Helicobacter pylori stimulates pepsinogen secretion from isolated human peptic cells

S Lorente, O Doiz, M Trinidad Serrano, J Castillo, A Lanas

Background: Different acid and peptic related gastrointestinal diseases are associated with both increased gastric secretion and Helicobacter pylori infection. Patients with H pylori associated gastritis or duodenal ulcer have increased serum pepsinogen levels which decrease after eradication. The mechanisms of H pylori induced gastric mucosal damage are not completely understood.

Aim: To determine the effects of H pylori on pepsinogen secretion from isolated human peptic cells.

Methods: Dispersed human peptic cells were prepared from endoscopically obtained biopsy specimens after collagenase digestion, mechanical disruption, and density gradient centrifugation. H pylori was obtained from gastric biopsies (antrum and body), and cultured in non-selective and selective media. Isolates of H pylori were used at different concentrations (1–20×10⁶ colony forming units (cfu)).

Results: H pylori (10⁶–2×10⁷ cfu) increased basal pepsinogen secretion in a concentration dependent manner. This stimulus was not observed with Escherichia coli. The increased secretion was in addition to that observed with 0.1 mM histamine and 0.1 mM dibutyryl-cyclic adenosine monophosphate. However, H pylori did not affect either carbamylcholine (0.1–1.0 µM) or cholecystokinin (1 µM) stimulated pepsinogen secretion. Addition of the nitric oxide synthase inhibitor Nω-monomethyl-arginine (1 mM) inhibited H pylori induced cGMP generation and pepsinogen secretion, which were also reduced in the absence of extracellular calcium. H pylori induced pepsinogen secretion was not affected by the absence/presence of the cagA gene.

Conclusions: H pylori increases pepsinogen secretion from human peptic cells through a calcium and nitric oxide mediated intracellular pathway. This effect is independent of the H pylori virulent cagA gene, and may be a mechanism of H pylori induced gastric mucosal damage.

METHODS

Materials

Chemicals were obtained from the following sources: bovine serum albumin (fraction V), carbamylcholine (carbachol), histamine, dibutyryl-cyclic adenosine monophosphate (db-AMP), cholecystokinin octapeptide (CCK-OP), HEPES, Percoll, crystalline porcine pepsinogen, soybean trypsin inhibitor, ethylene glycol tetraacetic acid (EGTA), Nω-monomethyl-arginine (L-NMMA), 8-bromo (8Br)-GMP and glucose from Sigma Chemical Co (St Louis, Missouri, USA); bovine haemoglobin from Gibco Diagnostics (Madison, Wisconsin, USA); and collagenase (type IV) from Worthington Biochemical Corp (Freehold, New Jersey, USA). All reagents for polymerase chain reaction (PCR) (primers, deoxynucleoside triphosphates, recombinant Taq DNA polymerase, and reaction buffer) were purchased from Boehringer Mannheim GmbH (Mannheim, Germany), and agarose was obtained from Bio Rad (California, USA).

Unless otherwise stated, the standard Ringer solution contained 82.2 mM NaCl, 4.0 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgCl₂, 0.8 mM NaSO₄, 17.8 mM NaHCO₃, 0.8 mM NaH₂PO₄, 11.5 mM glucose, and 0.2% bovine serum albumin. The medium was equilibrated with 95% O₂–5% CO₂, and pH adjusted to 7.25. Ca⁺⁺ low medium contained 0.1 mM CaCl₂ and no MgCl₂, and Ca⁺⁺ free medium contained no CaCl₂ or MgCl₂.

Human material

Gastric biopsy specimens from the oxyntic area were obtained from 76 patients undergoing upper gastrointestinal endoscopy (39 men/37 women). Mean age of this population was 47.1 years.

Abbreviations: cfu, colony forming units; db-AMP, dibutyryl-cyclic adenosine monophosphate; carbachol, carbamylcholine; CCK-OP, cholecystokinin octapeptide; EGTA, ethylene glycol tetraacetic acid; L-NMMA, Nω-monomethyl-arginine; PCR, polymerase chain reaction.
(21–72) years. In 21 of these patients, endoscopy was normal. 16 had an active duodenal ulcer, four a healed duodenal ulcer, eight oesophagitis, three a gastric ulcer, and 24 duodenitis or antritis without ulcer. Most were or had recently been receiving H2 receptor antagonists, and the rest were receiving antacids or were free from any treatment. *H pylori* status was determined by histological examination and/or the urease test (CLO test; Delta West LTD, Canning Vale, Bentley, Australia) in 68 patients (positive in 46). No biopsy specimens were obtained from patients who had recently used non-steroidal anti-inflammatory drugs. The study was approved by the Institutional Review Board and written informed consent was obtained from all patients.

