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Urease de *Helicobacter pylori*: ativação de plaquetas e neutrófilos

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Resumo

Ureases (3.5.1.5), enzimas níquel dependentes que catalisam a reação de hidrólise da uréia em amônia e dióxido de carbono, apresentam ampla distribuição em plantas, fungos e bactérias. A espiroqueta *Helicobacter pylori* causa úlceras pépticas e câncer gástrico por mecanismos ainda não totalmente conhecidos. *H. pylori* produz grande quantidade de urease, que neutraliza o meio ácido e permite sua sobrevivência no estômago. Nosso grupo demonstrou que as ureases de *Canavalia ensiformis*, soja e *Bacillus pasteurii* induzem agregação plaquetária independentemente de sua atividade ureolítica, por uma rota que requer ativação de canais de cálcio. ativação da rota do ácido araquidônico e secreção plaquetária. Estudos prévios mostraram ainda que a canatoxina, uma isoforma da urease de *C.ensiformis*, possui atividade pró-inflamatória, induzindo edema de pata em ratos. Neste trabalho, caracterizamos as propriedades ativadora de plaquetas e pró-inflamatória da urease recombinante de *H. pylori* (HPU). Em plaquetas, estudamos as vias recrutadas pela proteína na agregação plaquetária e comparamos com dados prévios para a canatoxina e a urease de *Bacillus pasteurii*. Em neutrófilos, demonstramos que a HPU, em doses nanomolares, induz quimiotaxia e produção de espécie reativas de oxigênio. A taxa de apoptose de neutrófilos ativados por HPU foi inibida, acompanhando alterações dos níveis de proteínas pró- e anti-apoptóticas. Por último, mostramos que a resposta dos neutrófilos a HPU envolve aumento dos níveis de lipoxigenase(s), sem, contudo, haver alterações das ciclo-oxigenase(s). Concluímos que as propriedades não enzimáticas aqui descritas para a HPU podem potencialmente contribuir para o processo inflamatório promovido por *H. pylori.*

Abstract

Ureases (EC 3.5.1.5), nickel-dependent enzymes that hydrolyze urea into ammonia and carbon dioxide, are widespread among plants, bacteria and fungi. The spirochete *Helicobacter pylori* is the etiological agent of gastric ulcers and gastric adenocarcinoma by mechanisms not yet fully understood. *H. pylori* produces high amounts of urease, which enables the bacterium to survive in the acidic medium of the stomach. We have previously reported that ureases from jackbean, soybean or *Bacillus pasteurii* induce blood platelet aggregation independently of their enzyme activity by a pathway requiring activation of calcium channels, lipoxigenase-derived eicosanoids and platelet secretion. We also showed that canatoxin, an isoform of *C. ensiformis* urease, presents pro-inflammatory property demonstrated by rat paw oedema. In this work we characterized the platelet aggregating and pro-inflammatory properties of the recombinant *H. pylori* urease (HPU). In platelets we studied the pathways recruited by the protein to induce platelet aggregation and compared the data to those previously reported for the plant urease canatoxin and for *Bacillus pasteurii* urease. Using neutrophils we demonstrated that nanomolar doses of HPU induce chemotaxis and production of oxygen reactive species in human neutrophils. The rate of apoptosis was decreased in HPU-treated neutrophils, accompanied by alterations in the levels of pro- and anti-apoptotic proteins. Moreover, we showed that the response of neutrophils to HPU requires increased levels of lipoxygenase(s) with no alterations of cyclo-oxygenase(s). We concluded that the non-enzymatic properties of HPU here described potentially contribute to the inflammatory process that underlies *H. pylori* infection.

1. Introdução

1.1. Estrutura e função das Ureases

Ureases (uréia amidohidrolase; EC 3.5.1.5) são enzimas níquel dependentes (Dixon *et al,* 1975) que catalisam a hidrólise de uréia a amônia e dióxido de carbono. As ureases de fungos e vegetais possuem unidades funcionais compostas por uma única cadeia polipeptídica com aproximadamente 90kDa. Já as ureases bacterianas possuem unidades funcionais compostas por duas ou três cadeias polipeptídicas diferentes, que são homólogas às cadeias únicas das proteínas vegetais ou fúngicas (Mobley, 1995; Sirko & Brodzik, 2000). A figura 1 ilustra as diferenças entre ureases vegetais e bacterianas, quanto às suas estruturas quaternárias.

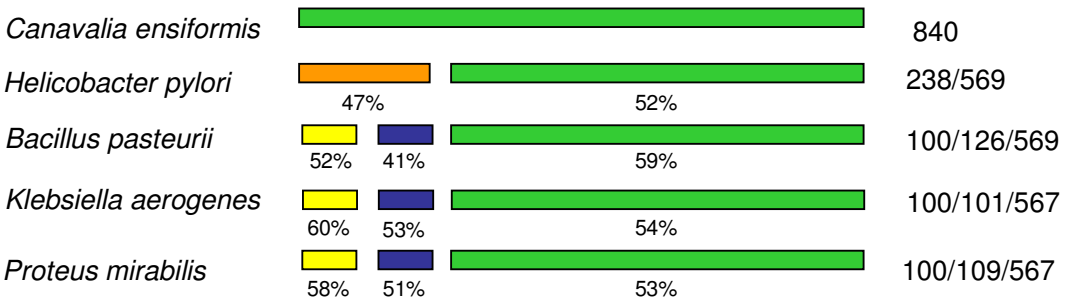

Figura 1. Estrutura das ureases**:** Ureases vegetais, como a de *Canavalia ensiformis*, possuem apenas um tipo de subunidade, enquanto que as ureases bacterianas possuem dois (*H. pylori*) ou três (*K. aerogenes; P. mirabilis; B. pasteurii*) de cadeias polipeptídicas formando suas "unidades funcionais". O número de aminoácidos de cada subunidade está indicado ao lado direito. A percentagem de identidade em relação à região correspondente da urease de *C. ensiformis* está indicada abaixo das barras.

Em seu estado nativo, as ureases são trímeros ou hexâmeros de suas unidades funcionais, formando complexos do tipo α_6, no caso das ureases vegetais e fúngicas, e oligômeros do tipo $[(\alpha\beta)_6]_2$ ou $(\alpha\beta\chi)_6$, no caso de ureases bacterianas.

Em bactérias, estas enzimas estão envolvidas em vários processos patogênicos, como, por exemplo, em casos de infecção por *Proteus mirabilis*, na formação de cálculos urinários, incrustação de catéter, pielonefrites, e inclusive coma hepático.

Em plantas, essas enzimas estão amplamente distribuídas, no entanto pouco se conhece sobre seu papel fisiológico. Uma possível função para urease em plantas superiores seria a biodisponibilização de nitrogênio (Polacco *&* Holland, 1993). Constatou-se que, em plantas e culturas de tecidos vegetais desprovidos de urease, quer induzidos geneticamente, na presença de inibidores de urease, ou por remoção do níquel, observa-se um acúmulo de uréia ou um comprometimento do emprego de uréia como fonte de nitrogênio (Polacco *&* Holland, 1993). O fato de a uréia ser uma forma de excreção de nitrogênio apenas em animais, ou seja, não é um metabólito majoritário nos vegetais onde esta enzima é abundante, constitui um entrave na argumentação de que a urease tenha como função principal a biodisponibilização de nitrogênio.

