Toxicon 69 (2013) 240–249

Contents lists available at SciVerse ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Pro-inflammatory properties and neutrophil activation by *Helicobacter pylori* urease

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ARTICLE INFO

Article history:
Received 11 September 2012
Received in revised form 25 December 2012
Accepted 5 February 2013
Available online 1 March 2013

Keywords:
Mouse paw edema
Chemotaxis
Inhibition of apoptosis
Pro-inflammatory eicosanoids
Reactive oxygen species
Urease inhibitor

ABSTRACT

The gastric pathogen *Helicobacter pylori* produces large amounts of urease, whose enzyme activity enables the bacterium to survive in the stomach. We have previously shown that ureases display enzyme-independent effects in mammalian models, most through lipoxygenases-mediated pathways. Here, we evaluated potential pro-inflammatory properties of *H. pylori* urease (HPU). Mouse paw edema and activation of human neutrophils were tested using a purified, cell-free, recombinant HPU. rHPU induced paw edema with intense neutrophil infiltration. In vitro 100 nM rHPU was chemotactic to human neutrophils, inducing production of reactive oxygen species. rHPU-activated neutrophils showed increased lifespan, with inhibition of apoptosis accompanied by alterations of Bcl-X_L and Bad contents. These effects of rHPU persisted in the absence of enzyme activity. rHPU-induced paw edema, neutrophil chemotaxis and apoptosis inhibition reverted in the presence of the lipoxygenase inhibitors esculetin or AA861. Neutrophils exposed to rHPU showed increased content of lipoxygenase(s) and no alteration of cyclooxygenase(s). Altogether, our data indicate that HPU, besides allowing the bacterial survival in the stomach, could play an important role in the pathogenesis of the gastrointestinal inflammatory disease caused by *H. pylori*.

1. Introduction

Helicobacter pylori infects at least half of the world's population and is a major cause of gastroduodenal pathologies. In 1994, the International Agency for Research on Cancer and the World Health Organization (WHO) classified *H. pylori* as a definite (group I) carcinogen (IARC-Working-Group, 1994). Gastric colonization by *H. pylori* is usually accompanied by an intense infiltration of polymorphonuclear leukocytes, macrophages and lymphocytes. The degree of mucosal damage correlates with an intense neutrophil infiltration (D'Elios et al., 2007). Neutrophils act

Abbreviations: rHPU, recombinant *Helicobacter pylori* urease; LTB_4, leukotriene B_4; ROS, reactive oxygen species; PMA, phorbol 12-myristate 13-acetate; CM-H_2DCFDA, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester; IL-8, interleukin-8; 5-LO, 5-lipoxygenase; fMLP, formyl-methionyl-leucyl-phenylalanine.

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0041-0101
http://dx.doi.org/10.1016/j.toxicon.2013.02.009

as the first line of defense against infectious agents, and the infiltration of gastric tissue by neutrophils is the hallmark of acute and chronic inflammatory disorders caused by the persistence of *H. pylori* in the gastric lumen (Elliott and Wallace, 1998). Prolonged inflammation can lead to tumor formation (Mantovani et al., 2008), and the persistence of ROS-producing neutrophils contributes to the amplification of inflammation.

H. pylori produces factors that damage gastric epithelial cells, among which are the vacuolating cytotoxin VacA, the cytotoxin-associated protein CagA, a neutrophil activating protein (HP-NAP) and a urease that neutralizes the acidic medium allowing its survival in the stomach. The gastroduodenal illness induced by *H. pylori* depends on the host inflammatory response elicited by the several virulence factors produced by the microorganism. There are reports showing that *H. pylori* whole cells or extracts of its water-soluble proteins promote inflammation, activate neutrophils and induce release of cytokines (Andrutis et al., 1995; Nielsen and Andersen, 1992). Infection by *H. pylori* may also induce impairment of DNA repair mechanisms, inducing gastric epithelial cells into a mutator phenotype (Machado et al., 2009). The biology of *H. pylori* and its involvement in stomach illness were reviewed recently (Herrera and Parsonnet, 2009; Polk and Peek, 2010).

The urease of *H. pylori* accounts for about 10% of total cell protein and is consistently present in all naturally occurring strains (Suzuki et al., 2007). It has been previously shown that genetically engineered urease-deficient *H. pylori* is unable to colonize either germfree piglets, ferrets, or mice (Andrutis et al., 1995; Eaton et al., 1991; Hu and Mobley, 1990). In vitro, purified *H. pylori* urease stimulates macrophages, eliciting the production of reactive species and cytokines, thus contributing to tissue inflammation and injury (Shimoyama et al., 2003). We have previously reported that a purified *H. pylori* urease activates platelets through a lipoxygenase-mediated pathway, leading to ADP exocytosis and, therefore, platelet aggregation (Wassermann et al., 2010).

In this study we aimed to evaluate the participation of *H. pylori* urease in the acute inflammatory process promoted by this bacterium. For that purpose we worked with a purified recombinant HPU (rHPU) produced in *Escherichia coli*. Our results showed that rHPU induces: (i) mouse paw edema; (ii) human neutrophil migration; (iii) protection of human neutrophils against apoptosis; (iv) production of reactive oxygen species by neutrophils, and (v) induction of expression of lipoxygenase(s) in human neutrophils.

2. Material and methods

2.1. *Recombinant* H. pylori *urease (rHPU)*

H. pylori recombinant urease (rHPU) was produced by heterologous expression (McGee et al., 1999) in *E. coli* SE5000 transformed with plasmid pHP8080, kindly provided by Dr. Harry T. Mobley, University of Michigan Medical School. rHPU was purified from bacterial extracts as previously described (Wassermann et al., 2010). For the experiments, the purified protein was concentrated using Centriprep cartridges (30 kDa cut-off) to give a 0.5 mg protein/mL solution (Suppl. Fig. 1) and dialyzed against 20 mM sodium phosphate, pH 7.5, 1 mM EDTA, 5 mM 2-mercaptoethanol. The buffer from the last dialysis change was used as a negative control in all bioassays.

