INTRODUCTION

Proteinase-activated receptors (PARs), a family of G-protein-coupled seven-transmembrane receptors, are activated by proteolytic cleavage of the N-terminal domain, and subsequent binding of the newly appeared N-terminus, a tethered ligand, to the receptor itself (1, 2). PAR1, PAR2 and PAR4, but not PAR3, can also be activated by synthetic PAR-activating peptides based on the tethered ligand sequences (2, 3). PARs, upon activation, induce various physiological/pathophysiological responses, including platelet aggregation, pro- or anti-inflammatory responses, gastric mucosal protection, exocrine secretion, accelerating proliferation, processing of pain signals, regulation of smooth muscle motility, etc. (2, 4, 5). Interestingly, PARs are activated by not only endogenous enzymes, e.g. thrombin, trypsin, tryptase, coagulation factors VIIa and Xa, plasmin, kallikrein, etc., but also exogenous serine proteinases, such as gingipains-R derived from Porphyromonas gingivalis, the pathogenesis bacteria of periodontitis, Der p3 and Der p9, house dust mite allergens, and cockroach proteinases (3, 6).

Helicobacter pylori (H. pylori), a microaerophilic Gram-negative bacterium, colonizes the human stomach. Infection with H. pylori is a well-known risk factor for gastroduodenal ulcers and for gastric adenocarcinoma and gastric mucosa-associated lymphoid tissue (MALT) lymphoma (7-9). H. pylori possesses various pathogenicity factors including CagA, VacA, peptidoglycan, lipopolysaccharide (LPS), urease and secretory proteinases, which trigger various signals for inflammation, immunity, proliferation, survival, and so on (9, 10). The secretory proteinases include HtrA and urokinase-type plasminogen activator (u-P A) (11, 12). Interestingly, there is evidence that H. pylori-derived proteinases cause interleukin-8 (IL-8) production through activation of PAR2 in gastric epithelial MKN45 cells (13, 14). On the contrary, it has also been reported that PAR1 protects the host against severe H. pylori-induced gastritis via suppression of production of macrophage-inhibitory protein (MIP)-2, the mouse IL-8 functional homologue, in mice (15). Another paper shows that PAR2 attenuates H. pylori-induced cell death and DNA fragmentation in gastric AGS cells (16). These reports strongly suggest that PARs may contribute to the H. pylori-related events in the gastric mucosa, although it is open to question whether PARs suppress or aggravate the H. pylori-induced pathological responses. In the present study, to examine if the H. pylori actually possesses proteinases that can activate PARs, we prepared the supernatant of H. pylori homogenate (H. pylori extracts), and evaluated the biological activity of the components in gastrointestinal and other cell lines that functionally express PAR1, PAR2 or PAR4 (17-19), and in rat platelets that express PAR4 and PAR3, like a PAR4-AP, H. pylori extracts induced aggregation when assessed in platelet rich plasma, an effect unaffected by the proteinase inhibitor, but did not cause aggregation of washed rat platelets that responded to the PAR4-AP or thrombin. The present study thus shows the biological activities of H. pylori extracts in A549 and HCT-15 cells or rat platelets, and suggests that they are not mediated by any PAR-activating proteinases, but may involve the other pathogenic factors including LPS.
MATERIAL AND METHODS

Materials

The PAR1-activating peptide (PAR1-AP) Thr-Phe-Leu-Leu-Arg-Arg-amide (TFLLR-NH₂), the PAR2-AP Ser-Leu-Ile-Gly-Arg-Arg-Leu-amide (SLIGRL-NH₂) and the PAR4-AP Ala-Tyr-Pro-Gly-Lys-Arg-amide (AYPGKF-NH₂) were synthesized and purified by high-performance liquid chromatography (HPLC), and their concentration and purity were determined by HPLC or mass spectrometry. Nafamostat mesilate was a gift from Torii Pharmaceutical Company (Tokyo, Japan). Polymyxin B sulfate, IRAK-1/4-inhibitor I (IRAK-1/4-I), genistein, pyrrolidine dithiocarbamate (PDTC), U0126 and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO), SP600125, SB203580 and BAY11-7082 were from Calbiochem (Darmstadt, Germany), and LY294002 was from Tocris Bioscience (Ellisville, MO). PAR-activating peptides were dissolved in saline. Polymyxin B and PDTC were dissolved in distilled water, and the other chemicals were in DMSO.

