

***Helicobacter pylori* urease activates blood platelets through a lipoxygenase-mediated pathway**

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Abstract

The bacterium *Helicobacter pylori* causes peptic ulcers and gastric cancer in human beings by mechanisms yet not fully understood. *H. pylori* produces urease which neutralizes the acidic medium permitting its survival in the stomach. We have previously shown that ureases from jackbean, soybean or *Bacillus pasteurii* induce blood platelet aggregation independently of their enzyme activity by a pathway requiring platelet secretion, activation of calcium channels and lipoxygenase-derived eicosanoids. We investigated whether *H. pylori* urease displays platelet-activating properties and defined biochemical pathways involved in this phenomenon. For that the effects of purified recombinant *H. pylori* urease (HPU) added to rabbit platelets were assessed turbidimetrically. ATP secretion and production of lipoxygenase metabolites by activated platelets were measured. Fluorescein-labelled HPU bound to platelets but not to erythrocytes. HPU induced aggregation of rabbit platelets (ED₅₀ 0.28 μM) accompanied by ATP secretion. No correlation was found between platelet activation and ureolytic activity of HPU. Platelet aggregation was blocked by esculetin (12-lipoxygenase inhibitor) and enhanced ~3-fold by indomethacin (cyclooxygenase inhibitor). A metabolite of 12-lipoxygenase was produced by platelets exposed to HPU. Platelet responses to HPU did not involve platelet-activating factor, but required activation of verapamil-inhibitable calcium channels. Our data show that purified *H. pylori* urease activates blood platelets at submicromolar concentrations. This property seems to be common to ureases regardless of their source (plant or bacteria) or quaternary structure (single, di- or tri-chain proteins). These properties of HPU could play an important role in pathogenesis of gastrointestinal and associated cardiovascular diseases caused by *H. pylori*.

Keywords: *Helicobacter pylori* • urease • platelet activation • eicosanoids • lipoxygenase

Introduction

Ureases (EC 3.5.1.5) are highly homologous nickel-dependent enzymes widespread among plants, bacteria and fungi, that hydrolyse urea into ammonia and carbon dioxide [1, 2]. Plant and fungal ureases are homotrimers or hexamers of a ~90 kD subunit, while bacterial ureases are multimers of two or three subunits complexes [3–4]. The N-terminal halves of plant or fungal urease single chain align with the primary sequence of the small subunits of most bacterial enzymes (*e.g.* β and γ chains of *Bacillus pasteurii* urease or the A subunit of *Helicobacter pylori* urease). The

C-terminal portions of plant and fungal chains resemble the large subunits of bacterial ureases (*e.g.* α chain of *B. pasteurii* urease or the B subunit of *H. pylori* enzyme). Considering the similarity in their sequences, all ureases are likely to possess similar tertiary structures and catalytic mechanisms indicating they are variants of the same ancestral protein [2]. *H. pylori* urease (1E9Z) and jackbean (*Canavalia ensiformis*) major urease (P07374), share about 50% identity despite differences in their quaternary structures. The 3D crystallographic structures of three bacterial ureases were successfully resolved: *Klebsiella aerogenes* (1FWJ), *B. pasteurii* (4UBP) and *H. pylori* (1E9Z).

Urease activity enables bacteria to use urea as a sole nitrogen source [2, 5]. Some bacterial ureases play an important role in the pathogenesis of human and animal diseases such as those from *H. pylori* or *Proteus mirabilis*. *H. pylori*, a Gram-negative bacterium that colonizes the human stomach mucosa, causes gastric ulcers and gastric adenocarcinoma by mechanisms not completely understood [6, 7]. This bacterium produces factors that damage gastric

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epithelial cells, such as the vacuolating cytotoxin VacA, the cytotoxin-associated protein CagA, and a urease (up to 10% of bacterial protein) that neutralizes the acidic medium permitting 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 release cytokines. The biology of *H. pylori* and its involvement in stomach illness were recently reviewed [2, 8–10].

The physiological role of urease in plants is still largely unknown despite its ubiquity in virtually all plants [3, 4]. Jackbean and soybean ureases display fungicidal [11] and insecticidal activity, suggestive of a role in plant defence [12, 13]. The insecticidal activity is due to a ~10 kD internal peptide released from plant ureases upon digestion by insect cathepsins [14, 15].

We have previously reported that canatoxin [16], an isoform of jackbean (*C. ensiformis*) urease [17], presents biological properties that are independent of its enzyme activity, as binding to sialylated glycoconjugates, activation of blood platelets [18–20] and pro-inflammatory effect [21]. Submicromolar concentrations of canatoxin-induced exocytosis in a number of cell system *in vitro* including platelets, synaptosomes, pancreatic islets, macrophages, neutrophils and mast cells [19, 22]. Canatoxin also induced hypothermia, bradycardia, hypoglycaemia, hyperinsulinemia, hypoxia and paw oedema in rats and mice, preceding convulsions and death of the animals [23]. Canatoxin disrupted Ca^{2+} transport across membranes [20, 24] and lipoxygenase metabolites were shown to modulate most of its pharmacological effects [18, 19, 21] either *in vivo* or *in vitro*. More recently we reported that jackbean, soybean and *B. pasteurii* ureases are also able to induce aggregation of platelets at nanomolar concentrations independently of enzyme activity [13, 25].

