Water Extract of Helicobacter pylori Inhibits Duodenal Mucosal Alkaline Secretion in Anesthetized Rats

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Background & Aims: The pathophysiology behind Helicobacter pylori–induced gastroduodenal dysfunction is incompletely understood. The aim of this study was to investigate if a water extract of H. pylori distorts acid-induced duodenal mucosal alkaline secretion. Methods: Chloralose-anesthetized rats were prepared for duodenal luminal perfusion and in situ pH-stat titration of mucosal alkaline secretion. Results: Mucosal bicarbonate secretion increased approximately 55%–60% after a 5-minute exposure to 10 mmol/L HCl. This response was absent when water extracts of three strains of H. pylori (protein content, 0.2–20 μg/mL) had been added to the perfusate. Presence of 3 mmol/L L-arginine, but not the stereoisomer D-arginine, in the luminal perfusate reversed the H. pylori extract blockade of acid-induced mucosal alkaline secretion. High-performance liquid chromatography–based analyses showed that the endogenous nitric oxide synthase inhibitor asymmetric dimethyl arginine (ADMA) increased fourfold in duodenal perfusate and fivefold in duodenal tissue after H. pylori extract exposure. In vitro proteolysis of H. pylori extract also resulted in a substantial accumulation of ADMA. Exogenously administered ADMA, giving similar tissue concentrations, inhibited the mucosal alkaline response to acid exposure. Conclusions: Water extracts of H. pylori inhibit acid-induced mucosal alkaline secretion via interference with mucosal NO synthase.

Materials and Methods

General

The experiments were approved by the Animal Ethics Committee of Göteborg University. Male Sprague–Dawley rats (Möllegard, Denmark) were anesthetized with methohexital injected intraperitoneally (75 mg/kg body wt) and α-chloralose injected intravenously (bolus, 50 mg/kg plus 25 mg·kg⁻¹·h⁻¹). The body temperature was maintained at 38°C with a heating pad and lamp. A catheter was inserted into the trachea to ensure free airways. A femoral artery and one vein were catheterized with polyethylene 50 tubes for subsequent blood pressure measurements and drug infusions, respectively. Blood pressure was recorded using a Statham P23Dc transducer (Statham, Hato Rey, Puerto Rico). To avoid with peptic ulcer is the ability of the duodenal mucosa to increase its alkaline secretion in response to acid exposure. The physiological mediation of acid-induced duodenal mucosal alkaline secretion involves several regulatory principles, e.g., local synthesis of prostaglandins and neurohumoral control. Furthermore, recent studies showed that the acid-induced response involves also the L-arginine/nitric oxide pathway. The question was raised if luminal factors, released from the H. pylori infection in patients with duodenal ulcer, disturb the mucosal responsiveness to luminal acid, via interference with the L-arginine/NO axis. Theoretically, this could be related to the microbe itself by excreted “toxins” disturbing NO formation or to indirect effects related to the inflammatory reaction. We decided to test the former possibility in an in vivo rat model by briefly exposing the duodenal mucosa to a water extract of H. pylori. Presence of H. pylori extracts (Hpx) markedly inhibited the mucosal responsiveness to acid because of local interference with NO synthase (NOS). Some of these data have been presented in abstract form previously.

Abbreviations used in this paper: ADMA, asymmetric dimethyl arginine; Hpx, Helicobacter pylori extract; NOS, nitric oxide synthase. © 1997 by the American Gastroenterological Association 0016-5085/97/$3.00
measured as described previously. Briefly, after a midline laparotomy, a 1.6-cm segment of the duodenum with intact vascular supply was isolated between two glass tubes connected to a water-jacketed (38°C) reservoir containing 150 mmol/L NaCl. The saline was circulated through the segment using a gas-lift (pure air). The common bile duct was catheterized to avoid contamination of the segment by bile and pancreatic juice. Alkaline secretion to the perfusate was continuously titrated with HCl by pH-stat equipment.

