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Helicobacter pylori-induced Prostaglandin E_2 Synthesis Involves Activation of Cytosolic Phospholipase A_2 in Epithelial Cells*

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Thomas Pomorski‡, Thomas F. Meyer, and Michael Naumann§

From the Max-Planck-Institut für Infektionsbiologie, Abteilung Molekulare Biologie, 10117 Berlin, Germany

Helicobacter pylori **initiates an inflammatory response and gastric diseases, which are more common in patients infected with *H. pylori* strains carrying the pathogenicity island, by colonizing the gastric epithelium. In the present study we investigated the mechanism of prostaglandin E_2 (PGE_2) synthesis in response to *H. pylori* infection. We demonstrate that *H. pylori* induces the synthesis of PGE_2 via release of arachidonic acid predominately from phosphatidylinositol. In contrast to *H. pylori* wild type, an isogenic *H. pylori* strain with a mutation in the pathogenicity island exerts only weak arachidonic acid and PGE_2 synthesis. The *H. pylori*-induced arachidonic acid release was abolished by phospholipase A_2 (PLA_2) inhibitors and by pertussis toxin (affects the activity of $G\alpha_i/G\alpha_o$). The role of phospholipase C, diacylglycerol lipase, or phospholipase D was excluded by using specific inhibitors. An inhibitor of the stress-activated p38 kinase (SB202190), but neither inhibitors of protein kinase C nor an inhibitor of the extracellular-regulated kinase pathway (PD98059), decreased the *H. pylori*-induced arachidonic acid release. *H. pylori*-induced phosphorylation of p38 kinase and cytosolic PLA_2 was blocked by SB202190. These results indicate that *H. pylori* induces the release of PGE_2 from epithelial cells by cytosolic PLA_2 activation via $G\alpha_i/G\alpha_o$ proteins and the p38 kinase pathway.**

The *Helicobacter pylori* infection induces the release of a number of proinflammatory cytokines and chemokines from the gastric epithelium (1) and plays a critical role in the development of gastritis, peptic ulcer disease, and rarely, in gastric carcinoma and B-cell mucosa-associated lymphoid tissue-associated carcinoma (2, 3). Evidence has been presented that an increase of prostaglandins (PGs)[1] in gastric tissue from patients may play a crucial role in *H. pylori* infection (4). In gastrointestinal epithelia, PGE_2 is implicated in maintaining the normal function and structure of the gastric mucosa by modulating diverse cellular functions such as secretion of fluid and electrolytes, mucosal blood flow, and cell proliferation (5, 6).

Studies have shown that *H. pylori* strains differ in their virulence and in their ability to trigger the induction of inflammatory mediators in gastric epithelial cell lines (1). The response is more intense to strains carrying the *cagA* gene. The analysis of the genomic region containing the *cagA* gene revealed a 40-kilobase DNA region, which represents a pathogenicity island (PAI) and codes for 31 genes (7). Upon contact with the gastric epithelium, PAI-encoded components contribute in a specialized type IV secretion machinery that translocates the CagA protein into the eukaryotic target cell where it is phosphorylated on tyrosine residues (8–12). *H. pylori* infection triggers by unknown bacterial factors multiple biochemical pathways in host cells including activation of transcription factors NF-κB and AP-1 (13–17), phospholipase C (PLC) (18), and the increase of the cytosolic free calcium concentration as well as the generation of adenosine 3′,5′-cyclic monophosphate and guanosine 3′,5′-cyclic monophosphate (19). The activation of the PGE_2 signaling pathway in *H. pylori*-colonized gastric cells has not been studied so far.

One of the mechanisms for increased PG production in response to *H. pylori* infection is an induction of COX-2 expression (20, 21). Another rate-limiting step in the control of PG production is the release of AA from membrane phospholipids, which is known to occur via a number of different pathways. One involves the activation of phospholipase A_2 (PLA_2), others involve the action of PLC or phospholipase D (PLD) (22–24). In this report, we studied the control of PGE_2 and AA production in response to *H. pylori* infection of epithelial cells after specific labeling of potential phospholipid precursors and selective inhibition of enzymes involved in the pathways of AA production. The presented results provide evidence that colonization of epithelial cells by *H. pylori* induces a release of PGE_2 and AA by activation of the cytosolic PLA_2 ($cPLA_2$) via pertussis toxin-sensitive heterotrimeric $G\alpha_i/G\alpha_o$ proteins and the p38 stress-activated kinase cascade. This process does not seem to involve PLC or PLD pathways. The identification of *H. pylori*-specific signaling pathways leading to the induction of putative anti-inflammatory immune response mediators is of substantial interest for therapeutic intervention to overcome *H. pylori*-induced diseases.

EXPERIMENTAL PROCEDURES

Bacteria—The isogenic *H. pylori* strains *P12* wild type, *cagA* (mutation affect *cagA* with a probable polar effect in the PAI), and *vacA* (25)

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‡ Present address: University of Amsterdam, Dept. of Cell Biology and Histology, Academic Medical Center L3, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands.

§ To whom correspondence should be addressed: Max-Planck-Institut für Infektionsbiologie, Abteilung Molekulare Biologie, Schumannstrasse 21/22, 10117 Berlin, Germany. Tel.: 49-30-28460410; Fax: 49-30-28460401; E-mail: naumann@mpiib-berlin.mpg.de.