Blood platelets are anucleated disc-shaped cells derived from megakaryocytes. Upon vascular injury or exposition to agonists such as adenosine diphosphate (ADP), collagen or thrombin, non-stimulated platelets become spherical (shape change) and adherent to each other and to surrounding tissues [26, 27]. Stimulated platelets may undergo release reaction, with exocytosis of α -granules and dense granules, whose contents contribute to haemostasis. Primary reversible platelet aggregation induced by direct agonists such as ADP, platelet-activating factor (PAF)-acether or thromboxane A₂ does not require the release reaction. When platelets secrete ADP it amplifies the aggregation response [26, 28]. Elevated intracellular levels of Ca^{2+} are necessary for platelet aggregation and secretion resulting from external Ca^{2+} influx through voltage-dependent channels, inhibition of Ca^{2+} ATPases and/or release of intracellular Ca^{2+} pools by the action of phosphatidylinositol-triphosphate [26–30].

Platelet membrane-bound phospholipase A₂ activated by agonist-coupled receptors hydrolyses phospholipids into free arachidonic acid, which serves as substrate for the synthesis of eicosanoids either resulting from the cyclooxygenase pathway, such as thromboxane A₂, or the lipoxygenase pathway, such as 12-hydroxyperoxy-eicosatetraenoic acid (12-HPETE), which in turn mediate platelet's response to the agonist [31, 32]. Platelets also synthesize PAF-acether (1-*o*-alkyl-2-acetyl-sn-glycero-3-phos-

phocholine) from arachidonic acid which interacts with its own receptors on platelets [33].

In the present work we characterized the platelet aggregating activity of a purified recombinant *H. pylori* urease (HPU), studied the pathways recruited by the protein to induce platelet activation and compared the data to that previously reported for the plant urease canatoxin and for *Bacillus pasteurii* urease.

Materials and methods

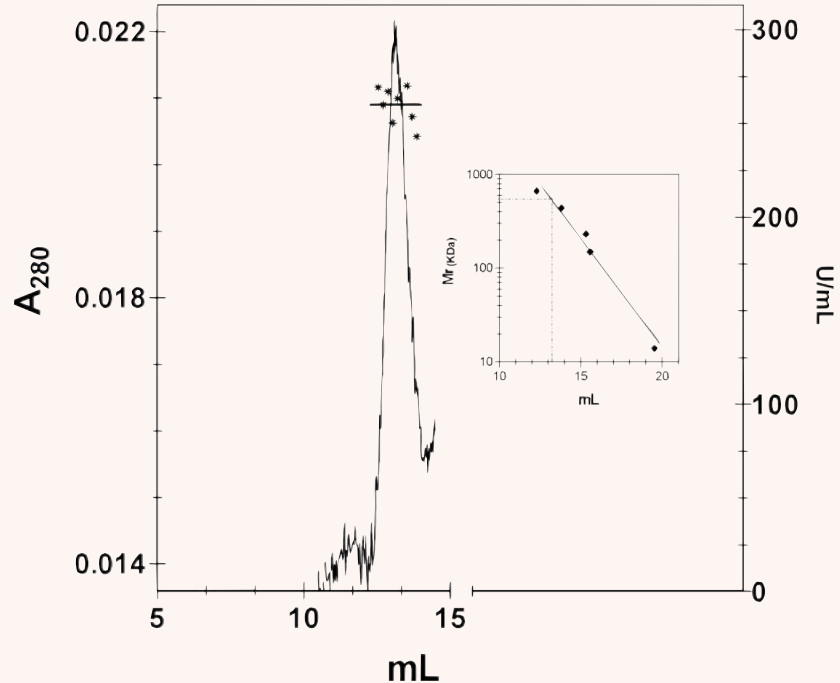
Materials

The following drugs were obtained from Sigma Chemical Co., St Louis, MO, USA: reagents for electrophoresis, ADP, potato apyrase (A-6535, a kind gift from Dr. Ana Maria O. Batastini, Department of Biochemistry, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil), esculetin, dexamethasone, indomethacin, bovine acid soluble collagen type I, luciferin and luciferase. PAF-acether (platelet-aggregating factor: 1-*o*-alkyl-2-acetyl-sn-glycero-phosphocholine) and Web 2170 (Bepafant; 5-(2-chloro-phenyl)-3,4-dihydro-10-methyl-3-[(4-morpholinyl) carbonyl]-2H, 7H-cyclopenta (4,5) thieno [3,2-*f*] [1,2,4 triazolo-[4,3-*a*] [1, 4] diazepine]) were a kind gift from Dr. João Baptista Calixto, Department of Pharmacology, Universidade Federal de Santa Catarina, Florianópolis, SC, Brazil. D-methoxy-verapamil (Verapamil hydrochloride) was from Sandoz Laboratory (Saluta Pharma GmbH, Germany). Solutions were prepared as follows: dexamethasone and esculetin were dissolved in absolute ethanol and diluted in saline to give final concentrations of ethanol in the platelet assay of no more than 0.2% v/v; indomethacin was first dissolved in 0.1 M Na_2CO_3 then diluted with saline and adjusted to pH 6.0; ADP was diluted in Tris buffer pH 8.2; PAF-acether was dissolved in 0.1 w/v% bovine serum albumin solution and used on the same day; Web 2170 and verapamil were dissolved in saline; apyrase was dissolved in phosphate buffered saline.