2.2. *Protein determination*

Protein content of samples was determined by their absorbance at 280 nm, or by the Coomassie dye binding method (Bradford, 1976).

2.3. *Urease activity*

Urease activity was measured colorimetrically by the alkaline nitroprussiate method (Weatherburn, 1967). For studies of urease inhibition, protein solutions were incubated overnight at 4 °C with 1 mM p-hydroxy-mercurybenzoate followed by extensive dialysis to remove excess of inhibitor (Follmer et al., 2001).

2.4. *Neutrophil isolation and culture*

Neutrophils were isolated from EDTA (0.5%)-treated peripheral venous blood of healthy human volunteers by Percoll gradient (Coelho et al., 2004) and suspended in RPMI medium (97% of viable cells, as assessed by trypan blue exclusion). Residual erythrocytes were removed by hypotonic lysis.

2.5. *Neutrophil migration assay*

Chemotaxis was assayed in 48-well microchemotaxis chambers (NeuroProbe, Gaithersburg, MD) using 5-μm polycarbonate filter (Coelho et al., 2004). Neutrophils (10^6 cells/mL in RPMI, 0.01% bovine serum albumin, BSA) were allowed to migrate toward formyl-methionyl-leucyl-phenylalanine (fMLP, 100 nM), rHPU (10 nM, 30 nM, 100 nM) or medium (random migration; 37 °C, 5% CO_2). After 1 h, filters were removed, fixed, stained, and neutrophils that migrated through the membrane were counted under a light microscope on at least 5 randomly selected fields (Coelho et al., 2004). To evaluate the participation of 5-lipoxygenase products, cells were treated with AA861 (10 μM) for 15 min prior to stimulation with rHPU. Each treatment was assayed in triplicate. Results are expressed as mean ± S.E.M.

2.6. *Morphological assessment of neutrophil apoptosis*

Cells were cytocentrifuged, stained with Diff-Quik (Dade Behring, Switzerland), and counted under light microscopy (×1000) to determine the proportion of cells showing characteristic apoptotic morphology (picnotic nuclei). Cells were either untreated or treated with AA861 (10 μM) alone, HPU (100 nM) alone, HPU plus AA861, or IL-8 (100 nM). At least 400 cells were counted per slide. The results were expressed as mean ± S.E.M.

2.7. *Preparation of cell extracts*

To obtain whole cell lysates, neutrophils (5×10^6 cells/mL) were resuspended in lysis buffer (50 mM HEPES, pH

6.4, 1 mM $MgCl_2$, 10 mM EDTA, 1% Triton X-100, 1 μg/mL DNAse, 0.5 μg/mL RNAse) containing a cocktail of protease inhibitors: 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, 1 μM leupeptin and 1 μM soybean trypsin inhibitor (all reagents from Sigma Chem. Co., St. Louis, MO, USA).

2.8. Western blot analysis

Cell lysates were denatured in sample buffer (50 mM Tris–HCl, pH 6.8, 1% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue) and heated in a boiling water bath for 3 min. Samples (30 μg total protein) were resolved in 12% SDS-PAGE gels and proteins were transferred to PVDF membranes (Hybond-P, Amersham Pharmacia Biotech). Rainbow markers (Amersham Pharmacia Biotech) were run in parallel to estimate molecular masses. Membranes were blocked with Tween-TBS (20 mM Tris–HCl, pH 7.5, 500 mM NaCl, 0.1% Tween-20) containing 1% BSA and probed with polyclonal antibodies: anti-Bcl-XL (Santa Cruz Biotechnology, 1:500), polyclonal anti-Bad (Santa Cruz Biotechnology, 1:500), polyclonal anti-5-LO (Cayman Chemicals, 1:500), or polyclonal anti-COX (Cayman Chemicals, 1:500). After washing in Tween-TBS, PVDF sheets were incubated with biotin-conjugated anti-rabbit IgG (1:1000; Santa Cruz Biotechnology) antibody for 1 h and then incubated with horseradish peroxidase-conjugated streptavidin (1:1000; Caltag Laboratories, Burlingame, CA). Immunoreactive proteins were visualized by 3,3′-diaminobenzidine (Sigma) staining and bands were quantified by densitometry using Scion Image Software (Scion Co., MD, USA).

2.9. Reactive oxygen species (ROS) measurement

The luminol-enhanced chemiluminescence of human neutrophils was measured using a microplate-reader Spectramax (Molecular Devices, CA, USA), as described previously (Shimoyama et al., 2002). Briefly, cells were stimulated with rHPU (10, 30 or 100 nM) or phorbol 12-miristate 13-acetate (PMA; 30 nM) and ROS production was measured for 60 min. Neutrophils were incubated for 30 min prior to stimulation. In order to measure intra- and extracellular ROS production, CM-H_2DCFDA (chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate; λ_{ex}470 nm, λ_{em}529 nm) and lucigenin (bis-N-methylacrydinium nitrate) were used, respectively. The protocol described by (Kujbida et al., 2008) was applied for luminol or lucigenin. For CM-H_2DCFDA, neutrophils were incubated with the dye (5 μM) for 15 min at 37 °C prior to stimulation (Espinosa et al., 2009).