[1] The abbreviations used are: PG, prostaglandin; PAI, pathogenicity island; AA, arachidonic acid; BIM, bisindolylmaleimide I; DAG, diacylglycerol; HELSS, haloenol lactone suicide substrate; MAFP, methyl arachidonylfluorophosphonate; MOI, multiplicity of infection; PA, phosphatidic acid; PC, phosphatidylcholine; PD98059, 2-(2-amino-3-methoxyphenyl)-oxanaphthalen-4-one; PE, phosphatidylethanolamine; PEt, phosphatidylethanol; PI, phosphatidylinositol; PLA_2, phospholipase A_2; $iPLA_2$, Ca^{2+}-independent PLA_2; $cPLA_2$, cytosolic PLA_2; PLC, phospholipase C; PLD, phospholipase D; PS, phosphatidylserine; SB202190, 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl) imidazole; SM, sphingomyelin; PTX, pertussis toxin.

were used for colonization of human epithelial cell lines. For cultivation, the bacteria were resuspended in brain heart infusion (Difco) medium. 10^3 bacteria were seeded on agar plates containing 10% horse serum and cultured for 48–72 h at 37 °C in a microaerophilic atmosphere (generated by Campy Gen, Oxoid, Basingstoke, UK). For stock cultures, *H. pylori* was resuspended in brain heart infusion medium supplemented with 10% fetal calf serum (FCS, Life Technologies, Inc.) and 20% glycerol, and maintained at −70 °C.

Cell Culture and H. pylori Infection—Gastric epithelial cells (AGS) and HeLa cells were grown in RPMI 1640 (Life Technologies, Inc.) supplemented with 4 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin (Biochrom KG, Berlin, Germany), and 10% FCS at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The cells were seeded into 35-mm-diameter Petri dishes or 6-well plates for 48 h before infection. 24 h before infection, the medium was replaced by fresh RPMI 1640 medium supplemented with 0.1% FCS. For the infection, the bacteria were harvested in PBS (pH 7.4) using sterile cotton swabs and diluted corresponding to the multiplicity of infection (MOI) as described in the figure legends. Cells were incubated for different periods of time in the absence (controls) or presence of the bacteria. To enhance the bacteria host cell interaction, bacteria were centrifuged onto the epithelial cell monolayer for 2 min at 500 × *g*. Infection with *H. pylori* was routinely monitored by light microscopy. Stimulation of the cells with 125 ng/ml melittin (Sigma) or 100 nM 12-*O*-tetradecanoylphorbol-13-acetate, (Sigma) was performed for the indicated periods of time.

Cell Labeling—Cells in 35-mm Petri dishes were metabolically labeled with 0.2 μCi of ^{14}C-labeled AA (specific activity 55 mCi/mmol), 0.5 μCi of [*methyl*-^{14}C]choline chloride (55 mCi/mmol), or 0.5 μCi of [^{14}C]palmitic acid (57 mCi/mmol) (Amersham Pharmacia Biotech) in 2 ml of RPMI 1640 medium containing 0.1% FCS for 24 h. Approximately 95% of the total AA radioactivity, 35% of the total choline radioactivity, and 90% of the total palmitic acid radioactivity added to the medium was incorporated into the cells during this time. Lysophosphatidylinositol (lysoPI) formation was studied in cells prelabeled with 2 μCi of *myo*-[^{3}H]inositol (16 Ci/mmol) (ICN Biomedicals, Eschwege, Germany) in 1 ml of RPMI 1640 medium containing 0.1% FCS for 24 h. Before infection, the medium was removed, and the cells were washed three times with nonradioactive RPMI 1640 medium containing 0.1% bovine serum albumin and incubated in the same medium for 30 min. In some experiments, various inhibitors of kinases or lipid-metabolizing enzymes were added to the cells during this incubation.

Lipid Analysis—After the incubations, the medium was carefully removed for analysis, and lipids were extracted from the cells by the addition of chloroform, methanol, 20 mM acetic acid (50:220:10, v/v) to the dishes. In the case of the incubation media, chloroform/methanol (1:2.2, v/v) was added to give a single phase. The phase were split according to the method of Bligh and Dyer (26). The chloroform phase was dried, and lipids were applied on high performance thin layer chromatography (TLC, Merck) plates. For determination of lysoPI, the cells and media were extracted twice with chloroform/methanol (1:2, v/v). The organic extracts were dried and then subjected to butanol/water partition. The radiolabeled lipids were recovered in the butanol phases, which were washed with water, dried, and analyzed by TLC. For phospholipid analysis, the plates were first developed in chloroform, methanol, 30% aqueous ammonium hydroxide, water (90:54:5.5:5.5, v/v) followed by chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5, v/v) for the second direction. For improved separation of phosphatidylinositol (PI) and phosphatidylserine (PS), the plates were first developed in chloroform/methanol/acetone/acetic acid/water (60:12:24:18:6, v/v) followed by 1-butanol/acetic acid/water (80:26:26, v/v) for the second direction. The separation of lysolipids was obtained by one-dimensional thin layer chromatography using chloroform, methanol, 4 M ammonium hydroxide (9:7:2, v/v). The ^{14}C-containing radiolabeled spots were imaged for 2 h on a ^{14}C-sensitive screen and quantified on a Fuji Imaging System imager (FUJIFILM BAS-1000, Raytest, Straubenhardt, Germany). The ^{3}H-containing radioactive lipids were detected by fluorography. The TLC plates were dipped in 0.4% 2,5-diphenyloxazol dissolved in 2-methylnaphthalene supplemented with 10% xylene (27) and exposed to Kodak X-Omat S films at −80 °C. The individual lipids were identified by comparison with commercial standards (Sigma). Phosphatidylethanol (PEt) standard was prepared as described in Huang and Cabot (28); LysoPI was prepared by PLA_2 (Sigma) treatment of PI. Lipids and standards were visualized with common lipid-locating agents such as iodine or molybdenum blue spray for phosphate on the same plates. When analysis of the choline release was required, radioactivity released into the medium and associated with the cells was measured in aliquots of the media and the cell extracts before phase splitting using a Wallac 409 β-counter (Berthold-Wallac, Bad Wildbad, Germany). The content of PGE_2 in the medium was measured by enzyme-linked immunosorbent assay (ELISA) kit according to the procedure indicated by the manufacturer (DRG Instruments GmbH, Marburg, Germany).