Helicobacter pylori recombinant urease

HPU was produced by heterologous expression in *Escherichia coli* SE5000 transformed with plasmid pHP8080 [34], kindly provided by Dr. Harry T Mobley, University of Michigan Medical School. HPU was purified from bacterial extracts as follows: after cultivation, cells were harvested by centrifugation, suspended in 20 mM sodium phosphate, pH 7.5 containing 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM 2-mercaptoethanol (extracting buffer, EB) and lysed using a Ultrasonic Homogenizer 4710, 10 pulses of 30 sec. in an ice bath. After centrifugation (20 min., 20,000 × *g*, 4°C), the supernatant was fractionated by ammonium sulphate precipitation. The precipitate formed between 0.3 and 0.7 saturation was dissolved in EB and dialysed to remove the excess of salt. This material was then submitted to anion exchange chromatography in Q-Sepharose (GE Healthcare, Uppsala, Sweden) at a ratio of 10 mg protein per 1 ml resin equilibrated in EB, pH 7.8. After removing the unbound proteins, the column was eluted stepwise and the urease-enriched fraction was recovered with EB containing 200 mM NaCl, pH 7.8. After dialysis to remove excess of salt and concentration on Centriprep (Millipore, Bedford, MA, USA) cartridges, the material was applied into a size exclusion Superose 6 HR column equilibrated in EB pH 7.8, mounted on a FPLC apparatus, at a flux of 0.3 ml/min. Figure 1 illustrates the elution pattern of purified HPU, with all fractions

Fig. 1 Purification of HPU. The figure shows the chromatographic pattern of HPU obtained in the last purification step consisting of a gel-filtration in a Superose 6 HR column. Fractions within the peak eluted at 13.2 ml all showed similar values of urease activity (asterisks), mean value of 264.4 ± 11.4 U/mg of protein, denoting the homogeneity and high purity of the enzyme. The inset shows the calibration curve of the Superose 6 HR column (Mr standard: tyreoglobulin 669 kD, ferritin 440 kD, catalase 232 kD, alcohol dehydrogenase 150 kD; cytochrome C 12.8 kD). A molecular mass of 540 kD was estimated for HPU.



within the peak showing similar specific activity with a mean value of 264.4 ± 11.4 U/mg of protein. SDS-PAGE of purified HPU showed two major bands of 60 and 30 kD (not shown). The fractions with urease activity were pooled and freeze-dried (in EB buffer). For the experiments, the freeze-dried protein was solubilized to give 0.5 mg protein/ml solution in 20 mM sodium phosphate, pH 7.5, containing 1 mM EDTA and 5 mM 2-mercaptoethanol.

For fluorescence microscopy experiments, HPU was labelled with fluorescein isothiocyanate (FITC, Sigma Aldrich, St Louis, MO, USA). HPU solutions (1 mg/ml) in EB buffer were incubated with 0.1% FITC for 2 hrs at 4°C. The mixture was exhaustively dialysed against EB buffer and then applied into a Fast-Desalting column (Amersham Biosciences, Uppsala, Sweden) to remove any unbound FITC.

Protein determination

The protein content of samples was determined by their absorbance at 280 nm or by the Coomassie dye binding method.

Urease activity

The ammonia released was measured colorimetrically by the alkaline nitroprussiate method [35]. One unit of urease releases one μ mol of ammonia per minute, at 37°C, pH 7.5.

Platelet aggregation

Platelet-rich plasma (PRP) was prepared from rabbit blood collected from the ear central artery in the presence of sodium citrate to a final concentration

of 0.313% (v/v). Blood samples were centrifuged at $200 \times g$ for 20 min. at room temperature, to give a PRP suspension. Platelet aggregation and shape change were monitored turbidimetrically as described [18], using a Lummi-Aggregometer (Chrono-Log Corporation, Havertown, PA, USA). Aggregation resulted in an increase in light transmission across PRP, registered on a chart recorder for 3 min. Platelet aggregation assays were also performed on a SpectraMax microplate reader (Molecular Devices, Sunnyvale, CA, USA) as described [36]. Briefly, urease samples in 96-wells flat-bottomed plates were completed to a final volume of 50 μ l with saline. Aggregation was triggered by the addition of 100 μ l of platelet suspension. The plate was incubated for 2 min. at 37°C before beginning of agitation and readings were performed at 650 nm every 11 sec., during 20 min. When testing potential inhibitors, platelets and the compounds were pre-incubated in the microplate wells for 2 min. at 37°C under stirring, or 10 min. at room temperature without stirring in the case of apyrase, and aggregation was triggered by addition of HPU or control inducer (ADP). Change in turbidity was measured in absorbance units, and results are expressed as area under the aggregation curves.

Fluorescence and scanning electron microscopy

Sample preparation for scanning electron microscopy was done using PRP samples pre-warmed at 37°C and then exposed to saline or HPU under low stirring for 2 min. Platelets were then fixed by adding glutaraldehyde in 0.1 M sodium cacodylate pH 7.2 to a final 2.5% concentration and incubated overnight at 4°C. The samples were washed twice for 30 min. in 0.1 M sodium cacodylate and filtered on 0.4 μ m polycarbonate membranes (Millipore). The fixed platelets were sequentially dehydrated in 30%, 50%, 70% and 90% (v/v) acetone, for 5 min. each,