Com a descoberta de duas isoenzimas de urease na soja (*Glycine max*) (Polacco *&* Holland, 1984), a urease ubíqua da soja, presente em todos os tecidos da planta, e uma urease embrião-específica, encontrada na semente madura, onde apresenta atividade ureolítica 1000 vezes maior que a ubíqua, surgiram algumas dúvidas a respeito da função dessas enzimas nas plantas (Polacco *&* Holland, 1984). Como a perda da urease embrião-específica não acarreta danos visíveis na planta, acredita-se que esta enzima não desempenha função fisiológica ligada ao metabolismo de nitrogênio na planta. O fato do embrião em desenvolvimento produzir altas quantidades

de uma enzima que praticamente não tem contato com o seu substrato, sugere que esta urease esteja envolvida em algum outro tipo de função, como por exemplo, a defesa da planta (Polacco & Holland, 1993).

1.2. Ureases como toxinas protéicas

A canatoxina é uma proteína tóxica encontrada nas sementes de *Canavalia ensiformis*, letal para ratos e camundongos por via intraperitoneal, mas inócua se administrada oralmente (Carlini & Guimarães, 1981). Essa toxina possui também atividade inseticida (Carlini *et al*., 1997; Ferreira-DaSilva *et al*., 2000; Stanisçuaski *et al,* 2005), e fungicida (Becker-Ritt *et al.*, 2007), o que reforça a hipótese de que as ureases estariam envolvidas nos mecanismos de defesa das plantas.

Vários peptídeos internos da canatoxina já foram sequenciados, obtidos por hidrólise tríptica ou por endoproteinase Lys-C, sendo que todos eles revelaram um alto grau de homologia com a sequência primária de urease da *C. ensiformis*. A composição percentual de aminoácidos é indicativa de uma grande semelhança das duas proteínas. A partir dessa evidência, a canatoxina foi caracterizada como uma variante da urease de *C. ensiformis*, apresentando-se em sua forma nativa como um dímero não covalente de cadeias de 95 kDa (Follmer *et al* 2001). As duas isoformas de urease podem ser separadas cromatograficamente, sendo que a canatoxina apresenta maior avidez por metais (Zn^{++} e Co^{++}) em cromatografia de afinidade em metal imobilizado, o que permitiu o estabelecimento de protocolos de purificação para a obtenção das isoformas altamente purificadas (Follmer *et al* 2004).

Apesar da alta homologia, a canatoxina purificada apresenta apenas 30-40% da atividade ureolítica da urease de *C. ensiformis*. Postula-se que essas ureases possuem domínios protéicos distintos, os quais são responsáveis por atividades biológicas diferentes: um domínio com atividade hidrolítica sobre uréia, suscetível de inibição por agentes quelantes e oxidantes; e pelo menos mais outro domínio, níquel e tiol independentes, que seria responsável pelos seus outros efeitos biológicos (Follmer *et al*, 2001; Follmer *et al*, 2004a).

1.3. Efeitos biológicos da canatoxina

Estudos anteriores do nosso grupo mostraram que a canatoxina apresenta uma série de efeitos biológicos que parecem estar relacionados com a capacidade da proteína em ativar os sistemas secretórios de diversos tipos celulares. Tal efeito secretagogo da canatoxina envolve mediação por metabólitos do ácido araquidônico via lipoxigenases. A tabela 1 expõe alguns dos efeitos descritos para canatoxina.

A canatoxina, quando administrada intraperitonealmente em ratos e camundongos (DL_{50} de 0.4-0.6 e 2-3 mg/kg respectivamente), induz alterações respiratórias, convulsões e morte (Carlini & Guimarães, 1981; Carlini *et al*, 1984). Em doses subconvulsivantes, a canatoxina promove um aumento dos níveis plasmáticos de gonadotrofinas (Ribeiro-daSilva *et al*., 1989), de insulina, de modo dose e sexo dependente em ratos (Ribeiro-daSilva & Prado, 1993), e apresenta também efeitos pró-inflamatórios em ratos, tanto em modelos in vivo como ex-vivo (Benjamin *et al*., 1992; Barja-Fidalgo *et al*., 1992).

TABELA 1. Efeito secretagogo da canat oxina: modulação por inibidores de lipoxigenase .

MODELO	EFEITO	DE_{50}	INIBIDOR	DOSE	INIBIÇÃO	Ref
Plaquetas, coelho	agregação	300 nM	NDGA	520 µM	50	(a)
			ETYA	19 µM	50	
			BW755C	50 µM	50	
	secreção: serotonina	300 nM	NDGA	500 µM	75	(b)
			Esculetina	100 µM	87	
Sinaptossomas, rato	secreção: serotonina	500 nM	NDGA	200 µM	90	(b)
			Esculetina	100 µM	90	
	secreção: dopamina	2 µM	NDGA	200 µM	42	
Ilhotas pancreáticas, rato	secreção de insulina	500 nM	NDGA	200 µM	76	(b,c)
			Esculetina	100 µM	36	
Mastócitos: rato	secreção de histamina	500 nM	não testado			(d)
macrófagos, camundongo	secreção: enzimas	200 nM	NDGA	150 µM	não inibe	(e)
Rato, *in vivo*	hipoglicemia	0,4 mg/Kg	NDGA	125 mg/Kg	100	(f)
			Esculetina	125 mg/Kg	100	
Rato, *in vivo*	hiperinsulinemia	0,4 mg/Kg	NDGA	125 mg/Kg	100	(g)
Rato, *in vivo*	hipoxia	0,4 mg/Kg	NDGA	125 mg/Kg	72	(h)
			Esculetina	125 mg/Kg	50	
Rato, *in vivo*	Edema de pata	0,4 mg/Kg	NDGA	125 mg/Kg	66	(i)
			Esculetina	125 mg/Kg	50	
Rato, *in vivo*	convulsões	0,4 mg/Kg	NDGA	125 mg/Kg	75	(h)

(a)(Carlini *et al* ., 1985); (b) (Barja-Fidalgo *et al* ., 1991a); (c) (Barja-Fidalgo *et al* ., 1991b); (d) (Grassi-Kassisse & Ribeiro-daSilva, 1992) ; (e) (Ghazaleh *et al*., 1992); (f) (Ribeiro-daSilva *et al* ., 1986); (g) (Ribeiro-daSilva & Prado, 1993) ; (h) (Ribeiro-daSilva *et al* ., 1992); (i) (Benjamin *et al*., 1992; Ribeiro-daSilva *et al*., 1992).

Em ensaios *in vitro*, a canatoxina apresenta uma potente atividade secretagoga quando administrada em doses nanomolares em diversos tipos de células, induzindo secreção de grânulos plaquetários e agregação plaquetária (Carlini *et al*, 1985), secreção de dopamina e serotonina em sinaptosomas de cérebro total de rato (Barja-Fidalgo *et al*., 1991b), liberação de histamina em mastócitos (Grassi-Kassisse & Ribeiro-DaSilva, 1992), secreção de insulina em ilhotas pancreáticas isoladas (Barja-Fidalgo *et al*, 1991), e liberação de enzimas lisossomais em macrófagos (Ghazaleh *et al*, 1992).

A maioria dos efeitos descritos para a canatoxina, tanto *in vivo* quanto *in vitro*, envolve mediação por metabólitos do ácido araquidônico via lipoxigenases, já que esses efeitos são bloqueados por inibidores de lipoxigenase, por exemplo, ácido nordihidroguaiarético e esculetina, e não por inibidores de cicloxigenases, como ácido acetilsalicílico e indometacina (Benjamim *et al*, 1992; Carlini *et al*, 1985; Barja-Fidalgo *et al*, 1991a,b; Ribeiro-Dasilva *et al*, 1989b).