Determination of [^{3}H]Inositol Phosphates—For analysis of the formation of inositol phosphates, cells in 6-well plates were incubated for 24 h in 1 ml of RPMI 1640 medium containing 0.1% FCS and 2 μCi of *myo*[^{3}H]inositol (ICN Biomedicals, Eschwege, Germany). Cells were washed two times with Hanks' balanced salt solution and preincubated with 10 mM LiCl (in this solution) for 10 min at 37 °C. In some experiments, PLC inhibitor was added to the cells during this incubation. Cells were then incubated with the bacteria for 120 min at 37 °C or stimulated either with lysophosphatidic acid (Sigma) or 0.1 mM ATP (Roche Molecular Biochemicals) as positive controls. The incubation was terminated by aspiration of the medium and the addition of 0.1 M NaOH to the dishes. Neutralization of the extracts was performed with 0.2 M formic acid. Total inositol phosphates were determined as described in Schulz *et al.* (29).

Immunoblot and Inhibitors—For the analysis of p38 activation, the cells were infected with *H. pylori* for the indicated periods of time, and the lysed cells were analyzed in an Immunoblot using a p38 phospho-specific antibody (sc-7973, Santa Cruz). The stripped blot (ECL kit, Amersham Pharmacia Biotech) was incubated with an anti-p38 antibody (sc-535, Santa Cruz) to indicate equivalent protein amounts in all lanes. For $cPLA_2$ detection an anti-PLA_2 antibody (sc-454, Santa Cruz) was used.

The inhibitors used in this study were as follows: mepacrine, sodium arsenite (Sigma); 1-(6-(17β-3-methoxyestra-1,3,5-(10)trien-17-yl)amino/hexyl)-1H-pyrrole-2,5-dione) (U73122), 1,6-bis-(cyclohexyloximinocarbonylamino) hexane (RHC80267), methyl arachidonylfluorophosphonate (MAFP) (Biomol, Hamburg, Germany); aristolochic acid, haloenol lactone suicide substrate (HELSS), 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra(acetoxymethyl)ester (BAPTA/AM), pertussis toxin (PTX), 2-(2-amino-3-methoxyphenyl)oxanaphthalen-4-one (PD98059), 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl) imidazole (SB202190), staurosporine, bisindolylmaleimide I (BIM), K-252a (Calbiochem). Stock solutions of U73122 and RHC80267 were prepared in ethanol and stored at −20 °C; mepacrine, aristolochic acid, HELSS, BAPTA/AM, PD98059, SB202190, BIM, and K-252a were prepared in dimethyl sulfoxide; PTX was prepared in double-distilled water. For all experiments, the effect of the appropriate vehicle was also determined.

RESULTS

H. pylori Increases AA Release from Epithelial Cells—To investigate whether *H. pylori* might elicit release of AA for PGE_2 formation (PGE_2 release was induced in HeLa and AGS cells infected with *H. pylori* wild type and an isogenic *vacA* mutant but not by an isogenic *cagA* mutant (data not shown)), ^{14}C-labeled AA-labeled AGS and HeLa cells were incubated for different periods of time in the absence or presence of different *H. pylori* strains. To trap the released radioactivity (free AA and its metabolites) in the extracellular medium, incubation was performed in the presence of 0.1% bovine serum albumin. Lipids were extracted from cells and their medium and analyzed by two-dimensional chromatography. As shown in Fig. 1, colonization of AGS (Fig. 1*A*) and HeLa cells (Fig. 1*B*) by the *P12* wild-type strain increased the intracellular accumulation and extracellular release of free radiolabeled AA as compared with controls. Typically, a 3–6-fold increase of free AA (intra- and extracellular ^{14}C-labeled AA) over the basal release was observed within 60 min at a MOI of 200 (Fig. 1*C*), which did not increase further, suggesting reincorporation and/or metabolism of the released AA. Incubation of cells with isogenic *vacA* mutant induced a comparable increase in AA release like the wild-type strain, whereas incubation with the isogenic *cagA* mutant caused a significant lower increase in AA release. Melittin, known to stimulate AA release by direct activation of PLA_2 (30), was used as a positive control and typically induced a 7–10-fold increase of AA over basal release.

The analysis of ^{14}C radioactivity in the cellular phospholipids revealed that the AA release induced by *H. pylori* (*P12*) was accompanied by a substantial decrease in the percentage of the total ^{14}C-labeled AA content in the PS/PI pool (Table I). The

