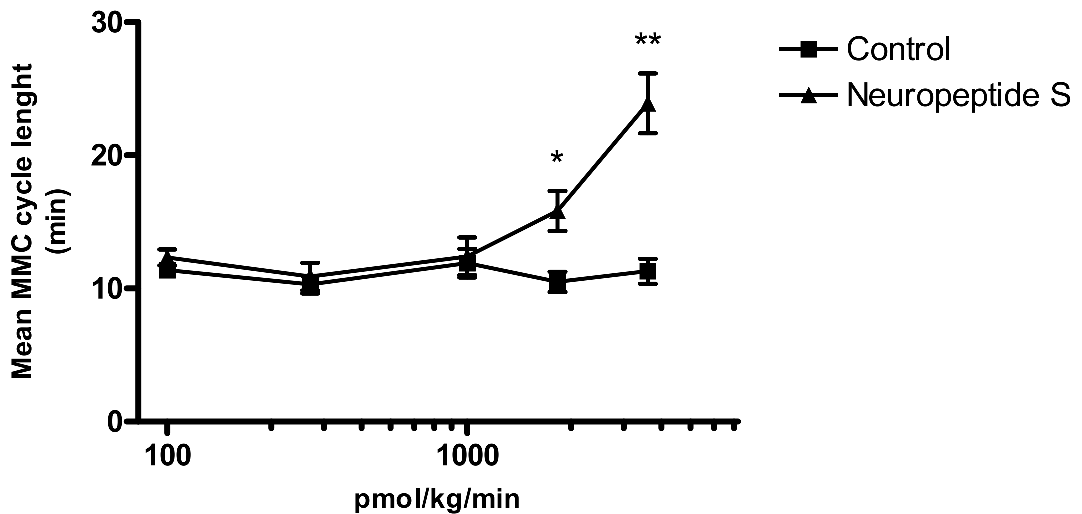

Fig. 2. Effect of infusions of neuropeptide S and saline on the cycle length of the migrating myoelectric complex in fasting small bowel motility. Infusions of saline or NPS (100, 300, 1000, 2000 or 4000 pmol $kg^{-1}min^{-1}$) were administered for 60 min. Results are mean ±SEM (**P*=0.0152; ***P*=0.0022).

Table 1. Primers of the target genes studied.

Gene product	Forward (5´-3´)	Reverse (5´-3´)
TNF	GACCCTCACACTCAGATCATCTTCT	ACGCTGGCTCAGCCACTC
IL-1β	GAAAGACGGCACACCCACC	AAACCGCTTTTCCATCTTCTTCT
iNOS	CACCTTGGAGTTCACCCA	ACCACTCGTACTTGGGATGC

iNOS, inducible nitric oxide; IL-1β, interleukine-1β; TNF, tumor necrosis factor

Table 2. Characteristics of MMC during infusion of neuropeptide S.

Study group	*Interval (min)*	*Duration (min)*	*Velocity (cm min^{-1})*
NPS Control period	11.4±0.4	3.9±0.3	1.9±0.2
Infusion of NPS (100 pmol kg^{-1}min^{-1})	12.3±0.6	3.7±0.2	1.7±0.2
NPS Control period	10.3±0.6	4.0±0.3	1.7±0.2
Infusion of NPS (300 pmol kg^{-1}min^{-1})	10.9±1.0	3.7±0.3	1.9±0.2
NPS Control period	11.9±1.1	4.1±0.5	1.8±0.2
Infusion of NPS (1000 pmol kg^{-1}min^{-1})	12.4±1.4	5.6±0.3	1.6±0.1
NPS Control period	10.5±0.8	4.4±0.2	2.0±0.2
Infusion of NPS (2000 pmol kg^{-1}min^{-1})	15.8±1.4*	11.2±0.4**	1.8±0.2
NPS Control period	11.3±0.9	3.6±0.2	2.0±0.2
Infusion of NPS (4000 pmol kg^{-1}min^{-1})	23.9±2.2**	12.4±0.7**	1.7±0.1

NPS, neuropeptide S. Values are mean ± SEM for all MMC cycles during the respective period. NPS = neuropeptide S. *P = 0.0152 (interval), **P = 0.0022 (interval and duration).

Table 3. Messenger RNA expression of target genes under challenge with neuropeptide S.

Gene	**Mean fold change in gene expression**
TNF	2.6 ±2.4
IL-1β	1.8 ±1.2
iNOS	2.9 ±0.9

Data analysis using the 2-ΔΔCt method (13). Effects of infusion of neuropeptide S (4000 pmol kg^{-1}min^{-1} IV) for 60 min on the mRNA expression of inflammatory biomarkers. Values are expressed as mean fold change of gene expression ±SEM.