**Bacterial Water Extracts**

*H. pylori* strains E50 (provided by Dr. J.-P. Butzler, WHO Collaborating Centre for Enteric Campylobacter, Brussels, Belgium); C21, a vacuolating cytotoxin negative strain (our clinical isolate from a patient with duodenal ulcer), were used. The strains were kept at −70°C until use and were then grown on Columbia agar plates with 8% horse blood in microaerophilic atmosphere of 10% CO₂ and 5% O₂ for 48–72 hours. Bacteria from agar plates were inoculated into *Brucella* broth supplemented with 1% dimethyl-β-cyclodextrin (a gift from Teijin Ltd., Tokyo, Japan), vancomycin, trimethoprim, and polymyxin. The liquid bacterial cultures were grown for 72 hours under microaerophilic conditions without shaking. *Escherichia coli* K12 strain C 600 was grown in Luria–Bertani broth at 37°C overnight. *H. pylori* from 1 L of liquid culture or 25 mL of *E. coli* culture was pelleted by centrifugation at 6000g and resuspended in 5 mL of distilled water. The bacterial suspensions were kept at room temperature for 20 minutes and shaken occasionally. Proteins extracted by this procedure were separated from bacteria by centrifugation at 20,000g. The protein contents were estimated by subtracting the absorbance value at 310 nm from the value at 280 nm, and protein concentration was expressed in milligrams per milliliter. The water extracts were stored at −70°C until use.

**Analyses of Arginine Analogues**

Occurrence of monomethyl arginine, asymmetric dimethyl arginine (ADMA), and symmetric dimethyl arginine in luminal perfusates and in the tissue of the duodenal segment under study was analyzed using a modified high-performance liquid chromatographic method with fluorescence detection. The luminal perfusates were frozen at −70°C until analysis. The duodenal segment, arranged for secretion study as described above, was dissected and immediately homogenized in 4 mL of 150 mmol/L NaCl (4°C) using a Polytron PT homogenizer (Kinematic AG, Lucerne, Switzerland). The homogenate was centrifuged at 23,000g for 20 minutes (4°C), and the supernatant was stored at −70°C until analysis.

**Drugs**

For anesthesia, methohexital (Brietal; Lilly Inc., Indianapolis, IN) and α-chloralose (Sigma Chemical Co., St. Louis, MO) were used. L- and D-arginine hydrochloride (Sigma) and proteinase K (Boehringer Mannheim Scandinavia AB, Bromma, Sweden) were freshly dissolved as stock solutions.

**Experimental Protocol**

The animals were left undisturbed for 1 hour after surgery. Acid exposure was performed after a 45–60-minute control period, and the circulating saline in the titration-chamber was then changed to body-tempered 10 mmol/L HCl (made isotonic with the addition of NaCl) during 5 minutes. In a separate series, without acid exposure, the acute effects of luminal administration of, e.g., bacterial extracts and L-arginine, were studied. The animals were divided into different groups (each 6 animals) depending on treatment. The experiments were terminated by induction of cardiac arrest with intravenous KCl.

**Statistics**

Analysis of variance (ANOVA) for repeated measurements and Bonferroni’s post hoc test were used to evaluate significance of changes within groups. Secretory data obtained during the control period immediately before any interference (e.g., acid exposure) were used as basal conditions. Responses to stimulations were analyzed by using the induced net change in secretion (prestimulatory value minus the value most different in secretory values could not be attributed to either the titration technique, because the acid buffering capacity was similar in the control solutions, or to any significant
difference in the physicochemical properties such as temperature and osmolality (data not shown).

**Effects on Acid-Induced Changes**

The mucosal alkaline secretion increased approximately 60% after 5-minute exposure to 10 mmol/L HCl. Interestingly, this response was absent when Hpx had been added to the perfusate (Figure 2). Hpx was added 45 minutes before the acid exposure at a concentration of 20 µg/mL protein content; this concentration was maintained also during and after the acidification. The three different *H. pylori* strains (E50, C21, and Hel73) showed identical blockade of acid-induced duodenal mucosal alkaline secretion. A similarly prepared extract of a nonenterotoxin-producing *E. coli* strain did not block the response, although the acid-induced response varied considerably between animals (Figure 2). Dose-response relations for strain E50-based Hpx showed that potent inhibition of the acid-induced mucosal alkaline secretion occurred at protein concentrations exceeding 0.2 µg/mL (Figure 3).