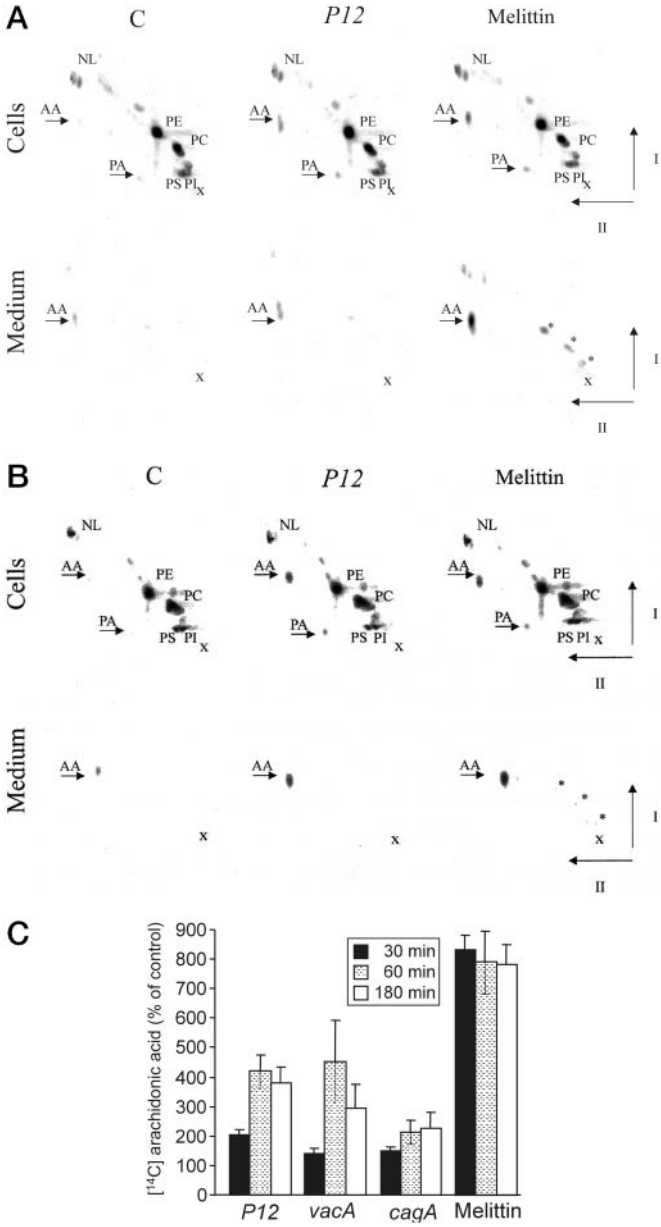

FIG. 1. **Effect of *H. pylori* on AA release and phospholipid hydrolysis in AGS and HeLa cells prelabeled with ^{14}C-labeled AA.** AGS and HeLa cells in 35-mm-diameter dishes were grown for 24 h in 2 ml of medium containing 0.2 μCi of ^{14}C-labeled AA and washed three times with nonradioactive medium containing 1 mg/ml bovine serum albumin before incubation with different *H. pylori* strains at a MOI of 200. At various times up to 180 min the supernatant was removed and cleared of detached cells by centrifugation. The lipids were extracted separately from labeled cells and their medium and analyzed by two-dimensional TLC. As a control, cells were stimulated with the PLA$_2$ activator melittin (125 ng/ml). *A*, AGS cells; *B*, HeLa cells. Representative two-dimensional TLC plates of total lipid extracts from untreated cells (control (*C*)), cells exposed to *H. pylori* (*P12*) for 60 min, and cells treated with melittin for 60 min are shown. The location of individual species was verified using lipid standards. *NL*, neutral lipids. Note that stimulation of cells with melittin for 60 min resulted in lysis of some cells, as determined by trypan blue-staining and, consequently, in the appearance of lipids in the supernatant (*asterisks*). *C*, time course of ^{14}C-labeled AA formation (sum of intracellular accumulation and extracellular release) by cells incubated with the *H. pylori* strains *P12* (wild type), *vacA*, and the PAI mutant *cagA* at a MOI of 200 and cells treated with melittin. Data are expressed as the percentage of control cells and represent the means ± S.E. of at least three independent experiments.

magnitude of the decrease ranged from 30 to 50% within the experiments. In terms of total counts, the amount of ^{14}C-labeled AA that disappeared from the phospholipid pool in *H. pylori*-colonized cells matched approximately the amount of radioactivity released. Two-dimensional phospholipid analysis, which separates PS from PI, revealed that PI was the exclusive source of AA in the PS/PI spot. The percentage of [^{14}C]phosphatidylcholine (PC) decreased slightly, although these changes were only significant after longer time points (12% after 180 min). At longer time points, *H. pylori* caused also a small increase (14% after 180 min) in the percentage of [^{14}C]phosphatidylethanolamine (PE) presumably by interconversion of intact phospholipids and reincorporation of released AA into PE specifically. In melittin-treated cells (positive control), the phospholipids whose radiolabeled content decreased were PC and PI. Exposure of cells to *P12* wild-type strain or treatment with melittin resulted also in a small but significant increase in ^{14}C-labeled AA-labeled PA and probably reflects the production of diacylglycerol (DAG) via activation of PLC (see below) and its phosphorylation by DAG kinase (31).

Involvement of Cytosolic Phospholipase A$_2$ in H. pylori-induced AA Release—As shown in Fig. 2, *H. pylori* (*P12*)-induced release of ^{14}C-labeled AA was significantly inhibited by both mepacrine (30 and 100 μM), a nonspecific inhibitor of the PLA$_2$ (32), and MAFP (10 and 50 μM), a potent inhibitor of cPLA$_2$ and Ca^{2+}-independent PLA$_2$ (iPLA$_2$) (33). In parallel with inhibition of AA release, both inhibitors abolished the decrease in the radiolabeled content of the PI pool while not affecting that of PC (data not shown), indicating that this lipid may serve as a major source for AA. To further delineate the type of PLA$_2$ that is involved in the *H. pylori*-induced AA release, the effect of the iPLA$_2$-specific inhibitor HELSS (33) and the secretory PLA$_2$ inhibitor aristolochic acid (34) was tested. *H. pylori*-induced AA release from the cells was not affected by aristolochic acid (50 μM) (data not shown). Likewise, HELSS at 10 μM did not affect the AA release, whereas higher concentrations (50 μM) inhibited both basal and *H. pylori*-induced AA release (data not shown), probably by inhibiting other important effectors in the signal transduction (35). These findings support a role of cPLA$_2$ in the AA release.

H. pylori induces an increase of cytosolic Ca^{2+} when colonizing epithelial cells (19), and intracellular Ca^{2+} regulates cPLA$_2$ activity; therefore, we studied the role of Ca^{2+} in AA release. Chelating intracellular calcium by preincubation of cells with BAPTA/AM (200 and 400 μM) abolished release of AA in response to *H. pylori* (Fig. 2*C*). Taken together, these results strongly suggest that AA release stimulated by *H. pylori* is Ca^{2+}-dependent.

The release of AA from membrane phospholipids could also occur through PLC activation followed by the action of the DAG lipase (23). However, *H. pylori*-induced AA release from the cells was insensitive to inhibition by U73122 (1 and 10 μM) (Fig. 2*D*), an inhibitor of the PLC (36). Unexpectedly, U73122 itself caused an increase in basal AA release, thereby increasing the AA formation in *H. pylori*-infected cells. Under the same experimental conditions, *H. pylori* caused a 1.2- and 2-fold increase of total inositol phosphates (mono-, bis-, and trisphosphate) in *myo*-[^{3}H]inositol-labeled HeLa and AGS cells, respectively, which was prevented by the PLC inhibitor U73122 (results not shown), demonstrating that the failure of the inhibitor to block *H. pylori*-induced AA release is not the result of U73122 failing to inhibit PLC. RHC80267 (40 and 80 μM), an inhibitor of the DAG lipase (37), slightly inhibited the basal release of AA from cells by about 30% but had no effect on *H. pylori*-induced AA release (Fig. 2*E*). Therefore, PLC and DAG lipase signaling pathways are not involved in the *H. pylori*-induced AA release.

