Paf-acether synthesis by *Helicobacter pylori*

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Abstract

Clinical studies suggest that *Helicobacter pylori* may play a role in the pathogenesis of gastroduodenal ulcers in man but direct evidence of mucosal injury by this micro-organism is still lacking. Paf-acether (paf) causes a number of disorders including ischaemic bowel necrosis and gastroduodenal ulceration. Since paf is produced by *Escherichia coli*, we investigated whether it could be synthesised by *H. pylori*. Five *H. pylori* isolates were collected from antral biopsy specimens from patients with gastritis and duodenal ulcer and cultured with selective antibiotics. Colonies obtained from both blood agar and brucella broth medium were used. Paf was determined by platelet aggregation assay after ethanolic extraction and subsequent purification by high performance liquid chromatography. Paf was detected in *H. pylori* in blood agar plates (680 (390) pg paf/1×10⁶ organisms) but not in bacteria cultured on brucella broth medium. Supplementation of the latter medium with lyso paf and acetyl-CoA, two paf precursors present in high amounts in the mammalian intestine, induced paf production in three of five isolates. The platelet aggregating material extracted from *H. pylori* exhibited biological and physicochemical characteristics identical to those of paf released from eukaryotic cells. These findings suggest that *H. pylori* may add to the local production of paf in inflamed gastric mucosa.

Clinical studies suggest that *Helicobacter pylori* may play a role in the pathogenesis of gastroduodenal ulcers in man¹ but there is still no direct evidence of mucosal injury by the bacteria. Recently, Slomiany *et al* speculated on gastric mucosal damage by the bacteria, based on the mucolytic power of *H. pylori* in vitro.² It has also been suggested that the lower relapse rate and superior microscopically observed healing in duodenal ulcer patients treated with bismuth or antibiotics, or both, are due to the disappearance of *H. pylori* after this therapy.³ In addition, Esplugues *et al* suggested that the local release of paf-acether (paf) may account for the gastric and mucosal damage that is associated with colonisation with *H. pylori*.⁴ Paf (first described as platelet activating factor), identified as 1-O-alkyl-2-O acetyl *sn*-glycero-3-phosphocholine,⁵⁻⁷ is synthesised by a wide variety of human cells including neutrophils, monocytes, macrophages, platelets, eosinophils, and vascular endothelial cells.⁸ This potent inflammatory mediator can produce severe pathological changes in various organs⁹ and, among its numerous harmful effects, causes

shock,¹⁰ gastric ulceration,¹¹ and ischaemic bowel necrosis.¹² Since paf has recently been shown to be produced by *Escherichia coli*,^{13,14} we investigated whether several *H. pylori* isolates, collected from antral biopsy specimens of patients with gastritis and duodenal ulcer, could produce paf in vitro. This could be of importance in light of the role of *H. pylori* in the development of gastritis and peptic ulcer.

Materials and methods

REAGENTS

The following reagents were used: BN 52021: 9H-1,7a-(epoxymethanol)-1H,6a-H-cyclopenta-(c) furo(2,3-b) furo(3',2':3,4) cyclopental(1,2-d) furan-5,9-12- (4H-trione,3-*tert*-butylhexahydro-4,7b,11-hydroxy-8-methyl (IHB, Les Ulis, France); CV 3988: 3-(N-n-octadecyl-carbomoyloxy) -2- methoxypropyl -2- thiazolioethyl-phosphate (Takeda Chemical Ind, Osaka, Japan); L652,731: (trans-2,5-bis) (3,4,5-trimethoxyphenyl)tetrahydrofuran (Merck, Sharp and Dohme, Rahway, NJ, USA); aspirin (Aspegic; Lab Egic, Amilly, France); creatine phosphate, creatine phosphokinase, phosphatidylcholine, lysophosphatidylcholine, sphingomyelin, fatty acid free bovine serum albumin (Sigma Chemical Co, St Louis, MO, USA); gelatin (E Merck, Darmstadt, FRG); paf (C18) 1-O-octadecyl-2-O-acetyl-*sn*-glycero-3-phosphocholine, lyso paf (C18) 1-O-octadecyl-*sn*-glycero-3-phosphocholine, acetyl-CoA (Bachem, Bubendorf, Switzerland); phospholipase A₂ from hog pancreas and lipase A₁ from *Rhizopus arrhizus* (Boehringer-Mannheim, FRG).

