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cagA+ *Helicobacter pylori* induces greater levels of prostaglandin E_2 than cagA− strains

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Abstract

cagA+ *Helicobacter pylori* (HP) infection is associated with an increased risk of distal gastric cancer. Previous studies investigating the effect of HP infection on prostaglandin E_2 (PGE_2) levels have not differentiated between cagA+ and cagA− strains and consequently have produced contradictory results. The aim was to investigate the effect of cagA+ strains on PGE_2 and enhance the understanding of the mechanisms leading to gastric diseases. Hundred patients without peptic ulcers and not on medication were recruited (one later excluded) from endoscopy clinics: six biopsies were obtained from each patient. PGE_2, colonization density and histology were determined. In addition, HP status was assessed by histology, CLOtest and culture with cagA+ being determined by PCR. Sixty-nine patients were HP− and 30 HP+ (10 cagA+, 18 cagA−, 2 undetermined). In age and sex-matched patients, PGE_2 was significantly greater ($P = 0.04$) in HP+ (37.2 ± 1.2 pg/mg per 20 min) than in HP− (22.6 ± 1.2). In patients without atrophy, those infected with cagA+ had significantly higher ($P = 0.03$) PGE_2 levels (53 ± 1.1) than HP−patients (22.6 ± 1.1) and greater levels ($P = 0.29$) than cagA− patients (35 ± 1.3). In conclusion, the increased levels of PGE_2 in the presence of cagA+ infection could be an important factor by which cagA+ strains enhance the gastric mucus layer protective functions leading to established colonization, gastritis and increased

Abbreviations: bp, base pair; cagA, cytotoxin-associated gene A; cag-PAI, cag-pathogenicity island; CFU, colony forming unit; CLOtest, Campylobacter-like organism; *H. pylori*, *Helicobacter pylori*; HP, *Helicobacter pylori*; IL, Interleukin; kb, kilobase; *n*, number; ng, nanogram; PCR, polymerase chain reaction; PGE_2, prostaglandin E_2; pg, picogram; (+), positive; (−), negative

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risk of gastric cancer. However, further evaluation with a large-scale multi-centre study is required to substantiate this hypothesis.
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1. Introduction

Prostaglandins are found in the stomach of many species including humans. They are not stored within the tissues, but with an appropriate stimulus are actively synthesised and released from the mucosal cells into the gastric lumen [1]. Prostaglandins have a variety of actions within the gastric mucosa that contribute to the mechanism of gastric cytoprotection including: stimulation of mucus secretion [2]; bicarbonate production [3]; stimulating the synthesis of surface-active phospholipids which increase the hydrophobicity of the mucus layer [4]; increasing the mucus gel layer thickness [5]; and the inhibition of acid secretion [6].

The role of prostaglandin E_2 (PGE_2) in the cytoprotection of the stomach mucosa, preventing it from becoming inflamed and necrotic when exposed to noxious agents, is well documented [7]. Cohen [8] showed that occult gastric bleeding, produced in healthy volunteers by administration of aspirin, was prevented by concomitant treatment with PGE_2.

Cytotoxin-associated gene A (cagA) is located within a 40-kb chromosomal region of *H. pylori* called the cag-pathogenecity island (cag-PAI) [9]. cagA+ *H. pylori* strains have been found to be associated with more severe gastric diseases, with individuals carrying cagA+ strains having more severe forms of gastric diseases compared to those carrying cagA− strains [10,11].

Since the discovery of *H. pylori* many studies have investigated its effect on PGE_2 levels and its role in gastric diseases. Unfortunately, most studies addressing these issues have not differentiated between *H. pylori* cagA+ and cagA− strains and have therefore produced contradictory findings (Table 1). The aim of this study was to investigate the effect of cagA+ strains on PGE_2 in order to assist in the understanding of their association with severe gastric diseases.

Table 1
Studies on the effect of *H. pylori* (HP) infection on human gastric prostaglandin E_2 (PGE_2) levels

Gastric PGE_2			
Increase with HP infection	*n*	Decrease (↓) or no change (→) with HP infection	*n*
Oderda et al. [16]	104	Goren [25] (↓)	64
Ahmed et al. [15]	28	Taha et al. [26] (→)	48
Avunduk et al. [17]	30		

n: total number of patients studied including *H. pylori* infected and control subjects.