Another potential pathway of AA release involves activation of PLD (24). To test this hypothesis, the release of water-

TABLE I

Effect of H. pylori on AA release and phospholipid hydrolysis in ^{14}C-AA-labeled HeLa cells

Results are expressed as a percentage of total lipid radioactivity and represent the means ± S.E. of five independent experiments for *H. pylori* (*P12*) and three independent experiments for melittin. Significant differences compared with the untreated control were determined by Student's paired *t* test. NL, neutral lipids.

Treatment	Lipid					
	PA	PS/PI	PC	PE	AA	NL
0-min control	0.06 ± 0.01	10.22 ± 0.93	33.07 ± 1.26	36.90 ± 3.40	0.54 ± 0.13	9.80 ± 3.33
30-min control	0.06 ± 0.01	8.66 ± 1.24	32.84 ± 1.25	37.97 ± 3.97	0.71 ± 0.13	9.87 ± 3.56
60-min control	0.05 ± 0.01	9.66 ± 1.06	32.00 ± 1.14	37.48 ± 3.37	0.85 ± 0.21	10.18 ± 3.60
180-min control	0.05 ± 0.01	9.77 ± 1.66	31.43 ± 0.51	37.79 ± 3.18	1.13 ± 0.22	7.87 ± 3.74
30-min (*P12*)	0.12 ± 0.02[a]	5.83 ± 0.99[a]	31.77 ± 0.71	41.67 ± 3.72	1.37 ± 0.24[a]	10.47 ± 3.50
60-min (*P12*)	0.15 ± 0.03[a]	5.75 ± 1.15[a]	30.96 ± 1.26	41.16 ± 3.85[a]	3.04 ± 0.33[a]	10.11 ± 3.07
180-min (*P12*)	0.19 ± 0.05[a]	7.85 ± 1.63[a]	27.36 ± 1.40[a]	43.18 ± 4.20[a]	3.69 ± 0.32[a]	6.99 ± 2.55
30-min melittin	0.28 ± 0.04[a]	6.48 ± 1.56[a]	30.97 ± 1.31[a]	39.39 ± 4.88	8.78 ± 1.46[a]	7.42 ± 2.48
60-min melittin	0.21 ± 0.03[a]	7.06 ± 1.43[a]	30.08 ± 1.43[a]	36.15 ± 4.48	9.80 ± 1.97[a]	6.83 ± 2.88
180-min melittin	0.14 ± 0.03[a]	5.43 ± 2.09[a]	28.53 ± 0.67	37.77 ± 3.37	9.00 ± 1.34[a]	8.95 ± 2.29

[a] $p < 0.05$.

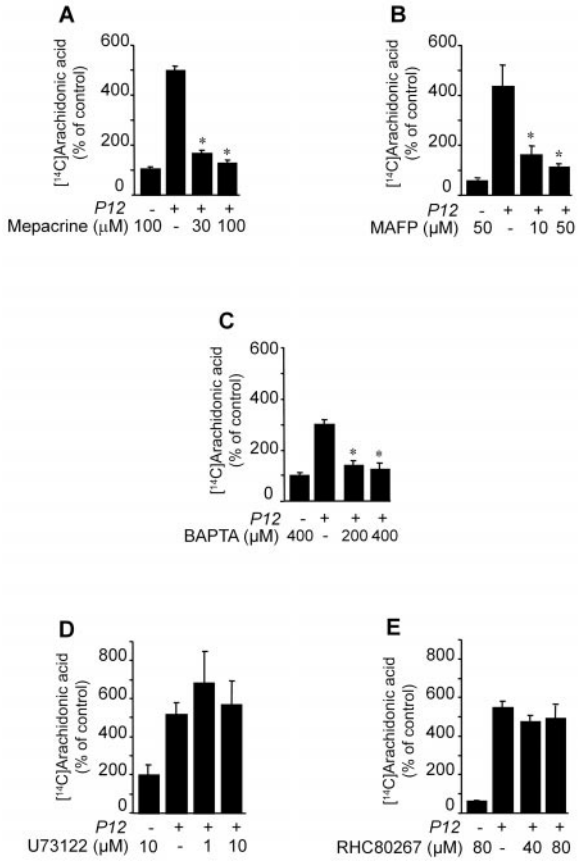

FIG. 2. **Effect of phospholipase inhibitors on *H. pylori*-induced AA release.** HeLa cells were labeled as described in the legend to Fig. 1 and then reincubated in nonradioactive medium containing 1 mg/ml bovine serum albumin. Mepacrine (*A*), MAFP (*B*), BAPTA/AM (*C*), U71322 (*D*), or RHC80267 (*E*) were added for 30 min. Cells were then exposed to *H. pylori* (*P12*) at a MOI of 200 or left untreated for another 60 min. The lipids were extracted from cells and their medium and analyzed by two-dimensional *H. pylori* TLC. Results are expressed as the percentage of AA formation in vehicle-treated cells (control) incubated under the same conditions and represent the means ± S.E. of three independent experiments (*panels A*, *B*, and *E*); results in *panel C* are the means ± S.E. from triplicate determinations in a representative experiment. The *asterisks* denote a significant difference compared with the respective response to *H. pylori* without drug pretreatment ($p < 0.05$).

soluble reaction products into the medium was measured in HeLa cells that had been labeled with [^{14}C]choline. Of the radioactivity incorporated into lipids, about 90 and 10% was found in PC and sphingomyelin, respectively. The addition of 12-*O*-tetradecanoylphorbol-13-acetate (100 nM), known to activate PLD in HeLa cells (38), resulted in a sustained release of radioactivity into the medium (about 2-fold over control), whereas incubation of cells with *H. pylori* up to 180 min did not increase the release of choline metabolites as compared with control cells (Table II). In addition, no significant decrease of [^{14}C]PC in cells incubated with *H. pylori* (*P12*) was observed. These results indicate that *H. pylori* cells do not activate hydrolysis of PC by PLD. To gain further evidence that *H. pylori* does not affect the PLD activity, the effect of wortmannin, known to inhibit activation of PLD (39), was tested. At a concentration of 100 nM, wortmannin had no effect on *H. pylori*-induced AA release (data not shown).