Culture of H. pylori and preparation of the extracts

H. pylori (ATCC 43504, Rockville, MD) were cultured on Trypticase soy agar (Becton, Dickinson and Company, Sparks, MD) with 10% defibrinated sheep blood (Japan Lamb, Hiroshima, Japan) at 37°C under microaerophilic conditions, and then harvested in the ice-cold sterilized phosphate-buffered saline (PBS). The suspension of H. pylori was sonicated, and then centrifuged. The supernatant was collected and filtered through a filter with 0.22 µm pore. The filtrate was used as the H. pylori extracts. The protein concentration of the extracts was measured with the Bradford protein assay method (Bio-Rad Laboratories, Tokyo, Japan).

Cell culture

Normal rat gastric epithelial mucosal RGM1 cells were cultured in DMEM F-12 Ham (Sigma-Aldrich) supplemented with 20% fetal calf serum (FCS) (Thermo, Melbourne, Australia) and 50 mg/L kanamycin (Meiji Seika Kaisha, Ltd., Tokyo, Japan). Human lung adenocarcinoma A549 cells and human colorectal adenocarcinoma HCT-15 cells were cultured in DMEM supplied with 10% FCS and 50 mg/L kanamycin.

Assay of PGE₂ and IL-8 release

RGM1, A549 and HCT-15 cells (1.5×10⁵ cells/well of 6-well plate) were cultured in the above-mentioned FCS-containing medium for 24 hours, and then, cultured in the FCS-free medium overnight. One hour after the culture was refreshed, the cells were stimulated with the H. pylori extracts or the PAR-APs. Inhibitors were added 30 min before the stimulation. Small volume samples were collected from the culture medium at appropriate points in time. The concentration of PGE₂ and IL-8 in the collected samples was assayed using the PGE₂ EIA kit (Cayman Chem., Ann Arbor, MI, USA) and IL-8 ELISA kit (Amersham Biosciences, Buckinghamshire, UK), respectively.

Western blotting

A549 cells were cultured, and then stimulated with the PAR2-AP SLIGRL-NH₂ or the H. pylori extracts, as mentioned above. After the stimulation, the cells were lysed in sodium dodecyl sulfate (SDS) buffer (2% SDS, 62.5 mM Tris-HCl, and 10% glycerol, pH 6.8). Protein samples (30 µg for COX-2, 10 µg for others) were separated by electrophoresis on 12.5% SDS-polyacrylamide gel (Wako Pure Chemicals, Osaka, Japan), and transferred onto polyvinylidene difluoride membrane (Millipore, Bedford, MA). After blocking the membrane with a blocking buffer (5% skim milk, 137 mM NaCl, 0.1% Tween-20 and 20 mM Tris-HCl, pH 7.6), the membrane was incubated with primary antibodies overnight at 4°C, and then washed and incubated with horseradish peroxidase-conjugated anti-rabbit or anti-goat antibodies (Cell Signaling Technol.). The primary antibodies used in the present study were: the anti-COX-2 and anti-GAPDH antibodies from Santa Cruz Biotech. (Santa Cruz, CA), anti-p44/42 MAP kinase and anti-phospho-p44/42 MAP kinase antibodies from Cell Signaling Technol. (Beverly, MA). Positive bands were developed by enhanced chemiluminescence detection Western blotting detection reagent (Amersham Biosciences).

Experimental animals

Male Wistar rats weighing 150–200 g were purchased from Japan SLC Inc. (Shizuoka, Japan) and used at the body weight of 300–400 g. All animals were used with approval by the Committee for the Care and Use of Laboratory Animals at Kinki University, and all procedures employed in the present study were in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 80-23, revised 1996).

Platelet aggregation

Under anesthesia with urethane at 1.5 g/kg, blood was collected from the abdominal aorta of rats into a plastic syringe containing one-tenth volume of 3.8% sodium citrate (Fuso Pharmaceutical Industries Ltd., Osaka, Japan). Platelet-rich plasma (PRP) and washed platelets at 4×10⁵ platelets/µL were prepared from the blood at room temperature as described previously (22, 23). Platelet aggregation was monitored as an increase in light transmission at 37°C a platelet aggregometer (SSR Engineering Co. Ltd., Tokyo, Japan).

Statistical analysis

All data are represented as mean ± S.E.M. Statistical analysis was performed by Tukey’s test. Significance was set at P<0.05 level.

RESULTS

Determination of the activity of H. pylori extracts in RGM1 cells expressing PAR1 and in A549 cells expressing PAR2

In normal rat gastric mucosal epithelial RGM1 cells that abundantly express PAR1, activation of PAR1 with the PAR1-AP TFLLR-NH₂ caused PGE₂ release (Fig. 1A), as reported previously (18). On the other hand, H. pylori extracts did not induce PGE₂ release in RGM1 cells (Fig. 1A).