**Effects of Arginine**

Short-term addition L-arginine (3 mmol/L) to the duodenal perfusate transiently lowered the alkaline secretion by approximately 50%. The secretion returned to baseline values within 20 minutes (n = 6; data not shown). Presence of L-arginine did not influence the response to 10 mmol/L HCl (n = 6; data not shown). When 3 mmol/L L-arginine had been added to the Hpx-containing (2 µg/mL) luminal perfusate, acid exposure increased the duodenal mucosal alkaline secretion to a level similar to one observed in untreated controls (Figure 4). A group of animals given the stereoisomer D-arginine (3 mmol/L) in the Hpx-containing luminal perfusate (2 µg/mL; n = 6) had similar steady-state basal secretion as the controls and L-arginine/Hpx–treated animals.

**Figure 1.** Duodenal mucosal alkaline secretion in the presence of various bacterial extracts with a protein concentration of 20 µg/mL. Data are means ± SEM; n = 6 for each group.

**Figure 2.** Effect of a 5-minute exposure to 10 mmol/L HCl on duodenal mucosal alkaline secretion in the presence or absence of bacterial extracts (20 µg/mL protein content). Data are means ± SEM; n = 6 for each group. ***P < 0.001, significant difference from control. n.s., nonsignificant difference.

**Figure 3.** Dose-response relationship between the presence of strain E50 Hpx at various concentrations and the increase in duodenal mucosal alkaline secretion elicited by a 5-minute exposure to 10 mmol/L HCl. The effect of acid exposure was tested in 6 animals for each Hpx concentration. A plotted value represents mean ± SEM from each group. *P < 0.05, ***P < 0.001; significant difference from control (equals zero concentration).
However, acid-induced stimulatory responses were absent when D-arginine (3 mmol/L) had been added together with Hpx (Figure 4).

**Direct Measurements of NOS Inhibitors**

In vitro circulated Hpx perfusate contained no detectable amounts of any of the analyzed methylated arginines. However, significant levels of ADMA but neither monomethyl arginine nor symmetric dimethyl arginine were recorded after intraduodenal circulation. Control perfusate (without Hpx) was shown to contain approximately 0.1 μmol/L of ADMA after 45 minutes of intraluminal circulation, whereas ADMA levels in Hpx-containing perfusates were fourfold higher (Table 1). Furthermore, tissue concentrations of ADMA in Hpx-exposed duodeni were fivefold higher than in control segments treated similarly, but without Hpx exposure (Table 1). In vitro proteolytic degradation of Hpx (protein content, 1.4 mg/mL) with proteinase K (200 μg/mL, 37°C) over 2 hours resulted in an increase in ADMA concentration from 0 to 6.3 μmol/L.

**Effects of Exogenously Administered ADMA**

Dose-response relationship between luminally administered ADMA and the mucosal alkaline response to 10 mmol/L HCl showed that a perfusate concentration of 50 and 500 μmol/L significantly (P < 0.05) inhibited the response (Figure 5). Furthermore, 45 minutes after receiving 50 μmol/L ADMA intraluminally, duodenal tissue concentration of the compound was 17 ± 2 nmol/g wet wt (n = 6), of the same order of magnitude as shown for the group exposed to Hpx during 45 minutes.

**Discussion**

This study shows that luminal administration of *H. pylori* water extracts inhibits duodenal mucosal alkaline secretion in response to acid exposure. Such an effect was not evident when an extract of a nonpathogenic *E. coli* strain was used, suggesting some specificity for the *H. pylori* species. Furthermore, data indicated a point of action for Hpx at the L-arginine/NO pathway in the duodenal mucosa. The involvement of NO in acid-induced mucosal alkaline secretion has been proposed by Bilski and Konturek based on systemic administration of NOS-inhibiting arginine analogues in awake dogs. We have shown that arginine analogues effectively inhibit the acid-induced mucosal alkaline response also when administered luminally. The present study showed that the Hpx inhibition of acid-induced mucosal alkaline secretion was counteracted by luminal administration of 3 mmol/L L-arginine, but not the stereoisomer D-arginine. These data indicate that Hpx acts as a false NOS substrate that can be competed with by high concentration of the true substrate, L-arginine. Thus, the factor in Hpx responsible for this effect is presumably an L-arginine analogue. A number of such synthetically derived arginine analogues, with specificity for NO, are cur-