Furthermore, PEt accumulation was measured in response to *H. pylori* in HeLa cells pretreated with 0.5% ethanol for 10 min (data not shown). In the presence of ethanol PLD catalyzed a transphosphatidylation reaction, resulting in the production of PEt and a consequent decrease in PA formation. As a positive control, HeLa cells prelabeled with ^{14}C-labeled AA or [^{14}C]palmitic acid were incubated for 60 min with 12-*O*-tetradecanoylphorbol-13-acetate in the presence of 0.5% ethanol. This substance caused an ethanol-dependent accumulation of [^{14}C]PEt and a decrease of PA production. In contrast, incubation of cells with *H. pylori* failed to stimulate the formation of PEt, and PA formation was only marginally decreased, indicating that *H. pylori* cells do not activate PLD. Moreover, ethanol treatment did not prevent *H. pylori*-induced AA release, thereby conclusively ruling out the involvement of PLD in *H. pylori*-induced AA release.

H. pylori Induces Generation of lysoPI in Epithelial Cells—To further validate that activation of cPLA$_2$ is involved in *H. pylori*-induced AA release, the generation of lysolipids was monitored in HeLa cells prelabeled with [^{3}H]inositol or [^{14}C]choline (Fig. 3). When prelabeled cells were treated with melittin (positive control), formation of both lysoPI and lysophosphatidylcholine was observed, consistent with preferential release of AA from PI and PC via the PLA$_2$ pathway (see above). When [^{3}H]inositol-prelabeled cells were incubated with *P12* wild-type strain, the ^{3}H radioactivity associated with lysoPI increased noticeably. Under the same experimental conditions, *H. pylori* (*P12*) did not stimulate the formation of lysophosphatidylcholine in [^{14}C]choline-prelabeled cells. The predominant formation of lysoPI in the presence of *H. pylori* (*P12*) is in agreement with the substantial decrease in the percentage of the total ^{14}C-labeled AA content in the PI pool (Table I) when ^{14}C-AA-labeled cells where infected with *H. pylori*.

H. pylori-induced AA Release Is PTX-sensitive and Involves the Activity of p38 Kinase—To explore the role of G-proteins in

TABLE II
Effect of H. pylori on the release of [^{14}C]choline metabolites in HeLa cells

The amount of radioactivity released is expressed as a percentage of total radioactivity associated with cells and medium. The data are the means ± S.E. of three independent experiments for *H. pylori* (*P12*) and the means ± range of duplicate determinations in one experiment for 12-*O*-tetradecanoylphorbol-13-acetate (TPA).

Treatment	Release of [^{14}C]choline metabolites		
	Control	*P12*	TPA (100 nM)
		%	
30 min	2.10 ± 0.39	2.06 ± 0.28	3.78 ± 0.11
60 min	3.44 ± 0.76	3.36 ± 0.47	9.48 ± 0.21
180 min	11.01 ± 4.36	10.97 ± 2.92	23.31 ± 0.24

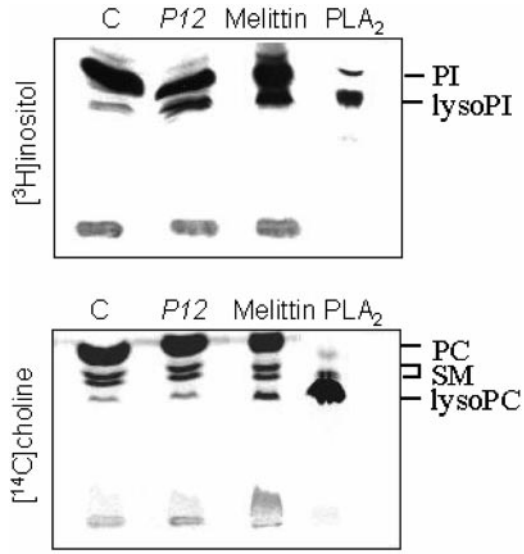

FIG. 3. **Effect of *H. pylori* on lysophospholipid formation in HeLa cells.** HeLa cells in 35-mm-diameter dishes were grown for 24 h in 1 ml of medium containing 2 μCi of *myo*-[^{3}H]inositol or 0.5 μCi of [*methyl*-^{14}C]choline and washed three times with nonradioactive medium containing 1 mg/ml bovine serum albumin before incubation with *H. pylori* (*P12*) at a MOI of 200. As a control, cells were stimulated with the PLA$_2$ activator melittin (125 ng/ml). After 60 min the supernatant was removed and cleared of detached cells by centrifugation. The lipids were extracted together from cells and their medium and analyzed by one-dimensional TLC. Representative TLC plates of total lipid extracts from untreated cells (control), cells exposed to *H. pylori* (*P12*) for 60 min, and cells treated with melittin for 60 min are shown. Lysolipid formation was confirmed by chromatography of PLA$_2$-treated total lipid extracts isolated from the labeled cells. The location of individual species was verified using lipid standards. *SM*, sphingomyelin.