2.10. Paw edema

Male Swiss mice (20–22 g), housed at 22 ± 3 °C with a 12/12 h light/dark cycle were used for the bioassays. On the day of the experiments, the mice received, under light ether anesthesia, a 30 μL intraplantar injection of different doses of rHPU into the right hind paw. The left hind paw of the same animal was used as control, receiving an injection of 30 μL of dialysis buffer. In some experiments the animals were pre-treated with anti-inflammatory drugs given subcutaneously 1 h (esculetin, 50 mg/kg, Sigma) or 4 h (dexamethasone, 0.5 mg/kg, Sigma) before rHPU administration. Increased paw thickness due to edema was measured with a micrometer (Mitutoyo, 0–25 mm, with 0.002 mm increments) at the indicated time intervals after the injections. Paw edema was expressed as the difference between the thickness of right and left paws of the same animal. Thus the results represent the net edema (in mm) induced by HPU.

2.11. Histological analyses

Mice paws injected with 45 μg HPU or 30 μL dialysis buffer were fixed in 10% formalin for paraffin block preparation. Sections of 5 μm were stained with hematoxilin–eosin, and studied under light microscopy at the Pathology Service of the Faculty of Veterinary, Universidade Federal of Rio Grande do Sul, Porto Alegre, RS, Brazil.

2.12. Animal experimentation

All procedures involving animals were conducted in strict accordance to Brazilian legislation (Law no. 6.638/1979) and in compliance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines (www.nc3rs.org.uk/ARRIVE), developed by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs).

2.13. Statistical analysis

Data were analyzed by ANOVA followed by the Tukey–Kramer test using the Instat Graph Pad software and values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Paw edema induced by rHPU

To investigate whether purified rHPU possesses pro-inflammatory activity the model of mouse paw edema was chosen. Fig. 1 shows the time course and dose-dependency curves of paw edema induced by subplantar injection of rHPU in mouse hind paws. As low as 0.5 μg (0.4 pmol) of injected protein produced an intense paw edema in some animals. At a dose of 45 μg, the rHPU-induced edema peaked at 4–6 h and lasted more than 24 h. Histopathological analysis of the paw edema showed an intense neutrophil infiltration (Fig. 2).

Pretreatment of mice with dexamethasone, or with the lipoxygenase inhibitor esculetin, produced significant reduction in the paw edema indicating that eicosanoids, particularly lipoxygenase metabolites, mediate the pro-inflammatory activity of rHPU (Table 1).

3.2. rHPU-induced human neutrophil chemotaxis

H. pylori infection induces an acute neutrophil-dominant inflammation and neutrophil density correlates with tissue damage (Nielsen and Andersen, 1992). *H. pylori* whole extracts were shown to stimulate chemokine production and

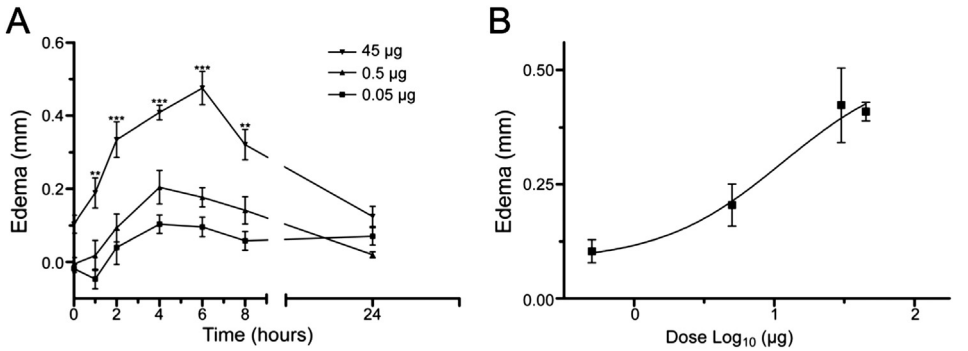

Fig. 1. Time course (A) and dose-response curve (B) of rHPU-induced mice paw edema. rHPU was injected into the right paw of mice in a final volume of 30 µL and the left paw of the same animal received vehicle (last change of buffer used in dialysis of rHPU). Results are expressed as net increase in thickness (mm) of the right paw as compared to the left. Each point represents mean ± SD from 9 animals. Values of $p < 0.05^{*}$, $p < 0.01^{**}$ or $p < 0.001^{***}$ were considered statistically significant.

activation of neutrophils in vitro (Shimoyama et al., 2003). Fig. 3A shows that rHPU stimulated human neutrophil migration in a dose-dependent manner. The chemotactic effect of 100 nM rHPU (55.6 ± 6.8 neutrophils/field) was equivalent to that induced by 100 nM fMLP (63 ± 7.2 neutrophils/field). This property of HPU is independent of its ureolytic activity, as rHPU treated with active-site inhibitors promoted the same migration profile (Fig. 3A). Treatment of cells with AA861, a lipoxigenase inhibitor, reverted the rHPU-induced chemotaxis, indicating the participation of lipoxygenases-derived products in neutrophil migration (Fig. 3B).

3.3. HPU induces production of reactive oxygen species by human neutrophils

It has been reported that *H. pylori* whole cells stimulate the generation of reactive oxygen species by neutrophils (Handa et al., 2010). Total ROS production comprises intra- and extracellular release and increase of ROS production is associated with an increased level of DNA damage/repair in epithelial cells (Machado et al., 2010). Here we evaluated the total, intra- and extracellular production of reactive oxygen species in rHPU-activated neutrophils. Cells were exposed to rHPU or PMA (positive control, not shown) and total ROS production was measured using luminol-amplified luminescence. Fig. 4, panels A–D, show that neutrophil exposed to 100 nM rHPU had a 2.5 fold increase in total ROS production as compared to controls. Extracellular ROS release was measured using lucigenin, a chemiluminescent probe that is more specific for superoxide anions released extracellularly, while CM-H_2DCFDA was used to measure intracellular ROS production (Abe et al., 2000; Espinosa et al., 2009). Data shown in Fig. 4E indicated that the increased ROS production induced by rHPU is entirely directed toward the extracellular medium.