**Study design**

**Cell isolation**

Dispersed peptic cells were obtained from endoscopically acquired biopsy specimens after collagenase digestion, filtration, and density gradient centrifugation, according to a method previously described.8–14 Eight to 10 regular sized punch biopsy specimens yielded approximately 10^6 cells which were suspended in Ringer’s solution under an atmosphere of 95% O2–5% CO2 and refrigerated overnight. The next morning, cells were allowed to warm at room temperature before they were used in experiments. No experiments were performed if cell viability was lower than 90% and experiments with a low total pepsinogen content were discarded.

**Culture of *H pylori***

*H pylori* were obtained from gastric biopsies (antrum and body of the stomach) of different patients (table 1). Specimens were placed in a sterile tube with 0.5 ml physiological serum, rapidly processed, and cultured in a non-selective medium (blood agar) or a selective *H pylori* medium (Dent medium; Oxoid, Madrid, Spain) with antibiotics (vancomycin, trimethoprim, cefsulodin, and amphotericin). Plates were incubated in a microaerobic atmosphere (5–10% CO2, 5–20% O2 and 80–90% N2) at 37°C with a relative humidity of 95% for up to seven days. Identification of the bacteria was performed by Gram stain and oxidase, catalase, and urease activity. Different *H pylori* strains were prepared in saline solution to obtain a heavy suspension (McFarland standard 6) of growth from plates. Isolates of *H pylori* were used at different concentrations (1–20×10^5 colony forming units (cfu)). Sonication of the bacteria was performed by ultrasonic homogenisation at 6 kHz for one minute (Bandelin Sonopuls, Berlin, Germany).

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**Detection of the cagA gene**

The presence of the cagA gene was determined from eight of 18 different *H pylori* strains used in this study (table 1). Genomic DNA was extracted from microbial cultures or gastric biopsy specimens according to standard proteinase K digestion and phenol/chloroform/isoamilalcohol extraction methods. DNA was amplified by PCR using previously described specific oligonucleotide primers.15 DNA samples were subjected to a previous three minute denaturation step at 95°C followed by 30 cycles comprising one minute of denaturation at 95°C, one minute of annealing at 55°C, and one minute of elongation at 72°C. After 30 cycles, a final 10 minute elongation step at 72°C was performed. The PCR product was subjected to gel electrophoresis (1.5% agarose) and ethidium bromide staining, revealing a 400 bp band when the cagA gene was present.

**Experiments and calculations**

Warmed cells were resuspended in fresh Ringer’s solution and counted (haematocytometer). Aliquots of 10^5 cells were then placed in 1.5 ml polypropylene test tubes containing different concentrations of *H pylori* and the appropriate agents, and incubated in a shaking water bath under an atmosphere of 95% O2–5% CO2 at 37°C for different periods of time. In general, cells from a single patient were sufficient for 9–11 aliquots (including duplicates). The experiments ended by centrifuging the tubes for 17 seconds at 14 000 g and pipetting off the supernatants. An aliquot of the supernatant was assayed for pepsinogen activity using a modified Anson-Milsky method using acid denatured haemoglobin as the substrate, as previously described.16 Secretory responses to stimuli were calculated as output per unit time with basal output subtracted. Mean (SEM) basal rate of pepsinogen secretion in the experiments was 0.18% (0.007) of total pepsinogen content per minute.

In some experiments carried out to study the role of calcium in basal or stimulated pepsinogen secretion, cells were washed once in calcium free Ringer’s solution, resuspended in the same solution containing 0.5 mM EGTA for three minutes to remove extracellular Ca++, washed again, and finally resuspended in calcium free Ringer’s solution plus the drugs to be tested. When the nitric oxide synthase inhibitor L-NMMA was used, this was added 15 minutes before the other agents.17 For determination of cGMP, chief cells ( aliquots of 10^5 ) were incubated alone or with 1 mM L-NMMA and then stimulated with *H pylori* or other agonists. After centrifugation at 14 000 g for 30 seconds, the supernatants were aspirated and the pellets were mixed with 0.5 ml iced 65% ethanol. cGMP was measured in 100 μl of supernatant by specific radioimmunoassay kit (Amersham Pharmacia Biotech, Barcelona, Spain).