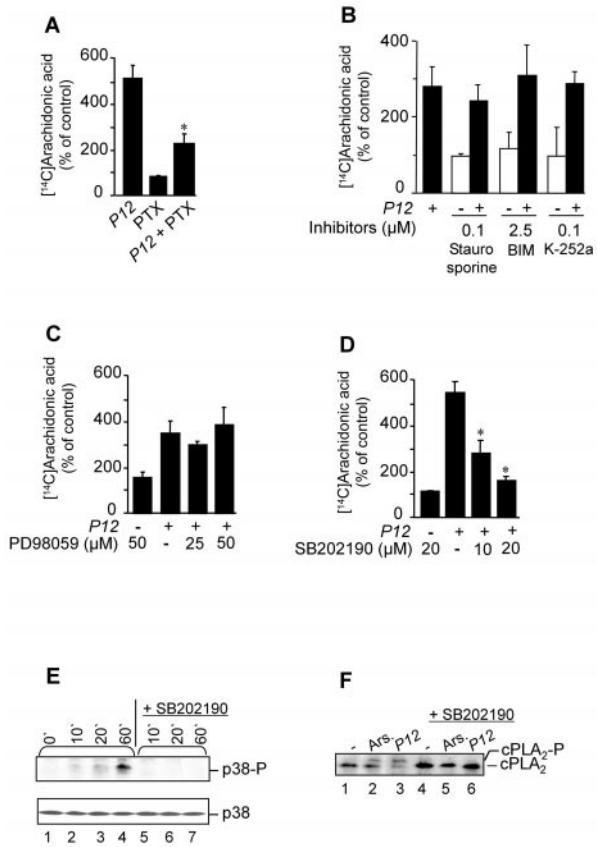

FIG. 4. ***H. pylori*-induced AA release is PTX-sensitive and involves the activity of p38 kinase.** HeLa cells were labeled for 24 h as described in the legend to Fig. 1 and pretreated with PTX (1 μg/ml, included during labeling) (*A*), with inhibitors of the protein kinase C (staurosporine, BIM, and K-252a (all for 30 min)) (*B*), with an inhibitor of the extracellular signal-regulated kinase-activating pathway (PD98059) (30 min) (*C*), or with a specific inhibitor of the p38 stress-activated protein kinase (SB202190) (30 min) (*D*). Thereafter, *H. pylori* (*P12*) was added at a MOI of 200, and incubation was continued for another 60 min. The lipids were extracted from cells and their medium and analyzed by two-dimensional *H. pylori* TLC. Data are expressed as a percentage of AA formation in control cells without drug pretreatment incubated under the same conditions. In *panels A* and C, data represent the means ± S.E. from three independent experiments. The *asterisks* denote a significant difference compared with the respective response to *H. pylori* without drug pretreatment ($p < 0.05$). In *panel B*, results shown are the mean of two experiments plus or minus the difference from the mean. *E*, HeLa cells were pretreated with SB202190 (20 μM, 30 min) and infected with *H. pylori* (*P12*) at a MOI of 200 for the indicated periods of time. Phosphorylation of p38 kinase was monitored from whole cell lysates using a p38 phospho-specific antibody (*upper panel*). As a loading control, the same blot was probed with an anti-p38 antibody (*lower panel*). *F*, the shift in electrophoretic mobility of cPLA$_2$, indicating cPLA$_2$ phosphorylation was monitored in an immunoblot using an anti-cPLA$_2$ antibody. *Lanes 1* and *4*, cell lysates prepared from nonstimulated; *lanes 2* and *5*, arsenite (*Ars.*)-stimulated (0.5 mM, 15 min); *lanes 3* and *6*, *H. pylori* (*P12*)-infected cells (MOI 200, 60 min).

modulating the *H. pylori* (*P12*) effects on AA release, HeLa cells were pretreated with 1 μg/ml PTX for 24 h. PTX alone did not have any effect on basal AA release, but PTX treatment significantly reduced the *H. pylori*-induced AA release (Fig. 4*A*). These results suggest that PTX-sensitive G-proteins are involved in mediating the stimulatory effect of *H. pylori* on AA release.

Next, we investigated whether protein kinase C and/or mitogen-activated protein kinases are involved in the *H. pylori*-induced AA release. Before colonization with *H. pylori* (*P12*), cells were pretreated with various inhibitors of protein kinase C: staurosporine (63), BIM (40), and K-252a (41*)*. As shown in Fig. 4*B*, staurosporine (0.1 μM), BIM (2.5 μM), and K252a (0.1 μM) failed to affect the AA release induced by *H. pylori*. Higher concentrations of BIM (10 μM) had no effect on *H. pylori*-induced AA release, whereas staurosporine (1 μM) and K252a (1 μM) increased the basal AA release and thereby the AA formation in *H. pylori*-infected cells (data not shown). Preincubation with a specific inhibitor of the extracellular signal-regulated kinase-activating pathway, PD98059 (42), had no significant effect on the AA release induced by *H. pylori* (*P12*) (Fig. 4*C*). We have shown previously that 50 μM PD98059 completely blocked the activation of the extracellular signal-regulated kinase pathway in response to *H. pylori* (17). In contrast, pretreatment of cells with SB202190 (10 and 20 μM), a specific inhibitor of the p38 stress-activated kinase (43), significantly decreased the release of AA induced by *H. pylori* (*P12*) (Fig. 4*D*). In agreement with the inhibition of the AA release by SB202190, *H. pylori*-induced activation (phosphorylation) of p38 kinase (Fig. 4*E*) and phosphorylation of cPLA$_2$ (Fig. 4*F*) were blocked in the presence of the inhibitor. As a positive control, cells were treated with arsenite (0.5 mM) known to

increase AA release through phosphorylation of cPLA2 via p38 kinase (44).