A canatoxina também apresenta um efeito inibitório sobre o acúmulo de Ca^{2+} em vesículas do retículo sarcoplasmático, resultante da atividade enzimática de uma Ca^{2+} Mg^{2+}-ATPase presente. A toxina parece desacoplar o transporte de cálcio, através da membrana, da atividade hidrolítica da enzima sobre o ATP, um dado relevante para o entendimento das propriedades secretagogas desta proteína (Alves *et al*., 1992).

A canatoxina promove influxo de cálcio através da membrana plasmática de plaquetas, e este parece ser um passo importante na ativação de fosfolipase A_2, secreção de ATP e agregação plaquetária induzidas por esta toxina. A agregação plaquetária é diminuída na presença de verapamil, um bloqueador de canais de cálcio voltagem-dependentes (Ghazaleh *et al*., 1997).

O efeito pró-inflamatório atribuído a essa toxina foi caracterizado em ratos pela indução de migração de neutrófilos e monócitos para as cavidades peritoneal e pleural, além de apresentar ação no modelo *air pouch*, mediada pela liberação de fatores quimiotáticos de macrófagos peritoneais de ratos (Barja-Fidalgo *et al*., 1992). Em ensaio de edema de pata em ratos, a inflamação é dose dependente com pico máximo após 6 horas da injeção intraplantar e com redução total em 48 horas, em doses de 50 µg e 100 µg de canatoxina por pata. Este fenômeno parece ser mediado por metabólitos das vias das lipoxigenases, provavelmente leucotrienos, que causam infiltração celular intensa no local da inflamação (Benjamin *et al*., 1992).

A urease de *C. ensiformis* apresenta efeitos biológicos em comum com a canatoxina, como a ativação de plaquetas, interação com glicoconjugados polisialilados e atividade inseticida, porém não é tóxica quando administrada intraperitonealmente em ratos e camundongos (Follmer *et al*, 2001; Follmer *et al*, 2004b).

Outros estudos mostraram que as várias atividades biológicas descritas para a canatoxina não são dependentes da atividade ureolítica da molécula. Assim, a canatoxina tratada com 200 µM de p-hidroximercuribenzoato perde totalmente a atividade ureolítica, mas mantém inalterada a sua atividade tóxica em camundongos, ainda induz agregação plaquetária, produz hemaglutinação indireta e mantém sua atividade inseticida. As mesmas observações foram feitas para a urease tratada com *p*-hidroxi-mercuribenzoato (Follmer *et al*., 2001). A Tabela 2 resume os dados comparativos disponíveis para as ureases de *C ensiformis*.

Tabela 2: Propriedades físico-químicas e biológicas da canatoxina e a urease clássica de *C. ensiformis*. Dados adaptados de Follmer et al (2001).

	Canatoxin	Urease
Physicochemical properties		
Molecular mass		
SDS–PAGE	95–100 kDa	90–97 kDa
Native form	Dimer	Hexamer
Urease activity		
K_m (pH 6.5–8.5)	2–5 mM	3–8 mM
V_{max} (pH 6.5–8.5)	4–10 U/mg	15–45 U/mg
Inhibitors, IC50		
p-Hydroxi-mercuribenzoate	5 μM	0.5 μM
Acetohydroxamic acid	3 μM	3 μM
Metal content (per 95 kDa)	Nickel-1 mol/mol	Nickel-2 mol/mol
	Zinc-1 mol/mol	Zinc-not detected
Biological properties		
Toxicity		
Mouse, i.p.	LD_{50}—2 mg/kg (*p*-OHMB treated, 100% active)	Not toxic with 10 LD50
C. maculatus, ingested	100% death—0.25% (w/w)	Not determined
R. prolixus, ingested	LD_{50}—0.4–0.8 mg/kg	LD_{50}—0.4–0.8 mg/kg
Indirect hemagglutination		
End-point (10^6 rabbit cells)	1–2 μg	1–2 μg
Inhibitors	Gangliosides-fetuin (*p*-OHMB treated-100% active)	Gangliosides-fetuin (*p*-OHMB treated-100% active)
Platelet aggregation (rabbit)	EC_{50}—2–3 μg (*p*-OHMB treated-100% active)	EC_{50}—2–3 μg (*p*-OHMB treated-100% active)

1.4. Plaquetas

Plaquetas sanguíneas são células discoides, anucleadas, originadas de megacariócitos. Possuem dois tipos de grânulos: os grânulos densos, que contém ADP, ATP, serotonina, histamina, e cálcio; e os grânulos-alfa, que contém fator V, fibrinogênio, vitronectina, trombospondina e fator de von Willebrand. Em exposição à injúria vascular ou a agonistas como ADP, trombina e colágeno, plaquetas estimuladas se tornam esféricas e aderentes umas às outras e ao tecido lesado (Andrews *et al*., 2004; Ruggeri *et al*., 2007). As plaquetas ativadas secretam os grânulos-alfa e grânulos densos, cujos conteúdos contribuem para a homeostase. O ADP secretado nos grânulos densos amplifica a agregação plaquetária. Níveis elevados de Ca^{2+} intracelular são necessários para que a agregação ocorra.

1.5. Eicosanóides

Os eicosanóides são autacóides derivados do ácido araquidônico por rotas metabólicas distintas, entre as quais a via das cicloxigenases e a via das lipoxigenases (figura 2), hoje reconhecidos como segundo mensageiros envolvidos na transdução de sinais numa vasta gama de fenômenos fisiológicos e patológicos.