In human lung adenocarcinoma A549 cells that abundantly express PAR2, H. pylori extracts significantly increased PGE₂ release (Fig. 1B), and caused upregulation of COX-2 (Fig. 1C) and phosphorylation of ERK (Fig. 1D), as the PAR2-AP, SLIGRL-NH₂, did (Fig. 1B-1D). The PGE₂ release induced by H. pylori extracts was resistant to nafamostat mesilate, an inhibitor of proteinases with a broad spectrum (Fig. 2A), and then harvested in the ice-cold sterilized phosphate-buffered saline (PBS). The suspension of H. pylori was sonicated, and then centrifuged. The supernatant was collected and filtered through a filter with 0.22 µm pore. The filtrate was used as the H. pylori extracts. The protein concentration of the extracts was measured with the Bradford protein assay method (Bio-Rad Laboratories, Tokyo, Japan).

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Assay of PGE₂ and IL-8 release

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Western blotting

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RGM1 (A) and A549 (B) cells were stimulated with 3D protein/ml and the released PGE$_2$ was quantified. **P<0.01 known to express PAR4.

Evaluation of the activity of H. pylori extracts in HCT-15 cells

In human colorectal adenocarcinoma HCT-15 cells that abundantly express PAR4, not only the PAR2-AP, SLIGRL-NH$_2$, but also the PAR4-AP, AYPGKF-NH$_2$, significantly caused IL-8 release (Fig. 3A), suggesting that PAR4 is also expressed in HCT-15 cells. *H. pylori extracts induced IL-8 release in HCT-15 cells (Fig. 3A), an effect being partially, but significantly suppressed by nafamostat mesilate, a proteinase inhibitor, or boiling of the extracts (Fig. 3B). The effect of the *H. pylori extracts was strongly suppressed by IRAK-1/4 inhibitor-I (Fig. 3D), but not by inhibitors of LPS (Fig. 3C) or NF-xB (Fig. 3E).

On the other hand, inhibitors of MEK (U0126), JNK (SP600125) and p38 MAP kinase (SB203580) significantly suppressed the activity of the *H. pylori extracts (Fig. 3F).

To determine whether *H. pylori extracts include PAR4-activating proteinases, we used rat platelets, since rat platelets express PAR4 and PAR3 that mediate the thrombin-induced platelet aggregation (21). In rat platelet-rich plasma (PRP), *H. pylori extracts caused aggregation, as the PAR4-AP, AYPGKF-NH$_2$, did (Fig. 4A). However, nafamostat mesilate, a proteinase inhibitor, did not suppress the activity of the *H. pylori extracts in PRP (Fig. 4A). Further, in the washed platelets that responded to AYPGKF-NH$_2$, the *H. pylori extracts failed to cause aggregation (Fig. 4B).

**Fig. 1.** Activity of *H. pylori extracts in the PAR1-expressing RGM1 cells and PAR2-expressing A549 cells. (A and B) RGM1 (A) and A549 (B) cells were stimulated with *H. pylori extracts, the PAR1-activating peptide (AP), TFFLR-NH$_2$, or the PAR2-AP, SLIGRL-NH$_2$, for 24 hours (A) or 12 hours (B), and the released PGE$_2$ was quantified. **P<0.01 vs. vehicle. Data show the mean ± S.E.M. from 4–8 experiments.

**Fig. 2.** Characterization of the PGE$_2$ release caused by the *H. pylori extracts in A549 cells. (A and B) nafamostat mesilate, a proteinase inhibitor, and boiling of the *H. pylori extracts did not affect the evoked PGE$_2$ release. (C, D and E) Effects of polymyxin B, an inhibitor of LPS, IRAK-1/4 inhibitor I (IRAK-1/4-I), or PDTC, an NF-xB inhibitor on the PGE$_2$ release by the *H. pylori extracts. Polymyxin B was incubated with *H. pylori extracts for 30 min, and then, the mixture was added to the cells. IRAK-1/4-I or PDTC was added 30 min before stimulation with *H. pylori extracts. *P<0.05, **P<0.01 vs. vehicle + vehicle (V+V). † P<0.05, †† P<0.01 vs. V+*H. pylori extracts. Data show the mean ± S.E.M. from 4–7 experiments.