2. Materials and methods

2.1. Subjects

One hundred patients were randomly recruited from consecutive endoscopy clinics at three hospitals in Yorkshire in the UK without prior knowledge of their *H. pylori* status. Prior to patient recruitment the study was approved by both the Leeds Health Authority clinical research ethics committee and the York research ethics committee. Written consent was obtained from each patient and all were given information sheets prior to participation. Patients were included in the study if they were not taking acid suppressive drugs, NSAIDs or receiving treatment for *H. pylori*, but excluded if they were known or found to have peptic ulcer disease or gastric cancer. Following recruitment into the study one patient was subsequently excluded due to the presence of duodenal ulcer. Of the remaining 99 patients, 42 (42%) were male and 57 (58%) female with mean ages of 46 ± 11 years and 51 ± 15 years, respectively. Six biopsies were taken from each patient (five from the antrum, one from the body of the stomach): one for PGE_2, one for polymerase chain reaction (PCR), one for culture of *H. pylori*, one for Campylobacter-like organism (CLOtest) and two for histology (antrum and body).

2.2. H. pylori status

H. pylori infection was assessed by histology, culture, PCR and CLOtest. Patients were considered to be *H. pylori* positive if two or more of the above tests were positive.

2.3. Polymerase chain reaction

Identification of *H. pylori* cagA+ strain was carried out by PCR using two pairs of primers, cagA (primer 1: 5′-CTC AAA TCC CCC TTA CCA AAC TC-3′, primer 2: 5′-GAG ATT AAG GAT TTC CAA AAA GAC TCT AA-3′, product size 130 bp) and Urease C (primer1: 5′-ATA AGG TGG CTC CGG TCG TT-3′, primer 2: 5′-TGG CTC AAT TGG TTA GGG TGT-3′, product size 120 bp), synthesised by Dr. F. Lewis (University of Leeds). DNA extraction and PCR were performed using standard protocols [12].

2.4. H. pylori culture

H. pylori culture was performed as described by Goodwin [13]. The bacterial viable count (colonization density) was assessed by direct counting of the bacterial colonies on the culture plates that were inoculated with the serial dilutions prepared from the bacterial homogenates using the method described by Collins and Lyne [14]. The bacterial viable count was expressed in colony forming unit (CFU)/mg of biopsy weight.

2.5. Histology

Two gastric biopsies (antrum and body) were routinely processed for light microscopy and stained with Haematoxylin and Eosin to determine the presence of gastritis. Colonizing *H. pylori* was identified by Giemsa stain and immunohistochemistry.

2.6. CLOtest (campylobacter-like organism)

The CLOtest (Delta-West Ltd., Australia) consists of an agar containing urea, phenol red, buffers and bacteriostatic agent in a sealed plastic slide. In the presence of *H. pylori* urease, the gel changes colour from yellow to red. The biopsy was inserted into the agar by means of a sterile needle after peeling the back label covering the well; then the test result was read at 1 and 24 h.

2.7. Assessment of prostaglandin E_2 levels

2.7.1. Sample collection and transport

The gastric biopsy obtained for PGE_2 measurement was immediately placed into a cryogenic tube (Nalgene, 1.2 ml) containing 0.5 ml ice-cold 0.9% saline and transported on ice. The biopsy was processed within 2–4 h from the time of collection without prior knowledge of *H. pylori* status.

2.7.2. Enzymeimmunoassay (EIA) reagents and standards preparation

Prostaglandin E_2 levels were measured using the PGE_2 Biotrak Enzymeimmunoassay system protocol 1 (RPN 222, Amersham Biosciences, UK). The preparation of reagents and standards were carried out as recommended by the manufacturer (http://www.amershambiosciences.com). The assay had the following components: (a) microplate, 12 × 8 well strips coated with goat anti-mouse IgG; (b) assay buffer, phosphate buffer concentrate containing 0.9% (w/v) bovine serum albumin and 0.5% (w/v) kathon, diluted with distilled water to give a 0.1 M phosphate buffer pH 7.5; (c) lyophilised PGE_2 antibody that was diluted by the assay buffer as recommended; (d) lyophilised PGE_2 conjugated to horseradish peroxidase that was diluted by the assay buffer as recommended; (e) wash buffer, phosphate buffer concentrate containing 0.05% (w/v) Tween20, diluted with distilled water to give 0.01 M phosphate buffer pH 7.5; (f) standards, PGE_2 at a concentration of 256 ng/ml in ethanol was serially diluted in the assay buffer in polypropylene tubes to give eight standard concentrations 2.5, 5, 10, 20, 40, 80, 160, and 320 pg/50 μl (pg per well), respectively; and (g) tetramethylbenzidine (TMB) substrate, a bottle containing 3,3′,5,5′-tetramethylbenzidine hydrogen peroxide.