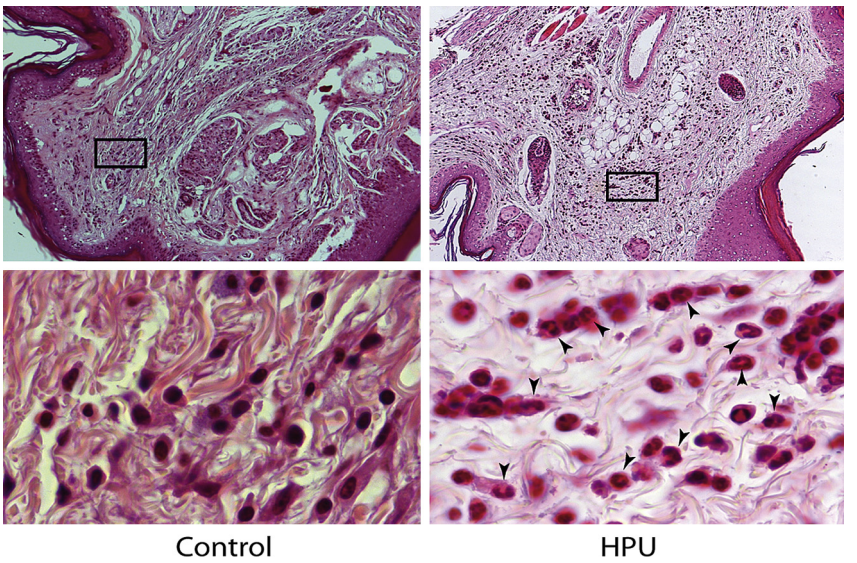

Fig. 2. rHPU induced neutrophil infiltration accompanying paw edema. Hematoxilin and eosin staining sections of mouse paw injected with rHPU (45 µg–3.6 pmoles) or control paws. The area in the boxes is enlarged in the panels below. Data are representative of 3 mice for each group. Neutrophils are indicated by arrowheads. Upper panels, magnification 100×; lower panels, 1000×.

Table 1
Involvement of lipoxygenases(s) metabolites in HPU effects.

Biological effect	Evidence of involvement of lipoxygenase metabolites
HPU-activated platelet	Inhibition by dexamethasone and esculetin treatment[a]
	Detection of 12-HETE production[a]
HPU-activated neutrophil	Inhibition of HPU-induced chemotaxis by AA861[b]
	Reversion of HPU anti-apoptotic effect by AA861[b]
	Increase of 5-lipoxygenase levels (Western blot)[b]
Mouse Paw Edema	Dexamethasone and esculetin treatment[b]

[a] (Wassermann et al., 2010).
[b] This paper.

3.4. HPU inhibits apoptosis of neutrophils by inducing Bad degradation and Bcl-x_L expression

The regulation of neutrophil apoptosis during an inflammatory response is a key point for its resolution (Serhan and Savill, 2005). As the intensity of gastric tissue damage in *H. pylori* infection correlates with the neutrophil density (Allen, 2001), we investigated the neutrophil viability after a 20 h culture in the presence of 100 nM rHPU or 100 nM IL-8. Fig. 5A shows that neutrophil apoptosis is significantly delayed when the cells are exposed to rHPU. The anti-apoptotic effect of rHPU persisted for the enzyme-inhibited protein after treatment with p-hydroxy-mercurybenzoate (not shown). Human neutrophils have a very short half-life, characterized by a constitutive expression of proapoptotic proteins, and almost undetectable levels of anti-apoptotic proteins (Akgul et al., 2001). Fig. 5C shows that rHPU-activated neutrophils had lower levels of Bad, a pro-apoptotic Bcl-2 member. On the other hand, rHPU induced the expression of the anti-apoptotic protein Bcl-x_L (Fig. 5B), increasing the survival rate of neutrophils.

We then investigated the involvement of 5-lipoxygenase products in the anti-apoptotic effect of rHPU. Data shown in Fig. 5D indicated that the protective effect is at least partly due to production of leukotrienes, given that pre-treatment of neutrophils with AA861 reverted this effect (Fig. 5D).

3.5. rHPU activates the arachidonic acid pathway in neutrophils

Two metabolites of the 5-lipoxygenase (5-LO) pathway, leukotriene B_4 and 5-hydroxyeicosatetraenoic acid, have been identified as important mediators of inflammatory processes in the gastrointestinal tract (Wang and Dubois, 2010). Considering our previous data showing that platelet activation by rHPU depends on eicosanoid(s) produced by a 12-lipoxygenase (Wassermann et al., 2010), here we studied the participation of 5-lipoxygenase in rHPU-activated neutrophil signaling. Western blot analyses of rHPU-activated neutrophils showed significantly increased levels of 5-LO (Fig. 6) while no alterations of cyclooxygenase-2 levels were observed (not shown), suggesting the possible involvement of leukotrienes or 5-HETE in neutrophil's response to rHPU. Contrasting to these results, stimulation of neutrophils by LPS (1 μg/mL) under the same experimental conditions did not alter their 5-LO content (not shown). Table 1 summarizes these data.