Unless otherwise specified, pepsinogen secretion obtained after different stimuli was expressed as percentage of total cell pepsinogen content with basal secretion subtracted. Data are reported as mean (SEM) and the “n” in each experiment is equal to the number of separate cell preparations. The Student’s t test for paired or unpaired data was used for statistical analysis and applied where appropriate.

**RESULTS**

**Effects of *H pylori* on basal pepsinogen secretion from isolated human peptic cells**

No differences were found in basal pepsinogen secretion from isolated cells in subjects with normal endoscopy or those with abnormal findings (see methods). Most patients were *H pylori* positive (46 of 68 in whom the status was determined) but no differences in basal pepsinogen secretion were found in experiments performed with cells obtained from *H pylori* positive and *H pylori* negative patients (basal secretion 5.5 (0.2) vs 5.8 (0.2)% of total pepsinogen content). Similarly, no differences were found in experiments performed with cells
obtained from duodenal ulcer and non-duodenal ulcer patients (basal secretion 5.5 (0.3)% vs 5.4 (0.3)% of total pepsinogen content).

*H pylori* stimulated basal pepsinogen secretion from isolated peptic cells in a concentration dependent manner (fig 1). Maximal stimulation was achieved with 10⁷ cfu (Δ secretion 2.7 (0.5)%). This was not observed with other Gram negative bacteria (*E coli*) at the same concentration (Δ secretion 0.2 (0.2)%). The presence of previous *H pylori* infection or duodenal ulcer did not influence the effect of the bacterium on pepsinogen secretion (Δ secretion 1.2 (0.1)% in *H pylori* positive v 1.3 (0.2)% in *H pylori* negative patients after *H pylori* 10⁷ cfu (n=35 and 17, respectively) and Δ secretion 1.4 (0.2)% in duodenal ulcer v 1.5 (0.2)% in non duodenal ulcer (n=16 and 15, respectively)). Moreover, *H pylori* induced pepsinogen secretion was not affected by the absence/presence of the *cagA* gene because no differences were found with the different strains (fig 2).

Bacterial sonicates also increased basal pepsinogen secretion but the stimulus was lower than that obtained with the whole bacteria at the same concentration (fig 3).

This effect was calcium dependent because removal of extracellular calcium inhibited the stimulating effect of *H pylori* on pepsinogen secretion (10⁷ cfu=1.1 (0.5)% with v 0.1 (0.4)% without calcium).

**Effects of *H pylori* on stimulated pepsinogen secretion**

*H pylori* increased histamine and db-AMP stimulated pepsinogen secretion. As previously shown,¹⁰ 0.1 mM concentrations of histamine and db-AMP significantly stimulated pepsinogen secretion from dispersed human peptic cells. When *H pylori* was combined with histamine, pepsinogen secretion was stimulated in an additive manner (fig 4). The addition of *H pylori* (10⁷ cfu) further increased pepsinogen secretion in db-AMP (0.1 mM) stimulated cells (fig 5).

Carbachol dose dependently increased pepsinogen secretion from dispersed human peptic cells but *H pylori* (10⁷ cfu) did not increase either carbachol (0.1–10 μM) or CCK (1 μM) stimulated pepsinogen secretion (fig 6).

**Role of nitric oxide on *H pylori* stimulated pepsinogen secretion**

Stimulating chief cells with 0.1 mM 8Br-cGMP, a cell permeable cGMP analogue, increased basal pepsinogen release (Δ secretion 2.4 (0.2)%; n=4; p<0.05). This suggests that nitric oxide is involved in the control of pepsinogen secretion in human chief cells as cGMP is the final effector of nitric oxide in target cells.

To study whether nitric oxide modulated the stimulatory effect of the bacterium on pepsinogen release, cells were preincubated alone or with 1 mM l-NMMA, an l-arginine derivative inhibitor of nitric oxide synthase, for 15 minutes and then
Helicobacter pylori increased dibutyryl-cyclic adenosine monophosphate (AMPc) stimulated pepsinogen secretion from dispersed human peptic cells. The combination of these agents potentiated pepsinogen secretion but did not have an additive effect (*p<0.05). The effect of AMPc on basal secretion was also significant (†p<0.05). Results are presented as mean (SEM) of four separate experiments. Basal secretion=5.9 (0.2)%. The H pylori strains used in these experiments correspond to strains HP25, HP31, HP32, HP33, and HP34.