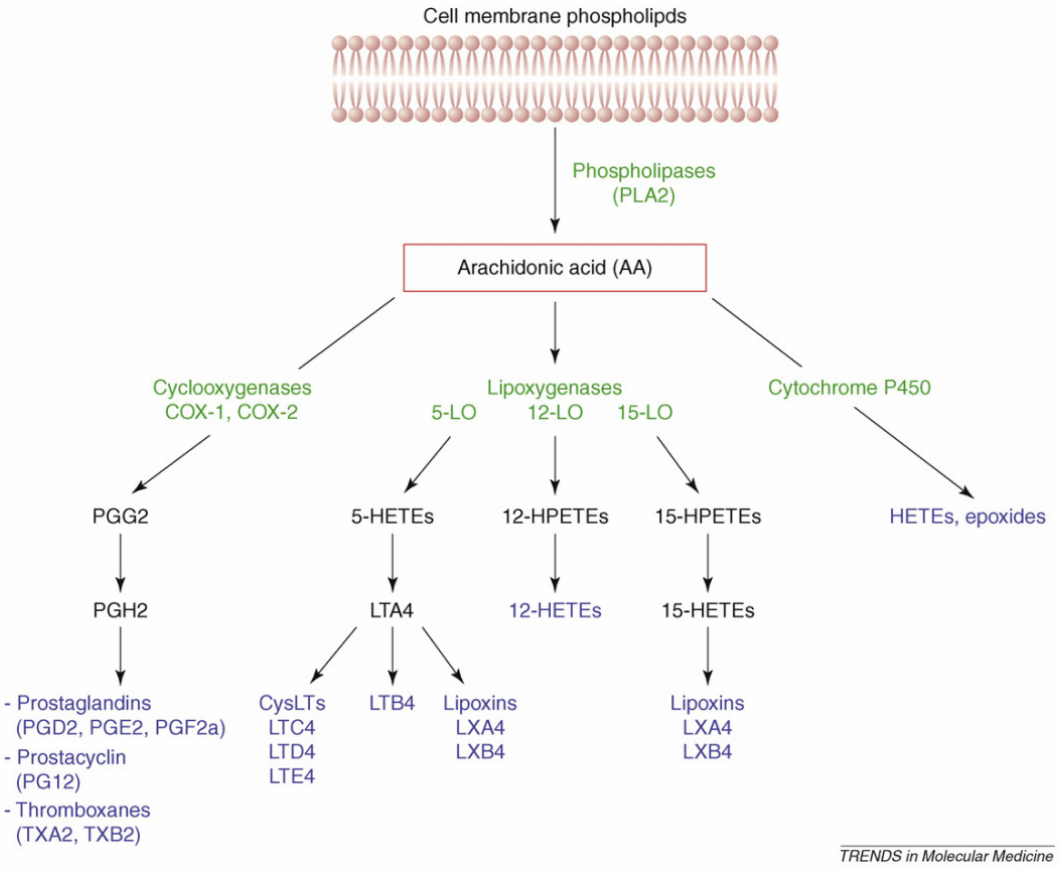

Figura 2: Principais vias do metabolismo dos eicosanóides. A liberação de ácido araquidônico, constituinte minoritário dos fosfolipídios de membranas, ocorre por hidrólise catalisada por fosfolipases tipo A2. Uma vez liberado, o ácido araquidônico será substrato para diferentes rotas metabólicas, particulares para cada tipo celular. Através da via da cicloxigenase formam-se prostaglandinas e tromboxanas, enquanto que a ação das diferentes lipoxigenases levará á formação dos hidroperóxidos correspondentes. Adaptado de Harizi *et al.,* 2008.

O ácido araquidônico, atuando diretamente ou na forma de seus metabólitos, eicosanóides, regula uma série de funções celulares (Sakata *et al*., 1987; Sumida *et al*., 1993). Prostaglandinas, produtos de cicloxigenases, e leucotrienos, produtos de lipoxigenases, são mediadores de atividades biológicas e várias doenças inflamatórias, e em condições crônicas de inflamação, os níveis de eicosanóides estão aumentados (Harizi *et al*., 2008).

Existem muito indícios de que os produtos de lipoxigenases, como os leucotrienos, estariam envolvidos nos processos secretórios de diferentes tipos celulares

(Snider *et al*., 1984; Metz, 1985b; Metz *et al*., 1983a; Metz *et al*., 1983b; Metz *et al*., 1983c). Além disso, a inibição de 5-lipoxigenase diminui o crescimento e promove morte celular em diversas linhagens transformadas (Massoumi & Sjölander, 2007). Sabe-se que a inflamação decorrente da infecção por *H. pylori* estimula a produção de metabólitos de 5-lipoxigenase, resultando em um aumento na produção de citocinas inflamatórias (Park *et al*., 2007).

Os produtos de ciclooxigenases estão envolvidos na patogênese de várias doenças inflamatórias, devido ao potencial inflamatório de PGE_2 e tromboxana A_2, (Linton *et al*., 2004), tendo sido descrito seu envolvimento na patogênese da aterosclerose e no desenvolvimento de problemas vasculares decorrentes da diabetes (Natarajan & Nadler, 2004).

1.6. Propriedades não enzimáticas de ureases

Desde 2004, nosso grupo vem demonstrando que propriedades biológicas independentes de ureólise não são exclusivas das ureases de *Canavalia ensiformis.* Follmer *et al*, 2004, mostraram que a urease embrião-específica de soja (*Glycine max*) e a urease da bactéria de solo *Bacillus pasteurii* também induzem ativação de plaquetas, de modo semelhante às ureases de *C. ensiformis*. Em Olivera-Severo *et al*, 2006, a rota de ativação da resposta das plaquetas à urease de *B. pasteurii* foi caracterizada, demonstrando-se o envolvimento da 12-lipoxigenase plaquetária e de canais de cálcio sensíveis a D-metoxi-verapamil.

Mais recentemente, Wassermann (2007) mostrou que a urease da bactéria *H. pylori* também ativa plaquetas sanguíneas e induz agregação plaquetária, recrutando a rota dos eicosanóides, através da via da lipoxigenase. Esta ativação é inibida por

dexametasona (inibidor de fosfolipase A2) e esculetina (inibidor de lipoxigenases), e potenciada por indometacina (inibidor de ciclooxigenases), como ilustra a Tabela 3.

Tabela 3. Envolvimento de fosfolipase A2 e eicosanóides na agregação plaquetária induzida por HPU.

Tratamento	Agregação Plaquetária % Média ± DP
Nenhum	100 ± 10,04
Dexametasona (50 µM)	62,64 ± 6,06
Esculetina (500 µM)	55 ± 6,06
Indometacina (150 µM)	160,74 ± 12,74
(300 µM)	313,26 ± 3,78

(Retirado de Wassermann *et al*., 2010)

1.7. *Helicobacter pylori*

Helicobacter pylori é uma espiroqueta, gram negativa, microaerófila, com 2.5 a 5.0 µm de comprimento e 0.5 a 1.0 µm de largura, que possui de 4 a 6 flagelos com aproximadamente 30 µm de comprimento (Goodwin *et al,* 1990). Em 1982, Marshall e Warren isolaram pela primeira vez esse microrganismo (Marshall e Warren, 1984). A partir de 1984, tornou-se cada vez mais evidente a associação de *H. pylori* com patologias gástricas e duodenais.

Atualmente, *H. pylori* é reconhecido como o agente patológico de gastrite crônica, úlcera péptica e câncer gástrico e duodenal (NIH Consensus Conference, 1994).

Estima-se que esse microrganismo pode ser encontrado em cerca de 50% da população mundial, e em países subdesenvolvidos, ocorrências de 70% a 90% são relatadas.

H. pylori causa dano ao tecido iniciando uma inflamação crônica na mucosa gástrica. Esta inflamação é mediada por uma gama de citocinas anti e pró-inflamatórias, amônia liberada pela urease e lipopolissacarídeo bacteriano (LPS) (Israel *et al*., 2001). A gastrite gerada reduz a produção de ácido clorídrico, e esta redução está diretamente ligada ao risco de desenvolver câncer gástrico (Collins *et al*., 2006). A infecção por *H. pylori* aumenta os níveis de IL-8, uma citocina ativadora de neutrófilos e linfócitos. O aumento da expressão de IL-8 parece estar diretamente relacionado a respostas inflamatórias mais severas (Machado *et al*., 2010).

Sugere-se que a transmissão desse microrganismo possa ocorrer a partir de três rotas. A primeira, e menos comum, está relacionada ao contato de pacientes com instrumentos endoscópicos contaminados (Akamatsu *et al*., 1996), podendo ser evitada com a esterilização dos instrumentos (Katoh *et al*., 1993; Tytgat, 1995). A transmissão fecal-oral é talvez a mais importante. Apesar de *H. pylori* ter sido isolado das fezes de crianças infectadas (Thomas *et al*., 1992), o isolamento desse microrganismo das fezes não é comum, o que sugere que deva ocorrer descamação gástrica intermitente. Água contaminada por material fecal pode ser também uma fonte importante de transmissão (Klein *et al*., 1991). Por último, a via de transmissão oral-oral foi identificada em alguns casos na África, onde em algumas tribos, as mães pré-mastigam o alimento dos filhos (Megraud, 1995).