2.7.3. Biopsy incubation and basal release of PGE_2

The biopsy incubation procedure was performed as described by Ahmed et al. [15]. In brief, the biopsy was transferred immediately to a pre-warmed tube (Epindrof, 1.5 ml) containing 0.5 ml modified Tyrode's buffer (contains (g/l): 8.0 NaCl, 0.2 KCl, 0.21 $MgCl_2 \cdot 6H_2O$, 1.0 $NaHCO_3$, 0.058 $NaH_2PO_4 \cdot H_2O$, 0.13 $CaCl_2 \cdot 2H_2O$ and 1.1 glucose) adjusted to pH 7.4. The tube containing the biopsy was oxygenated by a gas mixture of 95% O_2 and 5% CO_2 for 5 min and incubated in a water bath at 37 °C for 30 min. The biopsy was then removed from the tube and the supernatant (incubation medium) stored at −20 °C for PGE_2 determination. The biopsy dry weight was recorded.

2.7.4. Enzymeimmunoassay for PGE_2

The microplate was set up with sufficient wells for running all blanks, standards and samples in duplicate. The wells were filled with solutions as follows: (a) 100 μl diluted

assay buffer into the non-specific binding wells (NSB); (b) 50 μl diluted assay buffer into the zero standard wells (O); (c) 50 μl of each prepared standard (2.5–320 pg/50 μl) into the appropriate wells; (d) 50 μl of each unknown (sample supernatant) into the appropriate wells in duplicate; (e) 50 μl diluted antibody into all wells except the blank (B) and NSB; and (f) 50 μl diluted conjugate into all wells except the blank (B). The plate was then covered and incubated at room temperature for 1 h on a microplate shaker (Labsystems). All wells were then manually aspirated and washed four times with wash buffer. Immediately after washing, 150 μl of TMB substrate was pipetted into all wells. The plate was incubated again on the microplate shaker for 30 min at room temperature. The reaction was then halted by the addition of 100 μl 1 M sulphuric acid to all wells following which the optical densities (OD) at 450 nm were obtained using the plate reader (Labsystems, Multiskan Ascent).

2.7.5. Estimation of PGE_2

The levels of PGE_2 were calculated from the optical densities obtained using the following formula:

$$\frac{\%B}{B_0} = \left(\frac{\text{standard or sample OD} - \text{NSB OD}}{\text{zero standard OD} - \text{NSB OD}} \right) \times 100$$

where $\%B/B_0$ is the percent bound for each standard and sample, OD is the optical density and NSB is the non-specific binding. The standard curve was generated, for each patch of PGE_2 assay performed. The results of PGE_2 levels are expressed as pg/mg per 20 min.

2.8. Statistical analysis of data

Results were analysed using SPSS software version 10.1 and expressed as mean ± S.E.M. (standard error of the mean). Statistical significance was tested using analysis of variance (ANOVA). Data transformation (log) was used when required. The level of statistical significance was taken at P values <0.05. Spearman's correlation coefficient (r) was used in the determination of associations between various parameters.

3. Results

Of the 99 patients studied 69 patients were *H. pylori* negative (HP−) and 30 *H. pylori* positive (HP+): 10 cagA+, 18 cagA−, 2 undetermined. Nine patients had atrophic gastritis of which six were infected with cagA+. There was no significant difference ($P = 0.082$) in PGE_2 levels between HP+ (37.2 ± 1.2 pg/mg per 20 min, $n = 30$) and HP− (27 ± 1.1 pg/mg per 20 min, $n = 69$). In age and sex-matched patients the PGE_2 level was significantly higher ($P = 0.04$) in *H. pylori* infected patients (37.2 ± 1.2 pg/mg per 20 min, $n = 30$) than uninfected patients (22.6 ± 1.2 pg/mg per 20 min, $n = 30$). When patients with gastric atrophy were excluded the PGE_2 level was significantly higher ($P = 0.047$) in HP+ patients (37.6 ± 1.2 pg/mg per 20 min, $n = 22$) than HP− patients (22.6 ± 1.2 pg/mg per 20 min, $n = 29$). In patients without atrophy there was a significant ($P = 0.023$) positive linear correlation (Spearman's $r = 0.492$) between *H. pylori* colonization density and the level of PGE_2 (Fig. 1).