Carbachol (10 µM)+L-NMMA (mM) 4.28% (0.6)
Carbachol, carbamylcholine; L-NMMA, Nω-monomethyl-L-arginine.

*B*p<0.05.

Agent cGMP (%)

<table>
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<tr>
<th>Agent</th>
<th>cGMP (%)</th>
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<tr>
<td>Carbachol (10 µM)</td>
<td>17.13% (0.3)*</td>
</tr>
<tr>
<td>H pylori (10⁷ cfu)</td>
<td>9.86% (0.2)*</td>
</tr>
<tr>
<td>Carbachol (10 µM)+L-NMMA (mM)</td>
<td>4.28% (0.6)</td>
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<tr>
<td>H pylori (10⁷ cfu)+L-NMMA (mM)</td>
<td>1.48% (0.6)</td>
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</table>

Results are expressed as per cent of increase over basal values. Basal concentrations of cGMP were 5.37 (0.5) fmol/10⁶ cells (four experiments). *p<0.05. Carbachol, carbamylcholine; L-NMMA, Nω-monomethyl-L-arginine.

**DISCUSSION**

In this study, we showed that H pylori stimulated basal pepsinogen secretion from isolated human peptic cells in a concentration dependent manner. This stimulus was also observed with H pylori sonicates at the same concentration although the secretory response was lower than that achieved with the whole bacterium. The H pylori associated stimulating effect was independent of the presence of the cagA gene but dependent on the presence of extracellular calcium as cell incubation in a calcium free medium abolished increased pepsinogen secretion completely. The intracellular mechanisms involved in H pylori stimulated pepsinogen secretion were calcium and nitric oxide dependent as H pylori associated stimulating increase in histamine and db-AMP stimulated pepsinogen secretion was additive and was inhibited after preincubation with a nitric oxide inhibitor.

Maximal increase in basal pepsinogen secretion was obtained at a concentration of 10⁷ cfu but the effect was observed at any concentration (10⁶–2×10⁸ cfu) using both the whole bacterium and its sonicated products. Nevertheless, the pepsinogen stimulus obtained with sonicated H pylori was 50% lower than that obtained with the whole bacterium. These data agree with those reported by Cave and Cave who found that H pylori sonicates increased pepsinogen secretion in isolated rabbit gastric glands and suggested that a bacterial factor was the causative agent of the stimulus. The lipopolysaccharide contained in the membrane of H pylori might be the responsible factor as Young and...
colleagues showed that purified \textit{H pylori} lipopolysaccharide increased pepsinogen secretion by 50-fold while the \textit{E coli} lipopolysaccharide raised this 12-fold. However, the lower secretion obtained with \textit{H pylori} sonicates in our study only suggests that bacterial membrane integrity plays a role in \textit{H pylori} induced pepsinogen secretion. It is possible that in addition to lipopolysaccharide, proteins of the bacterial wall (for example, adhesins) could also be implicated, and the effect may be multifactorial. This however remains to be investigated.

Another reason for the lower level of pepsinogen secretion found in our experiments may be the different experimental model used. \(^1\) In their studies, Young and colleagues used gastric mucosal fragments from the guinea pig which include several cell types. However, we used isolated human peptic cells, and it is possible that our data show only interspecies differences.

It is well known that serum pepsinogen levels in patients with duodenal ulcer are higher than those observed in \textit{H pylori} gastritis, \(^2\) and that \textit{cagA} (+) strains are more prevalent in the former. \(^3\) This clinical information suggests a potential role for the \textit{cagA} gene as the bacterial factor involved in the increase in pepsinogen secretion. However, we did not find differences in pepsinogen secretion using either \textit{cagA} (+) \textit{H pylori} or \textit{cagA} (−) \textit{H pylori} strains, suggesting that other factors must mediate the increased secretion. A recent study carried out in vitro on pig chief cell monolayer cultures \(^4\) showed that VacA and CagA proteins do not affect \textit{H pylori} induced pepsinogen secretion. However, Chan and colleagues \(^5\) showed that pretreatment of human gastric adenocarcinoma cells with purified vacuolating toxin induced a concentration dependent release of pepsinogen. Differences between these studies can be explained by the use of either normal or immortalised cells whose phenotypic expression might be different. In the past it was indicated that hyperpepsinogenemia could be determined by genetic factors. \(^6\)

Another potential factor affecting pepsinogen secretion may be the characteristics of the patient whose cells were isolated to carry out different experiments (for example, the presence of duodenal ulcer, \textit{H pylori} status, etc.). However, our data showed that these characteristics did not affect the interexperiment variability observed in basal secretion or pepsinogen secretion obtained after the \textit{H pylori} stimulus.