Muitos fatores de virulência estão envolvidos no mecanismo patológico da infecção por *H. pylori*, incluindo várias enzimas (urease, catalase, lipase e algumas proteases) e toxinas proteicas, como a citotoxina vacuolizante, codificada pelo gene

vacA, e a proteína imunogênica Cag A, codificada pelo gene *cagA*, cujos genes estão localizados em uma ilha de patogenicidade (PAI; Pathogenicity Island). A PAI contém também diversos outros genes responsáveis pela virulência, expressão de citocinas pró-inflamatórias (IL-8) em células epiteliais, e expressão de um conjunto de proteínas formadoras de um sistema de secreção do tipo IV (T4SS), que transporta a proteína CagA para dentro de células eucarióticas (Censini *et al.* 1996; Figueiredo *et al.* 2005).

Estudos epidemiológicos mostram que a infecção por *H. pylori* também está associada a patologias não relacionadas ao trato gastrointestinal, como púrpura trombocitopênica idiopática, doenças cardiovasculares e cerebrovasculares (Atherton, 2005).

1.8. O processo inflamatório desencadeado pela infecção por *Helicobacter pylori*

Observações histológicas em humanos indicam que o grau de infecção por *H. pylori* e a severidade do dano celular estão diretamente associados com a extensão da infiltração de neutrófilos na mucosa gástrica. Embora esse organismo seja conhecido como não invasivo, a infecção por *H. pylori* leva a infiltração de células inflamatórias, especialmente neutrófilos (Montecucco *et al*., 1999; Shimoyama *et al*., 2003).

A exposição do mesentério a extratos aquosos de *H. pylori*, ricos em urease e sem contaminação significante por LPS da parede celular, resultou em aumento de três vezes na aderência de leucócitos nas vênulas, e de quatro vezes na migração destes para a região intersticial, contribuindo para o dano à mucosa gástrica (Yoshida *et al*., 1993).

A apoptose em neutrófilos e a subsequente atuação de fagócitos é crucial para a instalação de uma inflamação aguda (Lee *et al*., 1993; Savill *et al*., 2002). A apoptose é retardada em neutrófilos humanos ativados por IL-8, GM-CSF, LPS, ou leucotrieno B4, que modulam rotas de sinalização incluindo as MAPKs, especialmente as rotas ERK e PI3K/Akt (Hebert *et al*., 1996; Ward *et al*., 1999). A rota de ativação de NF-kB tem um efeito protetor, regulando a expressão de genes antiapoptóticos. Em neutrófilos humanos, a ativação de NF-kB parece regular a apoptose espontânea, bem como o efeito antiapoptótico de TNF-alfa. Vários estudos mostram que *H. pylori* é capaz de induzir apoptose em células do epitélio gástrico, tanto *in vivo* como *in vitro*, bem como em monócitos de camundongos (Cover *et al*., 2003; Galgani *et al*., 2004).

Espécies reativas de oxigênio (ROS) são subprodutos de processos metabólicos das células, e em altas concentrações podem estar implicados em processos inflamatórios. A gastrite associada à infecção por *H. pylori* estimula a geração de ROS por células inflamatórias presentes na mucosa (Smoot *et al*, 2000). Distúrbios no balanço oxidante-antioxidante podem aumentar os riscos de morte celular, ou ainda causar danos ao DNA, podendo representar um passo inicial da carcinogênese gástrica. Extratos aquosos de *H. pylori* induzem diretamente a síntese de ROS por células epiteliais gástricas, associado ao reparo de DNA (Obst *et al*, *2000*).

1.9. A urease de *Helicobacter pylori*

A urease é uma enzima altamente expressa por *H. pylori*, podendo compor de 10% a 15% das proteínas totais dessa bactéria. A urease nativa de *H. pylori* possui massa molecular de aproximadamente 1,1 MDa, é uma metaloenzima níquel dependente

e dodecamérica. Em solução também podem ser encontrados hexâmeros de HPU, bem como formas menores. Sua unidade funcional ("monômero") é composta por duas cadeias polipeptídicas (UreA [30kDa] e UreB [62kDa]) em proporção 1:1 (Dunn *et al*., 1990; Hu *&* Mobley, 1990). A afinidade dessa enzima pelo substrato uréia, com um Km ~ 0.3 mM, a torna cataliticamente eficiente até mesmo nas concentrações submilimolares de uréia presentes nos fluidos humanos (Dunn *et al*., 1990; Hu *&* Mobley, 1990). A figura 3 ilustra a estrutura cristalográfica do monômero da urease de *H. pylori* (Ha *et al*., 2001).

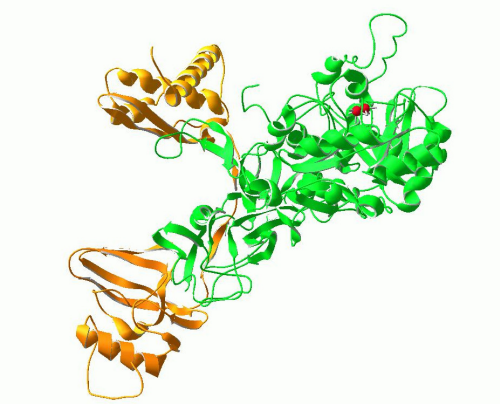

Figura 3: Estrutura cristalográfica da urease de *H. pylori.* A cadeia A está representada em laranja, e a cadeia B em verde, na qual os dois átomos de Ní (esferas vermelhas) marcam a posição do sítio ativo.

A urease de *H. pylori* foi clonada e expressa em *E. coli* em nível semelhante ao produzido pela bactéria selvagem (Hu & Mobley, 1993). Foram descritos pelo menos sete genes envolvidos na produção da urease de *H. pylori*. Os genes *ureA* e *B* codificam as duas subunidades que compõem a enzima, enquanto que os genes *ureE, F, G, H* codificam proteínas acessórias responsáveis pelo *folding* e pela incorporação do níquel no centro ativo da urease. O gene *ureI* codifica uma proteína que funciona como um canal na membrana externa da espiroqueta, atuando na internalização da uréia. Além

desses genes, uma proteína transportadora de níquel é expressa a partir do gene *nixA* (Mobley *et al.*, 1995).

A urease de *H. pylori* é considerada um fator de virulência, sendo a sua atividade um marcador utilizado amplamente para diagnóstico (Krogfelt *et al.*, 2005). Mutantes de *H. pylori* urease negativos são incapazes de colonizar o estômago de leitões gnotobióticos e tampouco camundongos *nude* (Eaton *et al*., 1991; Tsuda *et al*., 1994). Supõe-se que a principal função dessa enzima está relacionada com a formação de um microclima neutro no lúmen gástrico, possibilitando sobrevivência das bactérias em ambientes de pH desfavorável. Apesar de inibidores de urease terem sido utilizados no tratamento de algumas destas patologias, seu uso foi descontinuado pelo fato de muitos pacientes apresentarem reações colaterais adversas. O uso clínico de ácido acetohidroxâmico, um inibidor de urease, causa depressão na síntese de DNA, afetando a medula óssea, além de ser teratogênico em doses elevadas (Bailie *et al*., 1986)

Extratos de *H. pylori* induzem em macrófagos *in vitro* aumento da óxido nítrico sintase induzível (iNOS) (Wilson *et al*., 1996), resultando em uma grande liberação de óxido nítrico, o qual está associado à ativação de células do sistema imune no tecido lesado. A degradação de uréia, que difunde do leito capilar, pela urease e, consequentemente, a liberação de amônia resulta em danos celulares (Barer *et al,* 1988) e contribui na indução de vacuolização das células epiteliais gástricas, em conjunto com a toxina VacA. Além do efeito neutralizador da acidez gástrica, a urease de *H. pylori* está envolvida na ativação de fagócitos e na produção de citocinas inflamatórias (Harris *et al*., 1996). Esses dados sugerem que a urease seja importante também no desenvolvimento de lesões gástricas, e não só na manutenção da bactéria em pH desfavorável.