4. Discussion

Ureases (EC 3.5.1.5) are highly homologous nickel-dependent enzymes that hydrolyze urea into ammonia and carbon dioxide (Dixon et al., 1975; Mobley et al., 1995). We have previously reported that canatoxin (Carlini and Guimaraes, 1981), an isoform of jackbean (*C. ensiformis*) urease (Follmer et al., 2001), presents biological properties that are independent of its enzyme activity, including activation of blood platelets (Barja-Fidalgo et al., 1991; Carlini et al., 1985; Ghazaleh et al., 1997) and pro-inflammatory effect (Barja-Fidalgo et al., 1992; Benjamin et al., 1992). Submicromolar concentrations of canatoxin induced exocytosis in a number of cell systems in vitro such as platelets, synaptosomes, pancreatic islets, macrophages,

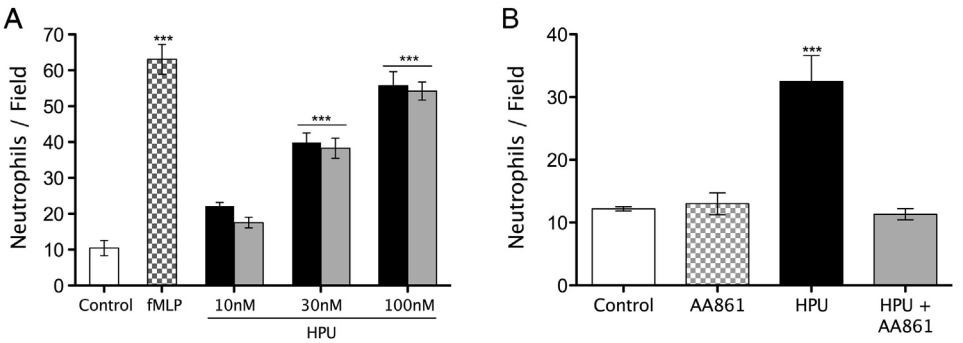

Fig. 3. Effect of rHPU on human neutrophil chemotaxis. (A) Chemotaxis was assayed in 48-well microchemotaxis chambers using 5-μm PVP-free polycarbonate filter. Neutrophils (10^6 cells/mL in RPMI-0.01% bovine serum albumin at 37 °C, 5% CO_2) were allowed to migrate toward rHPU (10 nM, 30 nM or 100 nM; untreated (black bars) or treated (gray bars) with the inhibitor p-hydroxymercury-benzoate 50 μM) and fMLP (100 nM), or medium alone (control – random migration). After 1 h, filters were removed, fixed, and stained, and neutrophils that migrated through the membrane were counted under a light microscope on at least 5 randomly selected fields. (B) Neutrophils were incubated with 10 μM AA861 prior to stimulation with 100 nM HPU. Each sample was assayed in triplicate. Results are expressed as number of neutrophils per field. All data show mean ± S.E.M. from at least three independent experiments done in triplicates. ***$p < 0.001$ compared to control.

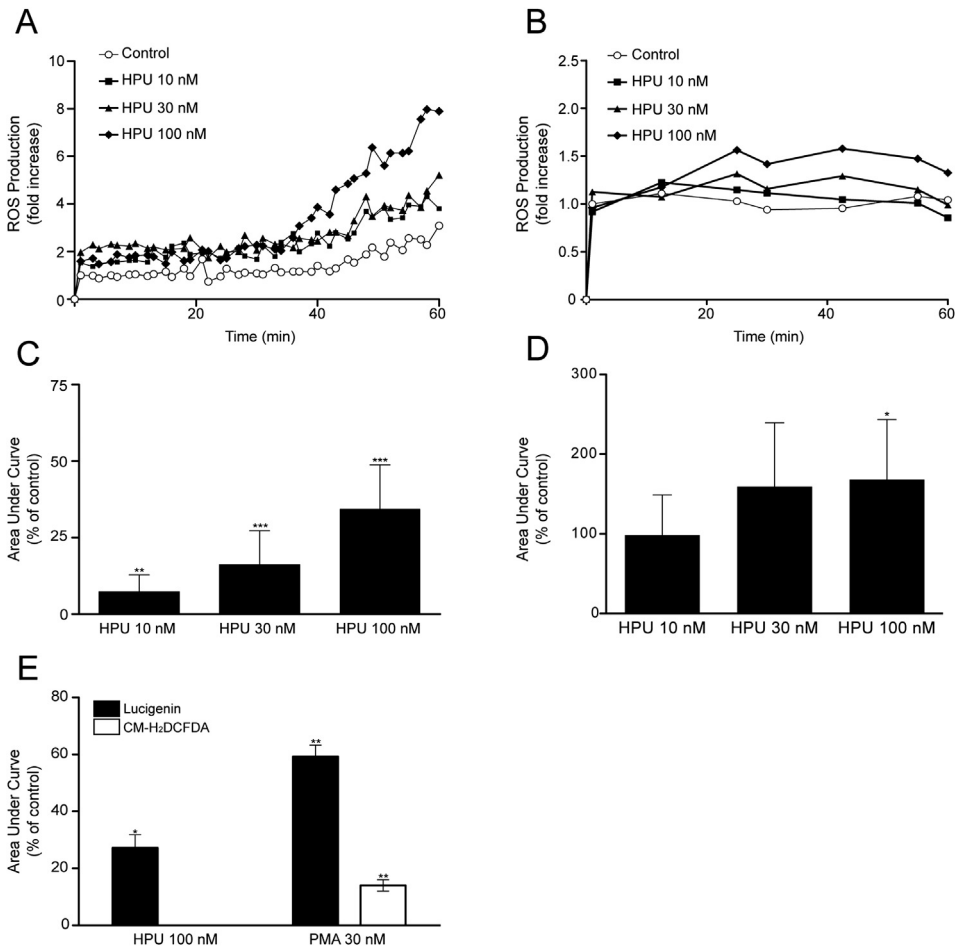

Fig. 4. rHPU induces neutrophil ROS production. (A) Time-course. Neutrophils (5×10^5 cells/well) were placed in a white flat-bottom 96-wells plate. Cells were not stimulated (circles) or stimulated with 10 nM (squares), 30 nM (triangles) and 100 nM (diamonds) of rHPU in the presence of luminol (500 mM). (B) Same experiment was performed using lucigenin as chemiluminescent probe (25 mM). Data are representative means of three independent experiments. ROS accumulation was evaluated calculating the area under curve using luminol (C), or lucigenin (D) over 60 min treatment. Results are mean + S.E.M. of three independent experiments. (E) rHPU promotes extracellular ROS production while maintaining the intracellular redox status. Neutrophils (6×10^5 cells/well) were placed in a white flat-bottom 96-wells plate for lucigenin chemiluminescence assay or in a black flat-bottom 96-wells plate for CM-H_2DCFDA fluorescence assay. Cells were then not stimulated or stimulated with rHPU 100 nM or PMA 30 nM in the presence of lucigenin (25 mM; gray bar) or CM-H_2DCFDA (5 mM; black bar). Accumulated ROS generation was evaluated calculating the area under curve of lucigenin-dependent emitted chemiluminescence or CM-H_2DCFDA fluorescence accumulation over 120 min treatment. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared to control.