**Table 1. Concentration of ADMA in Duodenal Perfusate and Tissue**

<table>
<thead>
<tr>
<th>Perfusion</th>
<th>Tissue</th>
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<tbody>
<tr>
<td><strong>Ex vivo</strong></td>
<td><strong>After 45 min of duodenal luminal perfusion (μmol/L)</strong></td>
</tr>
<tr>
<td>Control (saline)</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>Hpx (protein content, 20 μg/mL)</td>
<td>&lt;0.03</td>
</tr>
</tbody>
</table>

**NOTE.** Each value represents mean ± SEM from six experiments.

*P < 0.01, *P < 0.001; significant difference from control.
In an attempt to further characterize the factor in the Hpx-perfusate mediating the inhibition of alkaline mucosal secretion, we screened for a number of such methylated arginines. One of these, ADMA, was detected in the duodenal perfusates and tissue. This arginine analogue is a potent NOS inhibitor in various cardiovascular test systems and, when administered exogenously, inhibited the acid-induced alkaline secretory response in the present study.

The Hpx-treated perfusate contained approximately 0.4 μmol/L ADMA when the acid-induced mucosal response was completely blocked. The concentration was zero in the original Hpx, which had not been circulated in vivo. However, ADMA was detected also in duodenal perfusates not exposed to Hpx. These values were significantly lower than after Hpx and probably reflect the endogenous production as reported previously. The increased luminal ADMA concentrations after Hpx are probably caused by intraluminal protein degradation into free amino acid residues. It seems reasonable that membrane-bound epithelial peptidases are responsible for the occurrence of ADMA in the luminal perfusate. Such proteolytic enzymes, in addition to peptide degradation, facilitate transmembrane transport of the resulting dipeptides or free amino acids, which, therefore, accumulate within the epithelial cell layer. When ADMA was administered exogenously to the perfusate, a secretory inhibition was obtained with perfusate concentration of 50 μmol/L. The duodenal tissue concentration of ADMA at this luminal concentration was similar to those after Hpx, supporting a causal relationship.

It has been reported that ADMA is formed endogenously subsequent to nuclear lysis after cell death. A substantial accumulation of ADMA was evidenced in the present study by proteolysis of Hpx in vitro. However, with the presently used preparation of the bacterial extracts it cannot be distinguished whether it is lysated cell structures or specifically expressed proteins secreted by the bacterium that are degraded to ADMA. Independent of the source for mucosal ADMA formation, the present results explain why the NO-dependent mucosal alkalinization in response to luminal acid is defective in the presence of degraded H. pylori in our rat model. It remains to be investigated if this is the case also in patients with duodenal ulcer. An active H. pylori infection, by necessity, produces numerous apoptotic or otherwise killed bacteria that are degraded in close vicinity of the mucosal surface. A local H. pylori–derived blockade of gastroduodenal NOS may be an explanation to the decreased mucosal alkaline secretory capacity observed in

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**Figure 5.** Effect of ADMA administered intraluminally on the duodenal mucosal alkaline response to a 5-minute exposure to 10 mmol/L HCl (each value is the mean ± SEM from 6 separate experiments). *P < 0.05, significant difference from zero concentration.
patients with duodenal ulcer. Furthermore, NO is a regulatory factor of several principal gastrointestinal functions (e.g., local blood perfusion, mucosa-protective functions, epithelial permeability, motility), and NO is used as a cytotoxic agent in inflammatory reactions. Consequently, it may be of interest to consider inhibition of the mucosal L-arginine/NO system as a virulence factor for gastrointestinal pathogens other than *H. pylori*.

In summary, the present results suggest that the water extract of *H. pylori* contains certain peptides that, when in contact with the duodenal lumen, probably by means of the inherent digestive proteolytic systems, become degraded into free amino acid residues, accumulating within the mucosa. Some of these amino acid residues, e.g., the presently analyzed ADMA, are potent inhibitors of NOS that interfere with the mediation of the mucosal alkaline secretory response to luminal acid.

**References**


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