Plaquetas participam da resposta inflamatória como um local de armazenamento de substâncias vasoativas e mediadores inflamatórios, bem como a geração de peróxidos e radicais hidroxil, que podem induzir perturbações na microcirculação (Kalia *et al.* 2003; Elizalde *et al.* 1977). Nosso grupo observou que, assim como já descrito para ureases de *C. ensiformis*, da soja, e da bactéria *B. pasteuri,* a urease purificada de *H. pylori* também promove ativação de plaquetas, com secreção de grânulos densos que culminam em agregação plaquetária. Nesses estudos, verificou-se que inibidores da 12-lipoxigenase inibiam a resposta das plaquetas à HPU, sem ter havido demonstração da produção do metabólito ácido 12-hidroxieicosatetraenóico (12-HETE) (Olivera-Severo *et al*., 2006; Wassermann, 2007).

Concordando com um papel da urease de *H. pylori* como molécula pró-inflamatória, Wassermann (2007) mostrou que a enzima purificada produz, de maneira tempo e dose-dependente, edema de pata em camundongos, atuando de modo muito semelhante à canatoxina, para a qual esse mesmo tipo de efeito já foi previamente descrito (Benjamin *et al*., 1992).

1.10. Objetivos

Baseados nos estudos anteriores das propriedades farmacológicas da canatoxina, em especial o seu efeito pró-inflamatório, bem como suas ações secretagoga e indutora de ativação plaquetária, igualmente descritas para a urease de *Bacillus pasteurii*, no presente trabalhos exploramos a hipótese de que a urease de *H. pylori* também compartilharia essas propriedades.

Este trabalho teve como objetivos específicos:

Em plaquetas:

1. Caracterizar as rotas de ativação de plaquetas por HPU;
    a. Investigar o envolvimento de PAF e a dependência de influxo de cálcio na agregação plaquetária;
    b. Demonstrar a dependência do ADP secretado pela plaqueta como indutor da resposta de agregação;
    c. Investigar a ativação do metabolismo de eicosanóides e produção de metabólitos de 12-lipoxigenase;.
2. Estudos de adesão da urease de *H. pylori* a membranas de plaquetas;

Em neutrófilos:

1. Investigar um possível potencial quimiotático de HPU;
2. Estudar o efeito da HPU na apoptose de neutrófilos;
3. Verificar a produção de espécies reativas de oxigênio em neutrófilos ativados por HPU;
4. Verificar o recrutamento de enzimas da rota dos eicosanóides em neutrófilos ativados por HPU;

2. Materiais e Métodos

2.1. Manipulação bacteriana

2.1.1. Linhagem bacteriana

A linhagem *E. coli* SE5000 [F^- araD193 Δ(argF lac)U169 rpsL150 relA1 ftbB5301 deoC1 ptsF25 rbsR recA56] foi utilizada como vetor de expressão da urease recombinante de *H. pylori* (gentilmente cedida pelo Dr. Harry L.T. Mobley - University of Michigan Medical School)

2.1.2. Cultivo bacteriano

O meio de cultura utilizado para o cultivo de *E. coli* foi o LB (Luria-Bertani) em pH 7.0, sendo composto de triptona (10g/L), extrato de levedura (5g/L) e NaCl (10g/L). Para meio sólido, foi adicionado 1,5% m/v de ágar.

2.1.3. Tranformação bacteriana

A preparação de células competentes para transformação seguiu o protocolo adaptado de Sambrook & Russel (2001). As transformações foram feitas por choque térmico e as células transformadas foram plaqueadas em *urea segregation agar* (Hu *et al*, 1992), após uma hora de recuperação em meio LB a 37ºC.

2.1.4. Vetor plasmidial

Foi utilizado o plasmídeo pHP8080 (cedido gentilmente pelo Dr. Harry L.T. Mobley - University of Michigan Medical School). A figura 3 mostra a estrutura do plasmídeo.

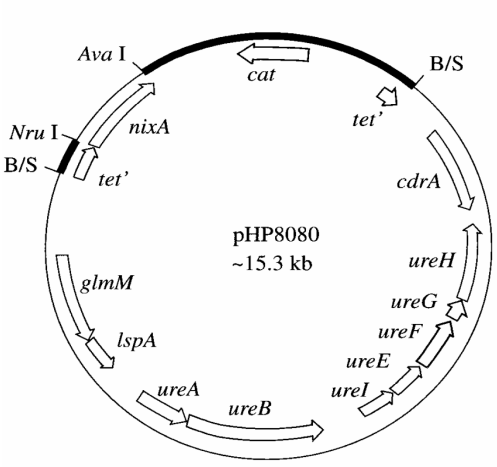

Figura 4: Estrutura do plasmídeo pHP8080, contendo o operon da urease de *H. pylori* linhagem *26695* (*ureABIEFGH*), o gene codificante para proteína transportadora de níquel (*nixA)* e marca de resistência para cloranfenicol (*cat*). Adaptado de McGee *et al*, 1999.

2.2.Expressão e purificação da urease recombinante de *H. pylori*

2.2.1. Pré-inóculo

Colônias mantidas a -196°C em nitrogênio líquido foram inoculadas em meio de cultura LB com cloranfenicol (20µg/ml). Este pré-inóculo foi cultivado *overnight* (O/N, ~16 horas) a 37ºC, e adicionado, na proporção 1:50, em LB contendo cloranfenicol (20 µg/ml) e Ni_2Cl (1 µM). Tipicamente, foram utilizados 5 mL do pré-inóculo. O cultivo foi incubado a 37ºC por cerca de 16 horas sob agitação (180 RPM).

2.2.2. Preparação de extratos brutos a partir dos cultivos

Após o desenvolvimento das cepas o cultivo foi centrifugado em Sorvall-Plus RC5b, a 15000 g, a 4°C, durante 10 minutos. O material sobrenadante foi desprezado e o precipitado suspenso em tampão 20 mM NaPB, 5 mM β-mercaptoetanol, 1 µM EDTA pH 7.0 (tampão de extração).

As células suspensas no tampão de extração foram lisadas, com a utilização de ultra-som (Ultrasonic Homogenizer 4710), com 10 pulsos (40 kHz) de 2 minutos, em banho de gelo. Em seguida esse material foi novamente centrifugado em Sorvall Plus RC 5b, a 15000 g, durante 20 minutos, o material insolúvel foi descartado e o sobrenadante dialisado e denominado como Extrato Bruto.

2.3. Purificação da urease recombinante de *H. pylori*

O método aqui utilizado foi desenvolvido por Wassermann, 2007.