PATIENTS

Five adult patients suffering from both duodenal ulcer and gastric erythema with erosions were investigated. For histological examination, *H. pylori* culture, and paf assay, biopsy specimens were obtained from the lesser curve midantrum, away from ulcers and as close together as possible, using forceps with an open outer diameter of 8 mm.

HISTOPATHOLOGY

Biopsy specimens were fixed in Bouin's aqueous solution. Sections (4 µm) were stained with haematoxylin and eosin and periodic acid Schiff and Masson's trichrom methods were used.

MICROBIOLOGY

Biopsy specimens were placed in 0.5 ml of saline for culture and were transported to the labora-

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tory within 15 minutes. Specimens were immediately cultured onto 10% horse blood agar supplemented with selective antibiotics¹ (trimethoprim, vancomycin, colistin). Plates were incubated at 37°C under microaerophilic conditions and examined for growth after three, five, and seven days. Subcultures for each patient, on brucella broth medium supplemented with 5% fetal bovine serum and selective antibiotics, were examined for growth at one, three, and five days. *H. pylori* were identified by standard methods as small (0.5–1 mm) translucent colonies of Gram negative motile curved rods that were positive for oxydase, catalase, and urease.^{15,16}

GASTRIC BIOPSY SPECIMENS

An antral biopsy (5 mg wet weight) from each patient was extracted in 80% ethanol to obtain the lipids from the tissue.¹⁷ Samples were frozen at –80°C until assayed for paf, lyso paf, and alkyl-acyl-glycerophosphocholine (A-A-GPC).

PAF PRODUCTION

Colonies obtained from blood agar after seven days incubation were harvested, gently washed at 4°C for five minutes with saline supplemented with 0.25% fatty acid free bovine serum albumin, and diluted in the same medium to a final concentration of 5×10^5 to 1×10^6 organisms/ml. Both cells and supernatant were then extracted with ethanol (80% final) and frozen immediately until paf assay.¹⁷ In other experiments, bacteria cultured for three, five, or seven days in brucella broth medium were harvested by centrifugation (600 g for 10 minutes at 4°C), gently washed with and diluted in saline supplemented with 0.25% fatty acid free bovine serum albumin to a final concentration of 5×10^5 to 1×10^6 organisms/ml. In some experiments, samples were incubated at 37°C in microaerophilic conditions for various periods of time (0, 10, 20, 30, 60, 120 minutes) with or without 0.1 mM lyso paf or 0.1 mM acetyl-CoA, or both. In

all cases both cells and supernatant were then extracted with ethanol and frozen until paf assay. The efficiency of ethanolic extraction always averaged 80%, as established by ethanolic extraction of known amounts of synthetic paf.

PURIFICATION OF PAF

The dried residues of ethanol extracted samples were dissolved in 500 µl of the high performance liquid chromatography solvent (dichloromethane/methanol/water, 60:50:5, v/v). Samples were put in a Microporasil column, 3.9 mm diameter × 300 mm length (Waters Associates, Milford, MA, USA),¹⁸ which was eluted at a flow rate of 1 ml/minute for 30 minutes. One ml fractions were dried under an air stream, resuspended in 50 µl of 60% ethanol, and then assayed for platelet aggregating activity.

PAF ASSAY

Washed rabbit platelets were prepared as previously described.¹⁹ Aspirinated platelets (1.6×10^8) in Tyrode's solution (300 µl) containing 2.5% gelatin and the adenosine diphosphate scavenger mixture, creatine phosphate (1 mM) and creatine phosphokinase (10 U/ml) were stirred in an aggregometer (Icare, Marseille, France). The aggregating activity of the samples was measured over the linear portion of the calibration curve obtained with 2.5–50 pg synthetic paf.

ASSAY FOR LYSO PAF AND A-A-GPC

Lyso paf was measured after its chemical acetylation into paf as previously described.²⁰ The amount of lyso paf was established as the difference between the quantity of paf measured before and after acetylation of the samples. Results are expressed in ng of lyso paf per mg of wet weight.

A-A-GPC in ethanolic extracts was submitted to alkaline hydrolysis and subsequent acetylation.²⁰ The amount of A-A-GPC was established as the difference between the quantity of paf measured before and after alkaline hydrolysis and acetylation, minus the value of lyso paf and paf detected in the same samples. Results are expressed in ng of A-A-GPC per mg wet weight.