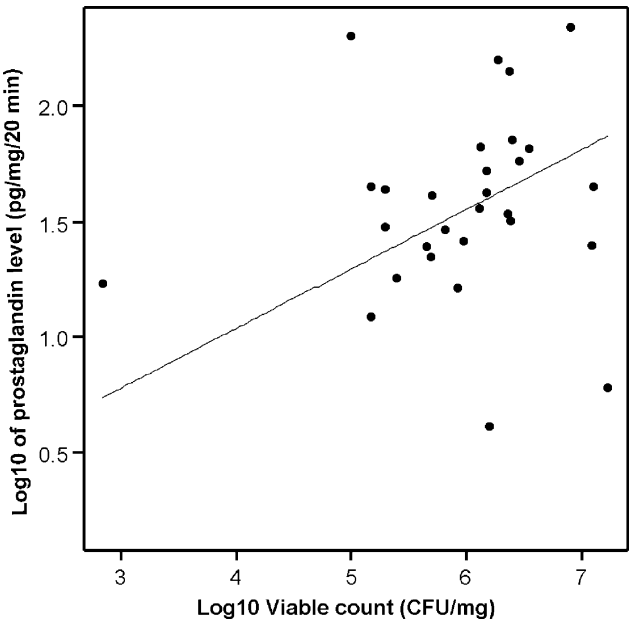

Fig. 1. Relationship between PGE_2 and *H. pylori* colonization density in patients without atrophy. The level of PGE_2 has a significant ($P = 0.023$) positive linear correlation ($r = 0.492$) with the colonization density of *H. pylori*.

In matched patients there was no significant difference ($P = 0.102$) in PGE_2 levels between cagA+ (44.7 ± 1.4 pg/mg per 20 min, $n = 10$), cagA− (33.9 ± 1.2 pg/mg per 20 min, $n = 18$) and HP− patients (22.6 ± 1.2 pg/mg per 20 min, $n = 30$). In matched patients without atrophy, cagA+ patients had significantly higher ($P = 0.031$) PGE_2 levels (53 ± 1.1 pg/mg per 20 min, $n = 4$) than HP− patients (22.6 ± 1.1 pg/mg per 20 min, $n = 30$). However, there was no significant difference ($P = 0.414$) in mean PGE_2 between cagA− (35 ± 1.3 pg/mg per 20 min, $n = 16$) and HP− (22.6 ± 1.1 pg/mg per 20 min, $n = 30$); or between cagA+ and cagA− patients ($P = 0.292$), although cagA+ patients had higher levels of PGE_2 compared to cagA− (Fig. 2).

4. Discussion

The findings of the present study are in agreement with the majority of previous studies that have reported increased levels of PGE_2 in the presence of *H. pylori* infection 15–17. In addition, the present study shows that cagA+ infection induces greater levels of PGE_2 than cagA− infection, although the difference was not significant. This lack of significance may be due to the relatively small sample size. It has also been shown that the PGE_2 levels are significantly increased with increased *H. pylori* colonization density. It should be noted that when patients with atrophic gastritis ($n = 9$) are excluded from data analysis significant