DISCUSSION

Infection of epithelial cells by *H. pylori* induced a rapid generation of AA and the production of PGE_2. The separation of cell-associated lipids by TLC demonstrated that the release of AA induced by *H. pylori* was accompanied by a substantial decrease in the PI pool in preference to that of PC, suggesting that PI could serve as a major source for AA in the PGE_2 synthesis. Consistent with this conclusion, lysoPI, which is characteristic for the hydrolysis of PI by PLA_2 (22, 45), was formed when the cells were incubated with *H. pylori.* Among the various types of mammalian PLA_2, the $cPLA_2$ has a key role in the release of AA from cell membranes to serve as substrate for the production of PGs. This enzyme has a high specificity for AA at the *sn*-2 position of phospholipids and requires for activation both elevation of the intracellular concentration of Ca^{2+} and a phosphorylation step (22, 46). We found, consistent with $cPLA_2$ involvement, that pretreatment of cells with the $cPLA_2$ inhibitor MAFP, which, although it also inhibits $iPLA_2$, is selective for $cPLA_2$ among known Ca^{2+}-dependent phospholipases (33), blocked the release of AA. In contrast, the *H. pylori*-induced AA release was not affected by HELSS or aristolochic acid, indicating that $iPLA_2$ and secretory PLA_2 are not involved. The inhibitory effect of the intracellular calcium chelator BAPTA confirmed the involvement of a Ca^{2+}-dependent PLA_2 in the AA release. In addition, exposure of cells to *H. pylori* resulted in a decrease in the electrophoretic mobility of $cPLA_2$, a finding consistent with $cPLA_2$ phosphorylation (46), which is known to increase the catalytic activity of $cPLA_2$ *in vitro* (22).

Several evidences were presented in this study ruling out the involvement of additional pathways in the generation of AA. First, although exposure of HeLa and AGS cells to *H. pylori* induced generation of *myo*-[^{3}H]inositol phosphates, confirming previous findings (18), inhibition of inositol phosphate production by the PI-PLC inhibitor U71322 had no effect on AA release, indicating that PI-PLC is not involved. Second, no significant release of choline metabolites or a decrease of PC was detected when [^{14}C]choline-labeled cells were exposed to *H. pylori*, ruling out the activation of PLD by *H. pylori*. This conclusion was supported by the observation that in the presence of ethanol, which substitutes for water in the transphosphatidylation reaction catalyzed by PLD, formation of PEt was not detectable in *H. pylori*-infected cells. Furthermore, formation of PEt would decrease the amount of PA, thereby inhibiting the AA release, if PA is the source for AA. However, inhibition of *H. pylori*-induced AA release was not observed for cells treated with ethanol. Moreover, wortmannin, known to inhibit PLD activation in a number of cell types (39), failed to affect the AA release induced by *H. pylori*. Finally, in the presence of the DAG lipase inhibitor RHC80267, *H. pylori*-induced AA release was unimpeded.

The *H. pylori*-induced predominant hydrolysis of PI, which is exclusively located in the inner leaflet of the plasma membrane (47), suggests that the host epithelial cell membrane was not damaged by the *H. pylori* phospholipases A_1, A_2, or C (48–50) or sphingomyelinase (51). Moreover, by using the isogenic PAI mutant strain, which does not induce $cPLA_2$ activation, we exclude the possibility that the bacterial phospholipases are able to activate host cell AA release from the epithelial cells, as has been shown in the case of the *Clostridium perfringens* α-toxin (52).

In a number of studies including ours, activation of $cPLA_2$ has been shown to be PTX-sensitive, implying that members of the heterotrimeric $G\alpha_i/G\alpha_o$ proteins are involved in its regulation (53–56). Although the underlying mechanism for the regulatory role of $G\alpha_i/G\alpha_o$ proteins on $cPLA_2$ activation is still unclear, it has been proposed that the $\beta\gamma$ subunits released from $G\alpha_i/G\alpha_o$ proteins can stimulate $cPLA_2$ activity and AA release (57). Furthermore, it has been shown that mitogen-activated protein kinases are involved in $cPLA_2$ activation (22, 46). Our studies, using specific inhibitors of protein kinase C and the extracellular signal-regulated kinase cascade, clearly demonstrated that the ability of *H. pylori* to cause a release of AA from epithelial cells does not depend on the activation of these kinases. This conclusion is supported by the observation that *H. pylori* induces the activation of the extracellular signal-regulated kinase cascade in a PAI-independent manner (14, 17), whereas in marked contrast to that, AA release induced by *H. pylori* is PAI-dependent. Recently, evidence for the involvement of p38 kinase in $cPLA_2$ activation has been presented (44, 58–60). In *H. pylori*-stimulated cells, activation of p38 kinase, the mobility shift of $cPLA_2$, and AA release were clearly abolished after pretreatment of cells with the p38 kinase inhibitor SB202190. These findings indicate the involvement of the p38 kinase in the signaling cascade leading to $cPLA_2$ phosphorylation and AA release. Notably, SB202190 has been used to inhibit p38 kinase activated by various stimuli (60, 61) and thereby shown to reduce phosphorylation of $cPLA_2$ in HeLa cells (59) as well as to block AA release in thrombin-stimulated platelets (61). Our findings are also consistent with the observation that activation of the p38 kinase by *H. pylori* in epithelial cells is strictly PAI-dependent (14). The release of AA was sensitive to both PTX and an inhibitor of p38 kinase, indicating that PTX-sensitive $G\alpha_i/G\alpha_o$ proteins could be involved in p38 kinase activation. Hence, additional work is required to elucidate the signaling upstream of p38 kinase involved in the activation of $cPLA_2$ by *H. pylori*.

In conclusion, in this study we have shown that colonization of epithelial cells by *H. pylori* promotes a rapid release of AA predominately from PI for PGE_2 production via activation of $cPLA_2$. The signaling pathway requires a pertussis toxin-sensitive G-protein and p38 stress-activated protein kinase but not the activation of PLC, PLD, protein kinase C, and extracellular signal-regulated kinase. The *H. pylori*-induced release of AA and PGE_2 from epithelial cells and their role in the support or prevention of physiological and/or inflammatory reactions in the stomach remain to be elucidated. Release of AA for PG production by activation of $cPLA_2$ could play an important role in mucosal defense to the bacterial infection (5, 62) since gastric epithelial cells are the first site of contact with *H. pylori*. On the other hand, prolonged activation of $cPLA_2$ is likely to be damaging to the gastric epithelia by excessive degradation of membrane phospholipids releasing AA and lysophospholipids.

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Helicobacter pylori-induced Prostaglandin E_2 Synthesis Involves Activation of Cytosolic Phospholipase A_2 in Epithelial Cells

Thomas Pomorski, Thomas F. Meyer and Michael Naumann

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