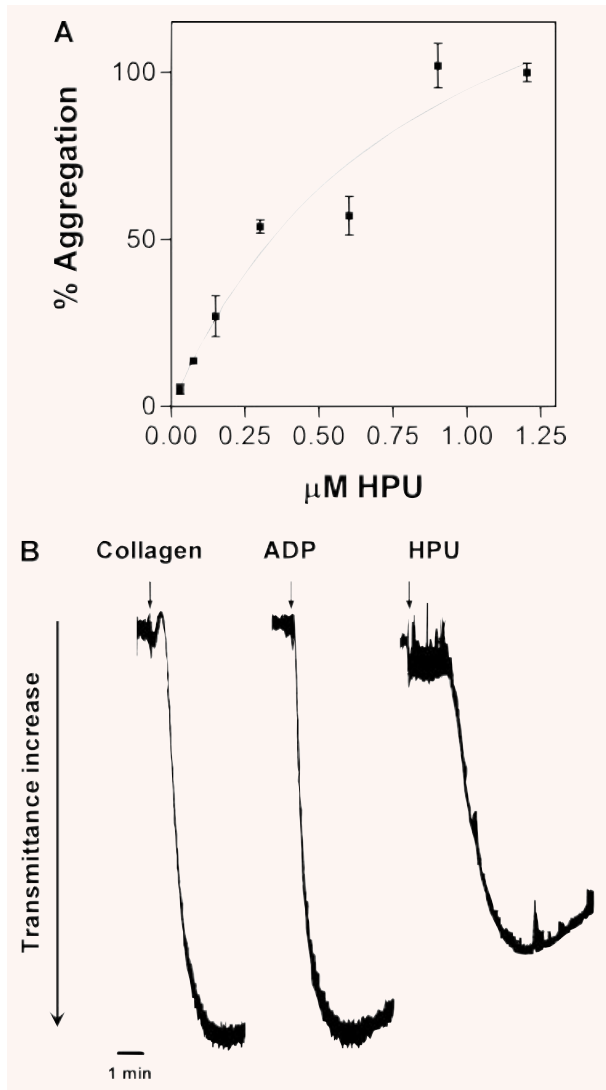


Fig. 2 Aggregation of rabbit PRP suspensions induced by purified HPU. (A) Rabbit platelet-rich plasma suspension in microwell plates were exposed to increasing concentrations of HPU or 5 μM ADP (100% aggregation). Aggregation of platelets was monitored every 11 sec. during 20 min. in a SpectraMax plate reader. Results (means \pm S.D.) are expressed as percentage of maximal aggregation for four replicates. (B) Comparison of aggregation tracings of platelets stimulated by collagen (30 $\mu\text{g}/\text{ml}$), ADP (5 μM) or HPU (0.3 μM). The arrows indicate addition of the agonists to the platelet suspension.

and finally twice in 100% acetone for 10 min. Critical-point drying and gold coating treatments were performed at our University's Center of Electron Microscopy (CEM-UFRGS, Brazil). Specimens were visualized in a JEOL-JSM 6060 scanning electron microscope with automated image digitalization and archiving.

For fluorescence microscopy rabbit PRP was incubated with 300 nM FITC-labelled HPU under vortex stirring for 5 min. at room temperature. PRP without exposition to HPU was submitted to the same stirring and filtering process and used as negative control. Platelet aggregates were recovered by centrifugation; the pellets were smeared on glass slides and observed under a Zeiss-Axiovert-200 (Carl Zeiss, Jena, Germany) fluorescence microscopy.

Platelet secretion

ATP release from PRP suspension was measured as a change in bioluminescence in the presence of the Chromolume[®] reagent containing a luciferin-luciferase mixture (Chrono-Log Corporation) according to manufacturer instructions.

12-HETE detection

The method described by Coffey and co-authors [37] was applied to investigate the activation of 12-lipoxygenase in HPU-aggregated platelets. Rabbit platelet rich-plasma suspensions (2 ml) were exposed to 300 nM HPU for 2 min. at room temperature under stirring. Platelet aggregates were harvested by centrifugation ($900 \times g$, 15 min.), washed three times in cold phosphate buffered saline and then lysed by suspending in 200 μl of cold 100% methanol, followed by centrifugation at $10,000 \times g$ for 10 min. The supernatant was collected and applied into a C-18 column (CLC-ODS(M), Shimadzu, Kyoto, Japan) mounted in a high-performance liquid chromatography system (Shimadzu). The column was previously equilibrated in 50% of solvent A (water-acetonitrile-acetic acid; 75:25:0.1 proportion) and 50% of solvent B (methanol-acetonitrile-acetic acid; 60:40:0.1 proportion). Elution consisted of a 50% to 90% gradient of solvent B in 20 min., at a flow rate of 1 ml/min., monitored at 235 nm. Authentic 12-hydroxideicosatetraenoic acid (12-HETE; Cayman Biochemicals, Ann Arbor, MI, USA) was dissolved in 100% methanol and submitted to chromatography under the same conditions.

Statistical analysis

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

Results

Purified *H. pylori* urease activates platelets

Figure 2 illustrates the pattern of aggregation of platelet-rich rabbit platelet suspensions triggered by purified *H. pylori* urease (HPU) and two physiological agonists, ADP and collagen. HPU induced aggregation of rabbit platelets with an ED₅₀ of ca. 150 $\mu\text{g}/\text{ml}$ (0.28 μM), with a time course and collagen-type shape change reaction very similar to those induced by canatoxin or the major jackbean urease (ED₅₀ 15.8 $\mu\text{g}/\text{ml}$) [13, 17, 18]. Scanning electron microscopy of platelets exposed to HPU

Fig. 3 Scanning electronic, light and fluorescence microscopy of platelets treated with HPU. **(A)** Scanning electronic microscopy of a single rabbit platelet after exposition to 0.3 μ M HPU for 2 min. under low stirring (to avoid aggregation). The morphology is typical of an activated platelet showing multiple pseudopodia. **(B)** Scanning electronic microscopy of a resting platelet. The white bars in **(A)** and **(B)** correspond to 2 μ m. In **(C)** and **(D)** rabbit platelets were aggregated by 0.3 μ M fluorescein-labelled HPU and aggregates (arrows) were observed under light **(B)** and fluorescence microscopy **(C)** at 400 \times magnification. Note that the few erythrocytes (stars) present in C do not appear as fluorescein-labelled in **(D)**.