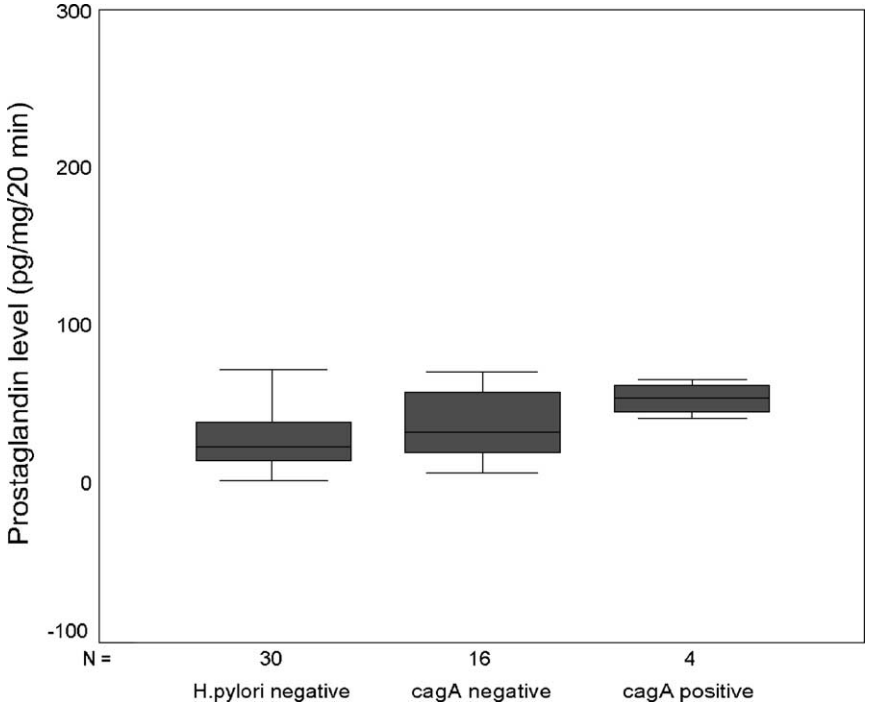

Fig. 2. Box plot of PGE_2 levels in *H. pylori* infected (cagA-positive and cagA-negative) compared to uninfected patients. Patients infected with cagA+ and without gastric atrophy had significantly ($P = 0.031$) higher levels of PGE_2 than uninfected patients. *N*, number of patients; boxes, median with 25–75% interquartile range; bars, 95% interquartile range.

differences between the groups were more evident, this is presumably due to physiological and structural changes obscured by atrophy.

It is interesting to note that *H. pylori* infection is associated with gastric ulcers and cancer, yet at the same time induces the production of the cytoprotective PGE_2, which was measured at higher levels with cagA+ compared to cagA− strains. It is not clear whether the increased levels of PGE_2 are just a response to mucosal damage by *H. pylori* infection or a primary initial step in cagA+ infection to enhance the gastric mucus layer protective functions favouring colonization of the gastric mucosa, as PGE_2 stimulation was found to increase the gastric mucus layer thickness [5]. However, PGE_2 level was found to be inversely related to the gastric mucus thickness in the presence of *H. pylori* gastritis [16]. Nevertheless, the findings of Oderda et al. [16] are true only in cagA− infected patients but not in cagA+ (unpublished data). Therefore, it is speculated that in the presence of cagA+ infection higher levels of PGE_2 may play a role in the establishment of its colonization leading to severe gastric diseases.

There are two possible mechanisms by which *H. pylori* increases PGE_2 production: (i) *H. pylori* has been found to stimulate IL-8 [18] leading to the accumulation of polymorphonuclear cells and macrophages [19], which in turn produce IL-1 leading to acute gastritis. IL-1

has been found to stimulate PGE_2 [20] leading to mucin secretion and gastric cytoprotection against infection; and (ii) it is believed that *H. pylori* increases gastric prostaglandin concentration through increased cyclooxygenase-2 (COX-2) expression [21]. This is supported by the finding that *H. pylori* infection induces the expression of COX-2 in the gastric mucosa [22], whereas eradication of *H. pylori* results in reduced gastric antral mucosal COX-2 expression [23].

It has to be noted that the limitation of the present study was the high cross reactivity of the PGE_2 antibodies to PGE_1, as the assay has a sensitivity of 2 pg per well (40 pg/ml) with intra-assay and inter-assay coefficient of variation 7.6 and 9%, respectively. The cross-reactivity of this assay with PGE_1 and $PGF_{2\alpha}$ are 25 and 0.04%, respectively. However, Redfern et al. [24] have shown that PGE_2 and PGF_2 are the two major prostaglandins produced in the human gastric mucosa.

In summary, the greater levels of PGE_2 in the presence of cagA+ *Helicobacter pylori* infection compared to cagA− infection may be an important factor by which cagA+ strains enhance the gastric mucus layer protective functions for its own protection, thereby enabling an established colonization to occur that induces severe gastritis leading to severe gastric disease and increased risk of gastric cancer. However, due to the relatively small sample size of cagA+ infected patients in the present study, the difference in PGE_2 levels between cagA+ and cagA− did not reach statistical significance and hence further evaluation with a large-scale multi-centre study is required.

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