CHARACTERISATION OF PAF

In addition to its adenosine diphosphate – and arachidonic acid – independent aggregating activity on rabbit platelets, the lipidic material obtained from bacteria was further characterised as paf on the basis of the following criteria:

(1) Study of the aggregating activity in the presence of 0.1 mM BN 52021, CV 3988, and L652,731 three specific paf receptor antagonists²¹;

(2) Same study after incubation of the samples with phospholipase A₂ from hog pancreas (10 µg/ml) as well as with cationic lipase A₁ from *R. arrhizus* (100 µg/ml)²²;

(3) Retention time during high performance liquid chromatographic analysis, using phospho-

Figure 1: Effect of exogenous paf precursors on paf formation by *Helicobacter pylori*.

Five *Helicobacter pylori* isolates cultured onto brucella broth medium for seven days (1×10^6 organisms/ml) were incubated in saline and bovine serum albumin, at 37°C, for 30 minutes, alone (■) or in the presence of 0.1 mM lyso paf (□) or 0.1 mM lyso paf + 0.1 mM acetyl-CoA (▨). Lipids were extracted, submitted to high performance liquid chromatography, and paf content was assayed as indicated in Materials and methods. Results are expressed in pg paf/1 × 10⁶ organisms, mean (SEM) of three experiments. *p values, $p < 0.05$ for *H. pylori* cultured in the presence of exogenous precursors compared with *H. pylori* cultured alone.

tidylcholine, sphingomyelin, lysophosphatidylcholine, and synthetic paf as standards.¹⁸

Results

PAF PRODUCTION BY *H. PYLORI*

Lipids extracted from *H. pylori* cultured for seven days on blood agar exhibited a paf-like activity. The amount of paf recovered from plates with *H. pylori* (mean (SEM) 680 (390) pg paf/ 1×10^6 organisms, $n=5$) was higher than in control plates without bacteria (50 (30) pg paf, $n=3$). No paf was detected in *H. pylori* cultured onto brucella broth medium at three, five, and seven days.

PAF PRODUCTION IN THE PRESENCE OF EXOGENOUS PAF PRECURSORS

We next investigated whether paf production in the medium could be limited by the available amounts of lyso paf or acetyl-CoA, or both, the immediate non-acetylated precursor and the acetate donor for paf synthesis respectively. Results in Figure 1 indicate that the exogenous precursors enhanced paf synthesis appreciably in three of five isolates of *H. pylori* on brucella broth medium. The combination of acetyl-CoA and lyso paf caused a twofold increase in paf production, when compared with bacteria supplemented with lyso paf alone. Acetyl-CoA alone did not stimulate paf formation.

One representative kinetic experiment of paf production in brucella broth medium is shown in Figure 2. In the presence of exogenous paf precursors, paf production began after 20 minutes, reached a maximum at 30 minutes, and decreased thereafter.

HISTOPATHOLOGICAL OBSERVATIONS

All patients had active chronic gastritis since increased numbers of mononuclear and polymorphonuclear leukocytes were found in the superficial or glandular epithelium.

PAF CONTENT OF BIOPSY SPECIMENS

No biopsy specimens ($n=5$) from the patients' stomachs contained paf but, interestingly, high amount of lyso paf (mean (SEM) 12.4 (1.02) ng/mg tissue) and A-A-GPC (mean (SEM) 121.6 (14.7) ng/mg tissue) were present.

STRUCTURAL ANALYSIS OF PAF

The platelet aggregating substance was indistinguishable from synthetic 1-O-alkyl-2-acetyl *sn*-3-phosphocholine by the following physico-chemical and biological criteria (Fig 3):

(1) It induced aggregation of washed rabbit platelets that were refractory to adenosine diphosphate and arachidonic acid/thromboxane A_2 ;

(2) During high performance liquid chromatographic analysis, the platelet aggregating activity was eluted from 18 to 21 minutes between sphingomyelin and lysophosphatidylcholine – that is, a similar retention time to that of synthetic paf;

Figure 2: Kinetics of paf formation by *Helicobacter pylori*. *Helicobacter pylori* (1×10^6 organisms/ml) were incubated in saline and bovine serum albumin at 37°C, for indicated time intervals, alone (●) or in the presence of 0.1 mM lyso paf (○) or 0.1 mM lyso paf + 0.1 mM acetyl-CoA (■). Lipids were extracted, submitted to high performance liquid chromatography, and paf content was assayed as indicated in Materials and methods. Results are expressed in pg paf/ 1×10^6 organisms. One representative experiment out of three is shown.