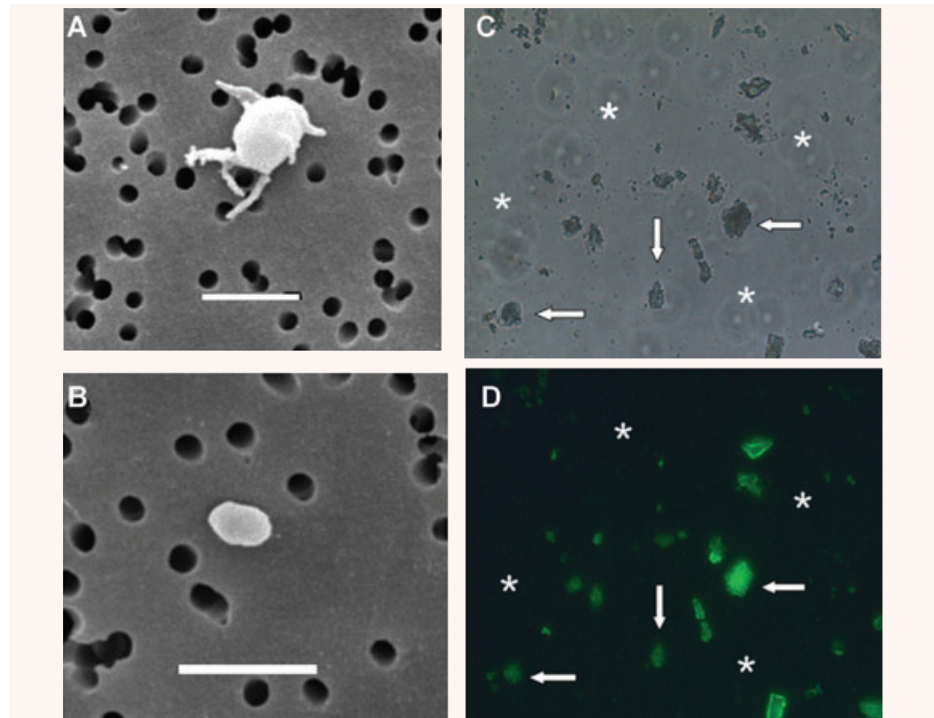
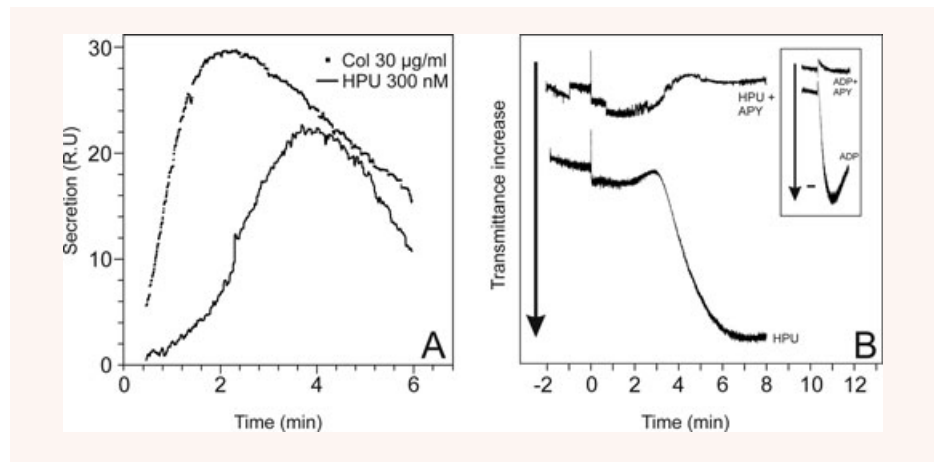


Fig. 4 HPU induces release reaction and ADP-induced aggregation of rabbit platelets. **(A)** Time course of release reaction of platelets activated by HPU (300 nM) or collagen (30 μ g/ml) was detected following ATP secretion as light emitted in the presence of a luciferin-luciferase mixture. L.R.U. – light relative units. A typical result out of three replicates is shown. **(B)** Platelet aggregation induced by HPU (300 nM) or 5 μ M ADP (inset) is completely abolished in the presence of 1 U/ml of potato apyrase. Platelets were pre-incubated with apyrase for 10 min. at room temperature without stirring, and then aggregation was triggered by addition of HPU or ADP (time zero) and aggregation was monitored turbidimetrically for 8 min. Typical results are shown.



showed a typical morphology of activated platelets with numerous pseudopodia (Fig. 3A). The cells clumped together (Fig. 3C) with no evidence of cell lysis and fluorescein-conjugated *H. pylori* urease were seen bound to platelets aggregates but not to erythrocytes (Fig. 3D).

Figure 4(A) shows that platelets stimulated by HPU undergo release reaction and secrete ATP from dense granules. Platelets

stimulated by 0.3 μ M HPU secreted about 60% of the ATP measured for a collagen-induced release reaction, with a slower kinetics and longer lag phase, peaking after 3 min. As previously described for canatoxin [18] and *B. pasteurii* urease [25] HPU-induced platelet aggregation is completely dependent on this secreted ADP and therefore inhibited by the scavenger activity of apyrase (Fig. 4B).

Table 1 Effect of inhibitors of phospholipase A2 and arachidonic acid metabolism on *H. pylori* urease-induced platelet aggregation

Platelet pre-treatment	% platelet aggregation mean \pm S.D. (N)
None	100.00 \pm 10.24 (4)
Dexamethasone (50 μ M)	62.64* \pm 6.06 (4)
Esculetin (500 μ M)	55.00** \pm 6.06 (4)
Indomethacin (150 μ M)	160.74*** \pm 12.74 (4)
(300 μ M)	313.26*** \pm 3.78 (4)

Rabbit PRP suspensions in microwell plates were incubated for 2 min. at room temperature in the absence or in the presence of the indicated concentrations of the drugs and aggregation was triggered by addition of HPU (0.3 μ M). Aggregation of platelets was monitored ever 11 sec. during 20 min. in a SpectraMax plate reader.

Values of $P < 0.05^*$, $P < 0.01^{**}$ or $P < 0.001^{***}$ were considered statistically significant.