neutrophils and mast cells (reviewed in Olivera-Severo et al., 2006b). Lipoxygenase metabolites were shown to modulate most of canatoxin's pharmacological effects, either in vivo or in vitro (Barja-Fidalgo et al., 1991; Benjamin et al., 1992; Carlini et al., 1985).

Jackbean, soybean and *Bacillus pasteurii* ureases induce aggregation of platelets in nanomolar concentrations independently of enzyme activity (Follmer et al., 2004; Olivera-Severo et al., 2006a). More recently, we demonstrated that purified recombinant *H. pylori* urease also promotes activation of rabbit platelets recruiting the lipoxygenase pathway (Wassermann et al., 2010). The fact that bacterial and plant ureases evolutionarily conserved the property of activating some cell types may shed new lights into the so far poorly understood biological functions of these proteins, which clearly are not restricted only to their ureolytic activities.

The data gathered here show that the cell-free, purified rHPU displays potent pro-inflammatory properties. Fig. 7 summarizes these and other previous results pointing to a relevant participation of HPU in the gastric inflammatory disease caused by *H. pylori*.

The time-course of HPU-induced mouse paw edema is very similar to that described for the rat paw edema induced by canatoxin (Benjamin et al., 1992). rHPU is at least 100-fold more potent than canatoxin in its ability to induce paw edema, although differences in inflammatory reactions of animal models have also to be considered. As described for canatoxin, eicosanoids derived from lipoxygenases(s) pathway(s) play an important role in the rHPU-induced inflammation, as evidenced by the reduction in paw edema in mice pre-treated with esculetin. More and more evidences are pointing to an important role of the arachidonic acid pathway in the development of chronic

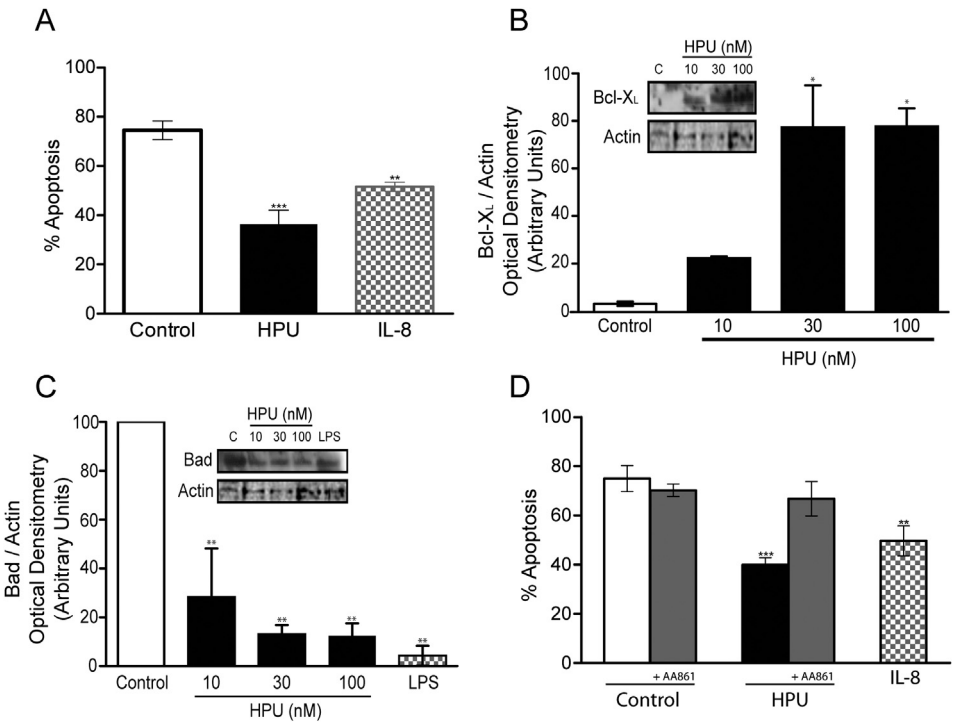

Fig. 5. rHPU inhibits human neutrophil apoptosis. (A) Neutrophils (5×10^6/ml) were incubated in the absence or in the presence of rHPU (100 nM) or IL-8 (100 nM). After 20 h, cells were centrifuged, and the number of apoptotic cells was determined in an optical microscope by counting the picnotic nuclei. $^{**}p < 0.01$; $^{***}p < 0.001$ compared to control. (B) rHPU induces the expression of Bcl-X_L in neutrophils. Human neutrophils (5×10^6 cells) were incubated with rHPU (10 nM, 30 nM and 100 nM) and LPS (1 μg/mL). After 4 h, Bcl-X_L protein expression was assessed by Western blot analysis. Results are mean ± S.E.M. of triplicates. Data shown in the inset are of a typical experiment. $^{*}p < 0.05$ compared to control. (C) Decrease in Bad levels in rHPU-activated neutrophils. Human neutrophils (5×10^6 cells) were incubated with rHPU (10 nM, 30 nM and 100 nM) and LPS (1 μg/mL). After 4 h, Bad protein expression was assessed by Western blot analysis. Results are mean ± S.E.M. of triplicates. Data shown in the inset are of a typical experiment. $^{**}p < 0.01$ compared to control. (D) Neutrophils were incubated with 10 μM AA861 prior to rHPU (100 nM) stimulation. IL-8 (100 nM), positive control. $^{**}p < 0.01$; $^{***}p < 0.001$ compared to control.

inflammation and gastric carcinogenesis (Wang and Dubois, 2010; Wymann and Schneiter, 2008). Lipoxygenase metabolites such as LTB_4 enhance the proliferation of epithelial cells and may induce oncogenes in these cells (Wang and Dubois, 2010).