(3) The platelet aggregation was completely inhibited by the paf antagonists BN 52021 (0.1 mM), CV 3988, and L 652,731 (the two latter not shown);

(4) For further characterisation of the platelet aggregating molecule, we used lipase from *R. arrhizus* (100 µg/ml), which hydrolyses exclusively the fatty acid ester bond at the 1-position of tri- and phosphoglycerides, and phospholipase A_2 (10 µg/ml), which specifically cleaves the acyl chain at the 2-position. The lack of effect of lipase from *R. arrhizus* and the sensitivity to phospholipase A_2 of the platelet aggregating factor recovered from *H. pylori* are in agreement with a 1-alkyl-2-acyl-3-glycero-phospholipid structure.

Discussion

This is the first report of the synthesis of paf, an endogenous proinflammatory mediator which is one of the most potent ulcerogens of gastrointestinal tract,¹¹ by *H. pylori*, a bacterium associated with the pathogenesis of duodenal ulcer and acute gastritis in man.¹ Given the numerous substances that can activate platelets, it was necessary to show that the platelet aggregating material produced by *H. pylori* was paf. To do this we used several experimental criteria to characterise and distinguish it from arachidonic acid, prostaglandin, collagen, thrombin or adenosine diphosphate, as described in the results sections. These data show that the *H. pylori* generated material was paf.

An interesting point is the production of paf in *H. pylori* colonies on blood agar medium. We could not obtain paf in liquid medium. One interpretation is that paf results from the bioconversion of paf precursors present in the culture media. Indeed, micromolar concentrations of lyso paf were detected in horse blood agar and supplementation of saline with synthetic lyso paf stimulated paf production from *H. pylori*. These results suggest that bacteria are capable of forming paf upon exogenous addition of limiting products, most probably via an acetyltransferase activity as previously reported for *E. coli*.¹³ The meagre amount of paf found in subcultures supplemented with paf precursors may be due to

Figure 3: Effect of lipase A_1 , lipase A_2 and a paf antagonist on platelet aggregation induced by synthetic paf, paf extracted from *Helicobacter pylori*.

A: dose response of platelet aggregation induced by synthetic paf. B: dose response of platelet aggregation induced by paf extracted from *H. pylori*. C: 2 μ l of the extract after treatment with lipase A_1 (100 μ g/ml) from *Rhizopus arrhizus*. D: 2 μ l of the extract after incubation with phospholipase A_2 (10 μ g/ml) from hog pancreas. E: 2 μ l of the extract in the presence of BN 52021 (0.1 mM) a specific paf receptor antagonist.

a low bacterial concentration (5×10^5 to 1×10^6 cells). By comparison, we showed paf production from *E. coli* using 1×10^9 bacteria/ml.¹³ We also speculate that liquid medium is not optimal for *H. pylori* growth.²³ In addition, these data may not reflect the magnitude of paf generation by bacteria in situ. Another explanation would be a paf degradation in the liquid medium by an acetylhydrolase, immediately after its release, as reported in some eukaryotic cells.²⁴ The fact that in kinetic experiments no paf was detected after 30 minutes incubation, could indicate the latter mechanism.

We were unable to detect paf in lipids from human biopsy specimens, a result that may be explained by their low weight. When three gastric biopsy specimens (15–20 mg) were extracted together ($n=2$), small amounts of paf could be detected (5–10 pg/mg tissue). This observation was similar to that of Eliakim *et al.*,²⁵ who studied paf release from rectal mucosal biopsy specimens obtained from patients with active ulcerative colitis. By contrast, we detected in gastric biopsy specimens high amounts of lyso paf and A-A-GPC, that could be used as precursor for paf by *H. pylori*. The latter micro-organism possesses phospholipase A_2 activity²⁶ (the enzyme that cleaves A-A-GPC into lyso paf) and, as shown in the present work, seems to be capable of acetylating exogenous lyso paf. There is also evidence to suggest that lyso paf resulting from the degradation of most eukaryotic cell membranes is abundant in the natural environment of the *H. pylori*, thus providing the 'raw material' to be acetylated into paf.

The erratic behaviour of *H. pylori* is striking since only three of five *H. pylori* isolates obtained from five patients could produce paf in the presence of exogenous precursors. Investigations on microbiological characterisation of these isolates are now in progress to attempt explaining this.

In conclusion, the present work shows that *H. pylori* is able to generate paf in vitro and therefore possesses at least the final enzymatic

pathways involved in paf synthesis. Because paf is a potent agonist in vivo, capable of inducing proinflammatory events such as cell chemotaxis, increase in vascular permeability, shock, gastric ulceration, and ischaemic bowel necrosis, it is tempting to speculate that its release from *H. pylori* may play a part in the development and perpetuation of gastric injury.

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