Involvement of 12-lipoxygenase in HPU-induced platelet activation

To elucidate the pathway(s) recruited by HPU to induce platelet aggregation we investigated the involvement of arachidonic acid metabolites in platelets pre-treated with dexamethasone (a phospholipase A₂ inhibitor), or indomethacin (a cyclooxygenase inhibitor) or esculetin (a 12-lipoxygenase inhibitor). Table 1 shows that dexamethasone treatment reduced HPU-induced aggregation indicating a requirement of free arachidonic acid. In indomethacin-treated platelets, HPU-induced aggregation was augmented up to 3-fold, excluding the participation of thromboxane A₂, an indirect product of cyclooxygenase activity in the aggregation response. On the other hand HPU-induced aggregation was reduced in esculetin pre-treated platelets, indicating that product(s) of the 12-lipoxygenase, which is inhibited by this compound [38], mediated the platelet's response to the protein (Table 1 and Fig. 5A). Likewise the observed enhancement of HPU-induced aggregation in indomethacin-treated platelets reflects the increased availability of arachidonic acid as substrate for the 12-lipoxygenase. HPU-activated platelets produced 12-HPETE, which could be measured as 12-hydroxy-eicosatetraenoic acid (12-HETE) in supernatants of lysed platelets (Fig. 5B). Thus, similar to what we described previously for canatoxin [18, 19], and *Bacillus pasteurii* urease [25], platelet aggregation induced by *H. pylori* urease is also mediated by lipoxygenase-derived eicosanoids.

Similar to what was previously seen for platelets aggregated by canatoxin (jackbean) or *B. pasteurii* urease, HPU also does not depend on PAF-acether synthesis. Table 2 shows that 2 μ M Web2170 inhibited only 16% the aggregation induced by HPU, while it blocked almost 90% of platelets response to a very high

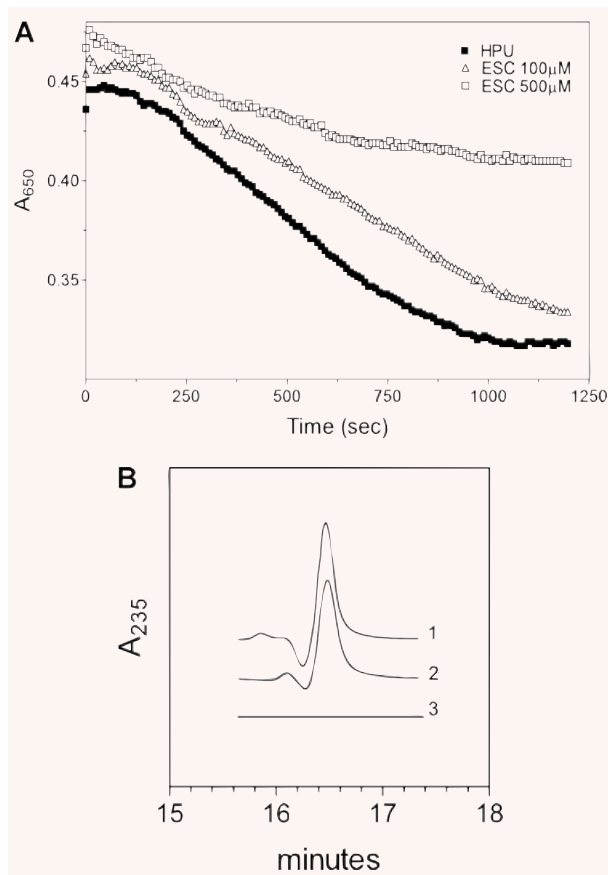


Fig. 5 Involvement of lipoxygenase-derived metabolite(s) in HPU-induced platelet aggregation. **(A)** Rabbit platelet-rich plasma suspensions in microwell plates were incubated for 2 min. at room temperature in the absence or in the presence of the indicated concentrations of esculetin and aggregation was triggered by addition of HPU (0.3 μ M). Aggregation of platelets was monitored as decrease in absorbance at 650 nm in a SpectraMax plate reader. A typical result out of four replicates is shown. **(B)** Fully aggregated platelets induced by 0.3 μ M HPU were lysed in 100% methanol and the supernatant was analysed by reverse-phase chromatography according to Coffey *et al.* [37]. Tracing 1 corresponds to a product isolated from HPU-activated platelets while the elution pattern of authentic standard 12-HETE (~200 ng) is shown in tracing 2, both peaks eluting at approximately at 16.5 min. Tracing 3 is the pattern of elution obtained for supernatant of non-activated platelets.

non-physiological concentration of PAF-acether. Thus, although statistically significant this inhibition is probably biologically irrelevant, reflecting secondary reactions of HPU-activated platelets. On the other hand, the response of platelets to HPU involved activation of voltage-dependent calcium channels as demonstrated by the inhibition caused by D-methoxy-verapamil (Table 2).

Table 2 Effect of a calcium channel blocker and of a PAF-acether antagonist on *H. pylori* urease-induced platelet aggregation

Platelet treatment	% platelet aggregation mean \pm S.D. (N)
0.3 μ M HPU	100.00 \pm 7.43 (4)
50 μ M Verapamil + HPU	80.83* \pm 1.58 (4)
75 μ M Verapamil + HPU	63.35*** \pm 1.19 (4)
2 μ M Web2170 + HPU	83.94* \pm 6.45 (4)
50 nM PAF-acether	100.00 \pm 3.21 (4)
2 μ M Web2170 + PAF acether	13.1*** \pm 1.62 (4)

Rabbit platelet-rich plasma suspensions in microwell plates were incubated for 2 min. at room temperature in the absence or in the presence of the indicated concentrations of the calcium channel blocker D-methoxy-verapamil or the PAF-acether antagonist Web2170. Aggregation was triggered by addition of HPU (0.3 μ M) or PAF-acether (50 nM) and monitored ever 11 sec. during 20 min. in a SpectraMax plate reader. Results are means \pm S.D. of four replicates of each condition.