Our data show that nanomolar doses of HPU directly activates human neutrophils. Chemotaxis induced by 100 nM rHPU was similar to that produced by 100 nM fMLP, a synthetic peptide that mimicks bacterial peptides (Niedel et al., 1979). The chemotactic effect of rHPU did not require its enzymatic activity. Additionally, histology sections of rHPU-induced edema showed an increased neutrophil infiltration. We have previously reported that the plant urease canatoxin induced neutrophil migration into rat pleural cavity and "air-pouches" and also that macrophages exposed to canatoxin released a neutrophil-chemotactic factor (Barja-Fidalgo et al., 1992).

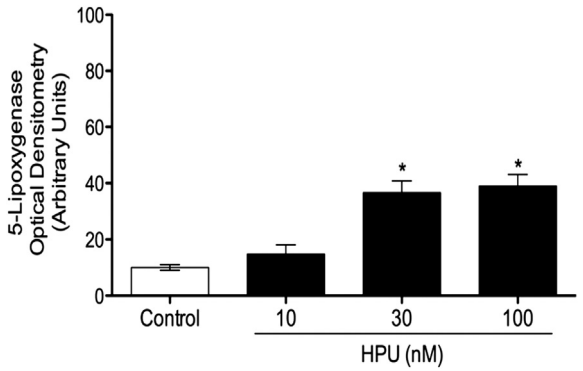

Fig. 6. HPU increases the content of 5-lipoxygenase in human neutrophils. Human neutrophils (5×10^6 cells) were incubated with HPU (10 nM, 30 nM and 100 nM) or buffer alone (control). After 4 h, 5-LO protein expression was assessed by Western blot analysis. Results are mean ± S.E.M. of triplicates. $^{*}p < 0.05$ compared to control.

Other studies have shown that purified *H. pylori* urease directly activated primary human blood monocytes and stimulated dose-dependent production of inflammatory cytokines (Harris et al., 1996).

The neutrophil activating protein HP-NAP is a dodecameric protein, structurally similar to bacterioferritines, which activates neutrophils by stimulating the production of reactive forms of oxygen (D'Elios et al., 2007; Evans et al., 1995; Zanotti et al., 2002). In monocytes HP-NAP induces activation and synthesis of cytokines, plasminogen activator inhibitor-2 and tissue factor (Montemurro et al., 2001). HP-NAP was shown to increase the lifespan of neutrophils and monocytes indirectly through the release of endogenous pro-survival factors (Cappon et al., 2010). Preliminary data suggest that rHPU is as powerful as HP-NAP in promoting activation of monocytes with induction