**Fig. 3A**

**Fig. 3B**

**Fig. 3C**

**Fig. 3D**

**Fig. 3E**

**Fig. 4A**

**Fig. 4B**
In the present study, we first found that the PAR1-AP but not *H. pylori* extracts caused PGE\(_2\) release in RGM1 cells that express PAR1 (18, 24), suggesting that the *H. pylori* extracts do not contain PAR1-activating proteinases. Next, we showed that *H. pylori* extracts, like the PAR2-AP, induced PGE\(_2\) release in A549 cells known to abundantly express PAR2 (17, 25, 26). However, this effect of the *H. pylori* extracts is not considered to be mediated by proteinases, since it was resistant to nafamostat mesilate or exposure to boiling. LPS, one of heat-resistant pathogenetic factors of *H. pylori*, might be responsible for the PGE\(_2\) release induced by *H. pylori* extracts, considering the findings that the effect of the *H. pylori* extracts was suppressed by polymyxin B, an inhibitor of LPS, and by inhibitors of IRAK-1/4 and NF-κB, downstream signals for toll-like receptor (TLR) 2, a receptor of *H. pylori*-derived LPS (9, 27, 28). The pathological roles of *H. pylori* LPS have been described in human peripheral blood mononuclear leukocytes (29), although it is not highly toxic.

**DISCUSSION**

In the present study, we first found that the PAR1-AP but not *H. pylori* extracts caused PGE\(_2\) release in RGM1 cells that express PAR1 (18, 24), suggesting that the *H. pylori* extracts do not contain PAR1-activating proteinases.

Next, we showed that *H. pylori* extracts, like the PAR2-AP, induced PGE\(_2\) release in A549 cells known to abundantly express PAR2 (17, 25, 26). However, this effect of the *H. pylori* extracts is not considered to be mediated by proteinases, since it was resistant to nafamostat mesilate or exposure to boiling. LPS, one of heat-resistant pathogenetic factors of *H. pylori*, might be responsible for the PGE\(_2\) release induced by *H. pylori* extracts, considering the findings that the effect of the *H. pylori* extracts was suppressed by polymyxin B, an inhibitor of LPS, and by inhibitors of IRAK-1/4 and NF-κB, downstream signals for toll-like receptor (TLR) 2, a receptor of *H. pylori*-derived LPS (9, 27, 28). The pathological roles of *H. pylori* LPS have been described in human peripheral blood mononuclear leukocytes (29), although it is not highly toxic.
Our group has reported that human colorectal adenocarcinoma HCT-15 cells release IL-8 in response to the PAR2-AP SLIGRL-NH₂ but not PAR1-AP TFLLR-NH₂ (19). In the present study, expression of functional PAR4 in addition to PAR2 in HCT-15 cells was demonstrated by the effectiveness of the PAR4-AP (see Fig. 3A). Our present findings that H. pylori extracts caused release of IL-8 in HCT-15 cells, an effect partially suppressed by nafamostat mesilate and exposure to boiling, could suggest the possibility of the presence of PAR4-activating proteinases in H. pylori extracts, which lack PAR2-activating proteinases, as shown in A549 cells (see Fig. 2A, 2B). The proteinase-independent and heat-resistant portion of the IL-8 release caused by H. pylori extracts in HCT-15 cells does not appear to involve LPS, since it was resistant to polymyxin B. The inhibition experiments suggest the involvement of IRAK1/4, MEK/ERK1/2, JNK and p38 MAP kinase, but not NF-κB, although the detailed signaling mechanisms are open to question. It has been reported that H. pylori increases H₂O₂ release via activation of NADPH oxidase, followed by activation of the Jak1/Stat3 pathway and RANTES release in gastric epithelial AGS cells (30). Since IL-8 release is enhanced by the reactive oxygen species (ROS) produced by NADPH oxidase (31), it is of interest to determine the involvement of the NADPH oxidase/ROS/Jak1/Stat3 pathway in the H. pylori extract-caused IL-8 release in HCT-15 cells.

Interestingly, H. pylori extracts caused aggregation of rat platelets in PRP, which express PAR4 and PAR3 (21). This effect of H. pylori extracts was resistant to nafamostat mesilate, being inconsistent with the nafamostat mesilate-sensitive IL-8 release by the H. pylori extracts in HCT-15 cells. Thus, H. pylori does not contain PAR4- or PAR3-activating proteinases, considering the finding that the H. pylori extracts did not cause aggregation in washed platelets, where the PAR4-AP (see Fig. 4B) and thrombin, an activator of PAR1, PAR3 and PAR4 (data not shown), are capable of causing aggregation. Our data also suggest that the H. pylori extracts require unknown plasma co-factor(s) in causing platelet aggregation. It has been reported that some strains of H. pylori induce human platelet aggregation through their binding to von Willebrand factor (vWF) followed by interaction with glycoprotein Ib (32). Similar mechanisms might be involved in the H. pylori extracts-induced aggregation in rat PRP.

In conclusion, our study demonstrates that H. pylori extracts mimic some of the actions of the PAR2-AP and/or PAR4-AP in A549 and HCT-15 cells or rat platelets, whereas any proteinases capable of activating PAR1, PAR2 and PAR4 are not involved in the activity of the H. pylori components.

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