Values of $P < 0.05^*$ or $P < 0.001^{***}$ were considered statistically significant.

Discussion

We previously reported on the platelet aggregating activity of jackbean [17, 18] and soybean [13] single chain ureases and of the tri-chain urease from *Bacillus pasteurii* [13]. The jackbean urease isoform canatoxin was shown to induce aggregation of rat, rabbit, guinea pig and human platelets, either in the presence or absence of plasma proteins, but it was inactive upon degranulated platelets [17]. Here we reported the same activity for the two-chain *H. pylori* urease. Thus, independent of their quaternary structures, the property of activating blood platelets inducing aggregation and release reaction (a model for exocytosis) is a common feature of bacterial and plant ureases (Table 3). The fact that bacterial and plant ureases conserved the property of inducing exocytosis in some cell types may shed new light into the so far poorly understood biological functions of these proteins.

Treatment of jackbean ureases [17] with the irreversible inhibitor *p*-hydroxy-mercury-benzoate abolished their ureolytic activity but did not affect their ability to induce platelet aggregation, clearly demonstrating that these two biological activities are not related. Platelet aggregation inducing property of the *B. pasteurii* urease did not correlate with the protein's enzyme activity [17], as

Table 3 Comparative data on physicochemical and biological properties of the isoform of jackbean urease canatoxin (CNTX), *B. pasteurii* urease (BPU) and a HPU

Physicochemical properties	CNTX*	BPU [†]	HPU [‡]
Molecular mass:			
SDS-PAGE	95 kD	11–13–61 kD (chains A, B and C, respectively)	30–62 kD (chains A and B, respectively)
Native form	Dimer	Trimer	Hexamer
Urease activity (U/ mg protein)	11.6	194.0	264.4
Binding to polysialogangliosides	Yes	ND	Yes
Biological properties			
Toxicity to:			
Mouse, i.p.	Toxic	Not toxic	ND
Treated with pHMB	100% active		
<i>D. peruvianus</i> , LD50	0.01% (w/w)	Not toxic	ND
Treated with pHMB	100% active		
Platelet aggregation, EC ₅₀ (rabbit)	22.2 μ g/ml	400 μ g/ml	150 μ g/ml
Treated with pHMB	100% active	ND	ND
Platelet secretion	Yes (serotonine)	ND	Yes (ATP)
Lipoxygenase inhibitors	Inhibition	Inhibition	Inhibition
Cyclooxygenase inhibitors	Potentialiation	Potentialiation	Potentialiation
ADP antagonists/scavengers	Inhibition	Inhibition	Inhibition
PAF-acether antagonists	No effect	No effect	No effect
Ca ²⁺ channel blocker	Inhibition	Inhibition	Inhibition

ND: not determined.

*[13, 17, 18].

[†][13, 25].

[‡][2], this paper.