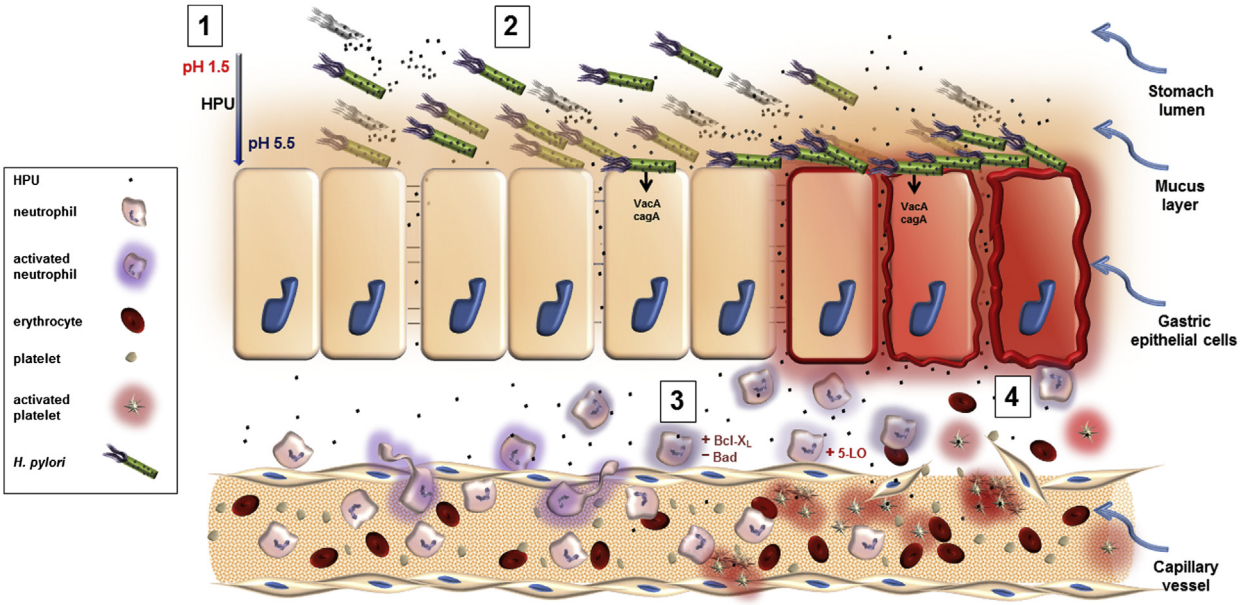

Fig. 7. Contributions of *H. pylori* urease to the gastric inflammation. The figure illustrates data from this work and from other groups on the cell-activating properties of HPU that potentially contribute to gastric inflammation. **1**. Infection by *H. pylori* starts when the bacterium enters the gastric mucus layer and promotes alkalization of its surrounding by action of its cytoplasmic urease (HPU), allowing bacterial proliferation and colonization; **2**. *H. pylori* adheres to the apical surface of epithelial cells and subsequently injects the cagA protein into the epithelial cells and releases other virulent factors such as vacA. Bacterial lysis releases HPU to the extracellular medium, part of which adsorbs onto the surface of viable *H. pylori* cells. HPU contributes to alterations of the gastric epithelial tight junction barrier function, increasing the epithelial permeability. **3**. HPU reaches the vascular lining and activates platelets and neutrophils through a lipoxygenase(s)-modulated process. Neutrophils migrate toward HPU released at the site of infection. HPU inhibits apoptosis of neutrophils by interfering on the levels of Bad and Bcl-xL; **4**. HPU-activated-neutrophils cause tissue damage by releasing reactive oxygen species. HPU-induced increase of neutrophil survival leads to a sustained focus of inflammation.

of mRNA synthesis for the cytokines IL1b, IL6, IL8, IL23 and TNFα (Olivera-Severo, D and De Bernard M, unpublished data). As proposed for HP-NAP (De Bernard and D'Elios, 2010), HPU is released most likely after lysis of *H. pylori* cells, reaching the underlying tissue and lamina propria where it would exert its pro-inflammatory effects, synergistically with other bacterial factors, recruiting neutrophils and monocytes, and activating platelets within nearby injured microcapillaries.

Enarsson et al., 2005, reported that *H. pylori* promoted significant T-cell activation and transendothelial migration in a model of human umbilical vein endothelial cells and that purified *H. pylori* urease induced a migratory effect similar to that of whole bacteria. Mutant *H. pylori* negative for the urease A subunit still promoted significant T-cell migration, an effect that was imparted as a contribution of the functional cag pathogenicity island (Enarsson et al., 2005). In face of our results, another possibility to consider is that this effect may rely on an ability of the bacterial urease B chain to induce leukocyte transmigration.

Handa et al., 2010, reported that *H. pylori* infection stimulates inflammatory cells within the gastric tissue to release ROS (Handa et al., 2010) which correlates with higher levels of DNA repair in gastric epithelial cells (Machado et al., 2010). Studies by Allen et al. (2005), showed that *H. pylori* infection interferes on the activity of human neutrophil NADPH oxidase leading to extracellular release of ROS (Allen, 2001). Here, we demonstrated that rHPU-activated neutrophils release ROS extracellularly potentially contributing to damage the gastric tissue.

The half-life of human neutrophils is typically of 12 h, as a result of the constitutive expression of pro-apoptosis proteins and almost non-detectable levels of anti-apoptosis proteins (Witko-Sarsat et al., 2011). Neutrophils release proteolytic enzymes and ROS that inflict local tissue damage and are removed from the inflammatory site by induction of apoptosis. Thus fine regulation of pro- and anti-apoptosis proteins that control neutrophil lifespan is a critical process for the resolution of inflammation. Our data show that rHPU delays neutrophils apoptosis, prolonging their survival and contributing to the underlying local tissue inflammation, as seen in the mouse paw edema assay. Increased lifespan of rHPU-activated neutrophils was accompanied by reduced levels of the pro-apoptotic protein Bad and induction of expression of the anti-apoptotic protein Bcl-X_L, a situation that would ultimately lead to a persistent inflammatory status. In agreement with our data, other groups have demonstrated that products of *H. pylori* exert an important role in maintaining inflammation, by suppressing human neutrophil apoptosis (Cappon et al., 2010; Kim et al., 2001). Interestingly, other studies demonstrated that *H. pylori* can induce apoptosis in gastric epithelial cell lines (Ashktorab et al., 2008), contributing to the worsening of the gastroduodenal illness.

Lipoxygenase products contribute to the anti-apoptotic property as well as to chemotaxis induced by HPU as indicated by the AA861 pretreatment of neutrophils. HPU-

treated neutrophils had increased levels of lipoxygenases(s) but no alteration of cyclooxygenase(s) content. Our data show that lipoxygenases play an important role in mediating the cell-activating property of HPU (Table 1).

Altogether our data demonstrate that HPU, besides its well known role in allowing bacterial gastric colonization, also displays potent pro-inflammatory activity modulated by lipoxygenase-derived eicosanoids. The increased lifespan of HPU-activated neutrophils and its ability to attract more neutrophils toward the injured tissues, acting together with other bacterial factors, potentially amplify the gastric inflammatory process. These findings could be particularly relevant to the mechanisms leading to gastrointestinal disease caused by *H. pylori* and should be taken into consideration in the development of more efficient therapeutic approaches.

Author contributions

A.F.U., D.O-S., G.E.W., A.S-G., J.A.M., P.B-S, have acquired all data and interpreted the results, C.B-F. and C.R.C. have conceived and supervised this study. C.R.C. wrote the manuscript whose final version was approved by all authors.

Ethical statement

The authors declare that: a) the material has not been published in whole or in part elsewhere, except in the form of an abstract or part of academic thesis; b) the work is not currently being considered for publication elsewhere; c) all authors have agreed upon the content and form of the manuscript; d) all relevant ethical safeguards have been met regarding animal experimentation.

Acknowledgments

This work was supported by the Brazilian agencies: Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq; Coordenação de Aperfeiçoamento de Pessoal do Ensino Superior – CAPES; Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul – FAPERGS and Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro – FAPERJ.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.toxicon.2013.02.009.

Conflict of interest

The authors declare that there are no conflicts of interest.

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