With regard to the effect of the bacteria on stimulated pepsin secretion, our data showed that \textit{H pylori} increased histamine and db-AMP stimulated pepsinogen secretion which supports the concept that \textit{H pylori} stimulated pepsinogen secretion is mediated by calcium. When \textit{H pylori} was combined with histamine, pepsinogen secretion was increased in an additive manner, indicating that the \textit{H pylori} stimulus was mediated by a different intracellular mechanism. In the same way, simultaneous addition of \textit{H pylori} and db-AMP potentiated pepsinogen secretion but it did not have an additive effect. This may be because the rise in db-AMP stimulated pepsin secretion depends mainly on adenylyl cyclase activation but also on calcium dependent intracellular signals. \(^7\)

Beil and colleagues \(^8\) showed that db-AMP stimulated secretion decreased with H-8, a protein kinase A inhibitor, but also with lanthanum and W-7, a calmodulin antagonist, suggesting that pepsinogen secretion induced by db-AMP was partially regulated by the calcium-calmodulin system.

In addition, when the effect of the bacterium on CCK-OP and carbachol stimulated pepsinogen secretion (both are agonist inducers of pepsin secretion by activating the calcium intracellular system) was studied, no change in stimulated pepsinogen secretion was observed, suggesting that \textit{H pylori} activates the same intracellular pathway as CCK and carbachol. On the other hand, no increase in pepsinogen secretion was found when cells and \textit{H pylori} were incubated in a calcium free medium or after calcium chelation with EGTA.

Three studies have been reported which focus on the effect of \textit{H pylori} on stimulated pepsinogen secretion with different agonists. In agreement with our results, Beil and colleagues \(^9\) showed that both db-AMP and 12-O-tetradecanoylphorbol-13-acetate, a phorbol ester activator of protein kinase C, increased the stimulus of \textit{H pylori} on pig chief cell monolayers. Moreover, secretion was inhibited with both lanthanum and W-7. Other authors \(^10\) have reported that \textit{H pylori} sonicates significantly increased basal and histamine stimulated pepsinogen secretion from human chief glands. However, Cave and Cave \(^11\) found an additive effect with carbachol and CCK-OP. However, as noted above, it is important to note that Cave and Cave carried out their studies in rabbit glands.

Finally, our results showed that cell preincubation with L-NMMA (a nitric oxide synthase inhibitor) significantly inhibited the pepsinogen response induced by \textit{H pylori}. These findings support the hypothesis that \textit{H pylori} induced pepsin secretion is calcium dependent and that nitric oxide acts as a second messenger in the pepsinogen secretion process induced by a Ca\(^++\) mediated agonist. \(^12\) To confirm these findings, we carried out a series of experiments where first we demonstrated that 8Br-cGMP which is the final effector of nitric oxide, significantly increased basal pepsinogen release. In a second step, we also showed an increase in the cellular concentration of cGMP in \textit{H pylori} stimulated pepsin cells, which was inhibited by preincubation with L-NMMA. We tried to quantify nitric oxide generation by chief cells after carbachol and \textit{H pylori} stimulation by colorimetric determination of citrulline \(^13\) but unfortunately we were unable to detect this coproduct of nitric oxide which was probably due to the scarce cell mass available in our experiments which was much lower than that reported in other experiments with guinea pigs. \(^14\)

In conclusion, our results showed that \textit{H pylori} induced a direct stimulating effect on pepsinogen release from isolated human peptic cells by nitric oxide and calcium dependent mechanisms. From a pathogenic point of view, this effect may be of clinical relevance as in addition to other mechanisms, \(^15\) the in vitro effect of \textit{H pylori} on pepsinogen secretion seen in our experiments was translated into an increase in intramusosal and/or intraluminal content of activated pepsin, which might eventually potentiate the mucosal damage associated with \textit{H pylori} infection.

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Authors’ affiliations
S Lorente, M Trinidad Serrano, A Lanas, Service of Gastroenterology, University Hospital, Zaragoza, Spain
O Dois, J Castillo, Service of Microbiology, University Hospital, Zaragoza, Spain

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