2.3.1. Cromatografia de troca iônica Q-Sepharose

O Extrato bruto foi submetido à cromatografia de troca iônica Q-Sepharose (Amersham Biosciences), na proporção de 1 mL de resina para cada 5 mg de proteína; a resina foi equilibrada em tampão de extração. Após a adsorção da amostra à resina, esta foi lavada com o tampão de equilíbrio e então eluída com gradiente descontínuo: Os tampões de eluição foram: 1ª eluição – Tampão de extração mais 100 mM NaCl; 2ª eluição - Tampão de extração mais 200 mM NaCl; 3ª eluição - Tampão de extração mais 300 mM NaCl; 4ª eluição - Tampão de extração mais 1 M NaCl.

2.3.2. Cromatografia de troca iônica Source 15-Q

A fração rica em atividade ureolítica oriunda da 2ª eluição da cromatografia em Q-Sepharose foi dialisada para a eliminação do NaCl e submetida a uma nova cromatografia de troca iônica em coluna Source 15-Q (Amersham Biosciences), esta adaptada em sistema de FPLC (Pharmacia). A coluna foi equilibrada com tampão de extração pH 7.5, e o sistema foi programado para gerar um gradiente contínuo de NaCl 25% a 75% em 20mL de tampão de extração, para eluição da amostra. Após a coleta

dos picos cromatográficos, foram realizados ensaios de determinação de atividade ureolítica.

2.3.3. Cromatografia de exclusão molecular

A fração rica em urease proveniente da cromatografia de troca iônica Source 15-Q foi submetida à cromatografia de gel filtração em coluna Superose 6 (GE Healthcare), equilibrada com tampão de extração, em sistema de FPLC (Pharmacia), obtendo-se assim a urease recombinante purificada.

2.4. Detecção de 12-HETE

Plaquetas agregadas com 300 nM HPU foram lavadas com tampão PBS e posteriormente lisadas, através da centrifugação dos agregados a 10.000 *g* em metanol gelado. O sobrenadante recuperado foi submetido à cromatografia de fase reversa em coluna C-18 (Shimadzu) em sistema HPLC (Shimadzu) segundo protocolo de Coffey *et al*. (2004). As amostras foram separadas usando um gradiente de 50% a 90% de B em (A = água:acetonitrila:ácido acético, 75:25:0,1; B= metanol:acetonitrila:ácido acético, 60:40:0,1), em 20 minutos com um fluxo de 1 ml/min. A absorbância foi monitorada a 235 nm.

2.5. Medida de conteúdo protéico e atividade enzimática

2.5.1. Conteúdo Protéico

A determinação do conteúdo protéico das amostras de HPU foi realizada a partir da absorção no ultravioleta em comprimento de onda de 280nm, utilizando cubetas de quartzo com passo óptico de 1 cm. No caso de lisados celulares, o conteúdo protéico foi medido através do método de Bradford, 1976.

2.5.2. Detecção de atividade ureásica

Alíquotas de amostras de todas as etapas de purificação foram incubadas com 10 mM de uréia, a 37°C, em tampão PBS 1X pH 7,0 (tampão fosfato 20 mM, 150 mM NaCl pH 7,0). A amônia liberada pela urease foi quantificada colorimetricamente pelo método de fenol-hipoclorito (Weatherburn MW, 1967), utilizando-se uma curva padrão de sulfato de amônio na faixa de 15 a 250 nM. Uma unidade enzimática de urease foi definida como a quantidade de enzima capaz de liberar 1 μmol de amônia por minuto, em pH 7,0 a 37°C.

2.6. Ensaio de agregação plaquetária

O plasma rico em plaquetas (PRP) foi preparado a partir de sangue de coelho coletado da artéria central auricular, na presença de citrato de sódio na concentração final de 0,313% (p/v). As amostras de sangue foram centrifugadas a 200 *g*, por 20 minutos a 18°C, para a obtenção de plasma rico em plaquetas. A agregação plaquetária e o *shape change* foram monitorados por turbidimetria usando um Lummi-agregômetro (Chrono-Log Co. Havertown, Pa.) e registrada por 10 minutos. A agregação plaquetária também foi monitorada utilizando leitor de microplacas SpectraMax (Molecular Devices, USA). Nesse caso, as amostras de urease foram adicionadas em placas de 96 poços com fundo plano e completadas para o volume final de 150 μL com solução

salina. O ensaio foi iniciado com a adição de 100 µL da suspensão de plaquetas. A placa foi incubada por 2 minutos a 37°C antes do início da agitação e leituras foram feitas a cada 11 segundos a 650nm, durante 20 minutos. A mudança da turbidez foi medida em unidades de absorbância e os resultados expressos como a área sob a curva de agregação (Born & Cross, 1963).

2.7. Ensaio de adesão de HPU a membranas de plaquetas

Para ensaios de fluorescência HPU foi marcada com isotiocianato de fluoresceína (FITC, Sigma-Aldrich). HPU foi colocada em contato com 0,1% FITC durante duas horas a 4°C em tampão de amostra. Após diálise exaustiva contra tampão de amostra, pH 7,5, a amostra foi submetida a uma cromatografia em coluna Fast-Desalting (Amersham Biosciences) para retirada do FITC que não interagiu com a proteína.

PRP de coelhos incubado com 300 nM HPU-FITC, sob agitação em vórtex por 5min. em temperatura ambiente. PRP sem HPU foi submetido às mesmas condições como controle negativo. Os agregados foram recuperados por centrifugação; *pellets* foram espalhados em lâminas e observados em um microscópio invertido Zeiss-Axiovert-200, para estudos de microscopia óptica (aumento 400 X) e de fluorescência (filtro FITC).

2.8. Isolamento de neutrófilos humanos

Neutrófilos humanos foram isolados de voluntários saudáveis, a partir de sangue venoso periférico, tratado com 0,5% de EDTA, utilizando-se 4 etapas de gradiente descontínuo de Percoll (Sigma), conforme Boyun, 1968. Após remoção das hemácias por lise hipotônica, neutrófilos foram suspensos em meio RPMI-1640 (Sigma) ou

DMEM (Dulbecco modified Eagle medium). A pureza e a viabilidade dos neutrófilos foi analisada pelo ensaio de exclusão do corante vital *trypan blue*.

2.9. Ensaio de migração de neutrófilos

A quimiotaxia foi medida em Câmara de Boyden (NeuroProbe, Gaithersburg, MD), com 48 poços, usando filtros de policarbonato de 5 µm. Neutrófilos (10^6 cells/mL em RPMI-0.01% albumina de soro bovino [BSA]) foram expostos ao fMLP (700 nM, Sigma), a HPU (10 nM, 30 nM, 100 nM) e ao meio (migração aleatória; 37°C, 5% CO2) (Arraes *et al*., 2006). Após 1 hora, os filtros foram removidos, fixados, e corados, e neutrófilos que migraram pela membrana foram contados ao microscópio, em pelo menos 5 campos aleatoriamente selecionados. Cada amostra foi medida em triplicata. Resultados foram expressos como o número médio ± DP de neutrófilos por campo.