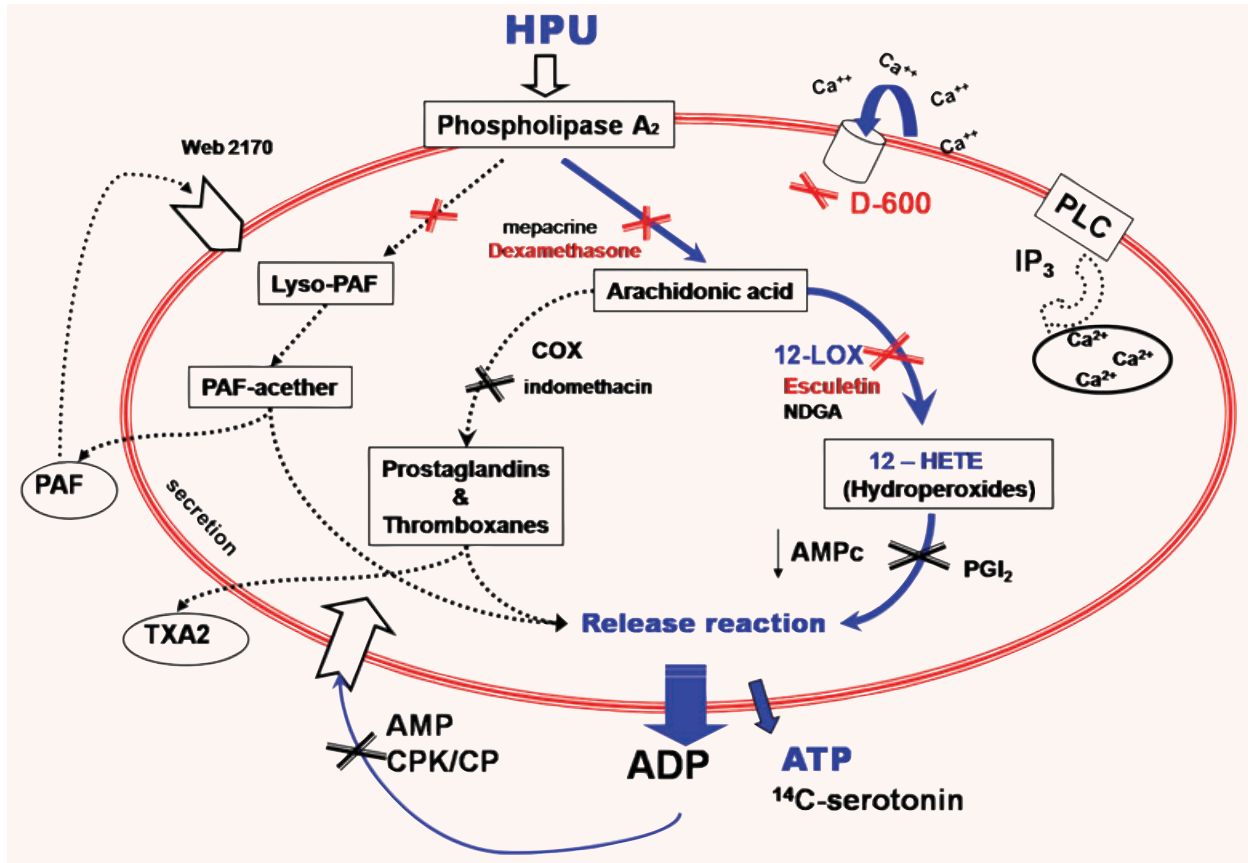


Fig. 6 Proposed mechanism of platelet aggregation induced by *Helicobacter pylori* urease. The biochemical pathways that underlie the platelet-aggregating activity of HPU as well as all ureases studied so far [13, 17–20, 25] are indicated as continuous lines. As depicted in blue, HPU activates platelets through a phospholipase A₂ and calcium-dependent pathway that makes arachidonic acid available for the 12-lipoxygenase enzyme and leads to secretion of platelet's dense granules. The released ADP then triggers aggregation of platelets. Dotted lines indicate other pathways tested for HPU or other ureases that are not relevant to platelet aggregation as induced by ureases. Inhibition sites of pathways are marked by (X). Modified from [25].

shown here also to be the case for HPU (Table 3). Platelet aggregation induced by all three ureases is consequent to the release of ADP from dense granules, stored together with serotonin [18, 19] and ATP (this work) which can be thereafter detected in the medium. As expected, HPU-induced aggregation was completely abolished in the presence of apyrase. PAF-acether is not relevant for urease-induced platelet aggregation [18] (this work). Platelet activation by ureases requires influx of external calcium through voltage-gated Ca²⁺ channels inhibited by verapamil [18] (this work), and occurs without activation of phospholipase C or production of IP₃ [20]. The responses of platelets to all three ureases (jackbean, *B. pasteurii* or *H. pylori*) were inhibited by different inhibitors of the endogenous phospholipase A₂ and of the platelet 12-lipoxygenase and metabolite(s) of this enzyme were produced by HPU-activated platelets. In agreement with an activation induced by lipoxygenase metabolites, indomethacin pre-treated platelets, in which more arachidonic acid is available as substrate for the

lipoxygenases, showed significantly enhanced reactivity to ureases [18, 20] (this work). This same increase of platelet reactivity following indomethacin treatment was seen in platelets activated by *Chlamydia pneumonia*, a human respiratory pathogen linked to cardiovascular disease [39]. Figure 6 summarizes our present knowledge on the signalling pathway recruited by ureases to induce platelet aggregation and release reaction.

In some aspects, urease induced-platelet aggregation resembles collagen-activated platelets. As demonstrated by Coffey and coworkers, collagen interaction with its platelet receptor, glycoprotein VI, results in activation of platelet 12-lipoxygenase with 12-H(P)ETE synthesis [37, 40]. Interestingly, we previously observed that canatoxin-stimulated platelets become refractory to a second exposure to this protein or to collagen, but are still responsive to ADP, PAF-acether or arachidonic acid [18], suggesting that ureases and collagen may be recruiting the same signalling cascade to exert their actions.

It is well known that platelets participate in the inflammatory response by modulating the activity of other inflammatory cells and ischemic lesions due to vascular insufficiency may lead to ulcers within the gastric mucosa [41]. Fluorescent *in vivo* microscopy studies have shown that *H. pylori* infection alters blood flow, the endothelial lining of the vessels, leucocyte activity and often induces the formation of circulating or adherent platelet aggregates [42–46], consistent with epidemiological studies that suggest a possible association between *H. pylori* infection and the incidence of cardiovascular diseases [43]. *H. pylori* aqueous extracts (which contain HPU) were shown to aggregate platelets [46]. Through a mechanism very different from the one we describe here for purified HPU, whole cell *H. pylori* also promotes platelet aggregation binding directly to von Willebrand factor and to platelet glycoprotein GPIb [47].

To our knowledge this is the first study demonstrating a direct effect of purified *H. pylori* urease on platelets. A recent report [48] showed that an active *H. pylori* urease is pivotal to the gastric epithelial barrier dysfunction that underlies inflammation leading to tissue damage. This mechanism could also lead to liberation of HPU into the vascular bed, where it would directly stimulate platelets.

H. pylori urease is a cytoplasmic enzyme. Upon bacterial lysis urease is released and adsorbed onto the extracellular surface of viable bacteria where it represents about 30% of the total cell urease content [49]. The cell wall bound enzyme facing the external

acidic medium is enzymatically inactive and the distribution of urease within the bacterium is dependent on external pH [45, 46]. It is not known whether cell wall bound urease *H. pylori* displays any biological activity. On the other hand, purified *H. pylori* urease has been shown to bind mucin and other glycoconjugates [50] and to contribute directly to the gastric inflammatory process by stimulating macrophages and cytokine production [41, 49, 51, 52]. An important aspect to be investigated is whether or not these other biological activities of *H. pylori* urease depend on its ureolytic activity. Moreover, our finding of a direct platelet-activating activity of *H. pylori* urease and the modulation of its platelet-activating properties by lipoxigenase-derived eicosanoids could be particularly relevant to the elucidation of mechanisms leading to the gastrointestinal and associated cardiovascular diseases caused by this bacterium and may have to be taken into consideration in the development of more efficient therapeutic approaches.

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