2.10. Medida da apoptose

Neutrófilos foram expostos a IL-8 (100 nM, Sigma), HPU (10 nM, 30 nM, 100 nM), ou somente ao meio de cultura, durante 24 horas. As células foram centrifugadas, coradas com Diff-Quik (Dade Behring – Suíça), e contadas em um microscópio de luz (x 1000) para determinação da proporção de células exibindo núcleo picnótico, resultado da condensação irreversível da cromatina, morfologia característica de células em apoptose (Arruda *et al*., 2004). Pelo menos 400 células foram contadas por amostra. Resultados foram expressos como média ± DP.

2.11. Preparo de extratos celulares

Para obter lisados celulares, neutrófilos foram suspensos em tampão de lise (50 mM HEPES, pH 6.4, 1 mM MgCl2, 10 mM EDTA, 1% Triton X-100, 1 μg/mL DNAse, 0.5 μg/mL RNAse) contendo os seguintes inibidores de proteases: 1 mM PMSF, 1 mM benzamidina, 1 μM leupeptina e 1 μM SBTI (Sigma).

2.12. Western Blot

Lisados celulares foram desnaturados em tampão de amostra (50 mM Tris–HCl, pH 6.8, 1% SDS, 5% 2-mercaptoethanol, 10% glicerol, 0.001% azul de bromofenol) e aquecidos em "banho-maria" fervente por 3 minutos. As amostras (30 μg proteína total) foram resolvidas em 12% SDS-PAGE e as proteínas foram transferidas para uma membrana de PVDF (Hybond-P, Amersham Pharmacia Biotech). Marcadores "Rainbow" (Amersham Pharmacia Biotech) correram em paralelo para estimar as massas moleculares. As membranas foram bloqueadas com Tween-TBS (20 mM Tris–HCl, pH 7.5, 0,9% NaCl, 0.1% Tween-20) contendo 1% BSA e como sondas foram usados os seguintes anticorpos desenvolvidos em coelho: policlonal anti-Bcl-XL (Santa Cruz Biotechnology, 1:500), policlonal anti-Bad (Santa Cruz Biotechnology, 1:500), policlonal anti-5-LO (Cayman Chemicals, 1:500), policlonal anti-COX (Cayman Chemicals, 1:500). Após extensiva lavagem com Tween-TBS, as membranas de PVDF foram incubadas com o anticorpo secundário IgG anti-coelho conjugado a biotina por uma hora, e então incubado com estreptavidina conjugada a peroxidase (1:1000; Caltag Laboratories, Burlingame, CA). Proteínas imunorreativas foram visualizadas após coloração com 3,3′-diaminobenzidina (Sigma). As bandas foram quantificadas por densitometria usando Scion Image Software (Scion Co., MD, USA).

2.13. Medida da produção de ROS por neutrófilos humanos

Conforme Pereira *et al*., 2001. Luminol (5-amino-2,3-dihidro-1,4-ftalazinedione, Sigma) foi utilizado como uma sonda permeável sensível a espécies reativas de oxigênio, sendo a quimioluminescência medida em leitor de microplacas Spectramax (Molecular Devices, CA, USA). Para os ensaios, 10^6 neutrófilos foram estimulados com HPU (10 nM, 30 nM e 100 nM) ou PMA (acetato de forbol miristato)(1 mg/mL, Sigma) e a produção de ROS foi medida durante 60 minutos. Neutrófilos foram pré-incubados no meio por 30 minutos antes da aplicação do estímulo, para diminuir uma eventual ativação decorrente da manipulação das células.

Para determinar a proporção de ROS extracelular e intracelular, foram utilizadas as sondas lucigenina (Sigma) e CM-H_2DCFDA (diacetato de diclorofluoresceina, Sigma) (λ_{ex}470nm/λ_{em}529nm), respectivamente. Para a lucigenina foi utilizado o mesmo protocolo do luminol. Para o CM-H_2DCFDA, neutrófilos foram incubados com a sonda por 15 minutos, a 37 C°, antes da aplicação do estímulo.

2.14. Análise estatística

Os dados foram analisados por ANOVA seguido por Turkey-Kramer utilizando o programa GraphPad Prism 3.0 e o valor de $p < 0.05$ foram considerados estatisticamente significativos.

3. Resultados

Os resultados dessa dissertação foram incorporados em dois manuscritos submetidos à publicação.

Capítulo 1. Estudos com a HPU em Plaquetas de Coelho

Wassermann, G. E., Olivera-Severo, D., UBERTI, A. F., Carlini, C. R. (2010)
Helicobacter pylori urease activates blood platelets through a lipoxygenase-mediated pathway.
Journal of Cellular and Molecular Medicine 14: 2025- 2034. E-pub Sept 2009.

Capítulo 2. Estudos com a HPU em Neutrófilos Humanos

UBERTI, A. F., Olivera-Severo, D., Wassermann, G. E., Moraes, J. A., Barcellos-de-Souza, P., Barja-Fidalgo, T. C., Carlini, C. R.
Mouse Paw Edema and Human Neutrophil Activation by a Recombinant Urease from *Helicobacter pylori*
Manuscrito a ser submetido ao periódico Helicobacter (ver anexo)

Capítulo 1. Estudos com a HPU em Plaquetas de Coelho

Wassermann, G. E., Olivera-Severo, D., UBERTI, A. F., Carlini, C. R. (2010) *Helicobacter pylori* urease activates blood platelets through a lipoxygenase-mediated pathway.
Journal of Cellular and Molecular Medicine 14: 2025- 2034. E-pub Sept 2009.

Os dados obtidos nessa dissertação estão contidos no artigo como:

figuras 3C e 3D,

figura 4B,

figura 5B,

figura 6

tabela 2

Resumo

A bactéria *Helicobacter pylori* causa úlcera péptica e câncer gástrico em humanos por mecanismos ainda não totalmente elucidados. *H. pylori* produz urease, que neutraliza o ambiente ácido, permitindo sua sobrevivência no estômago. Nosso grupo já demonstrou que as ureases de *Canavalia ensiformis*, soja e *Bacillus pasteurii* induzem agregação plaquetária independentemente de suas atividades enzimáticas por uma via que requer secreção plaquetária, ativação de canais de cálcio e produção de eicosanoides derivados de lipoxigenase. Nós investigamos se a urease de *H. pylori* ativa plaquetas e definimos a rota envolvida neste fenômeno. Para isso os efeitos da urease recombinante de *H. pylori* em plaquetas de coelhos foram monitorados turbidimetricamente, e a secreção de ATP e a produção de metabólitos de lipoxigenase por plaquetas ativadas foram medidos. HPU marcada com FITC ligou-se a plaquetas, mas não a hemácias. HPU induziu agregação de plaquetas de coelhos (ED_{50} 0,28 μM) acompanhada por secreção de ATP. Não foi observada correlação entre ativação plaquetária e atividade ureolítica de HPU. Agregação plaquetária foi bloqueada por esculetina (inibidor de lipoxigenase) e aumentada aproximadamente 3 vezes por indometacina (inibidor de ciclooxigenase). Um metabólito de 12-lipoxigenase foi produzido por plaquetas expostas a HPU. Agregação plaquetária ativada por HPU não envolveu o fator ativador de plaquetas, sendo dependente da ativação de canais de cálcio inibíveis por verapamil. Esta propriedade parece ser comum às ureases independente de sua origem (vegetal ou bacteriana) ou estrutura quaternária. Estas propriedades de HPU podem desempenhar um importante papel na patogênese de doenças gastrointestinais e cardiovasculares associadas com *H. pylori.*

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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 (ED_{50} 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-eicosatetranoic 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-phosphocholine) 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 $\times$ *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

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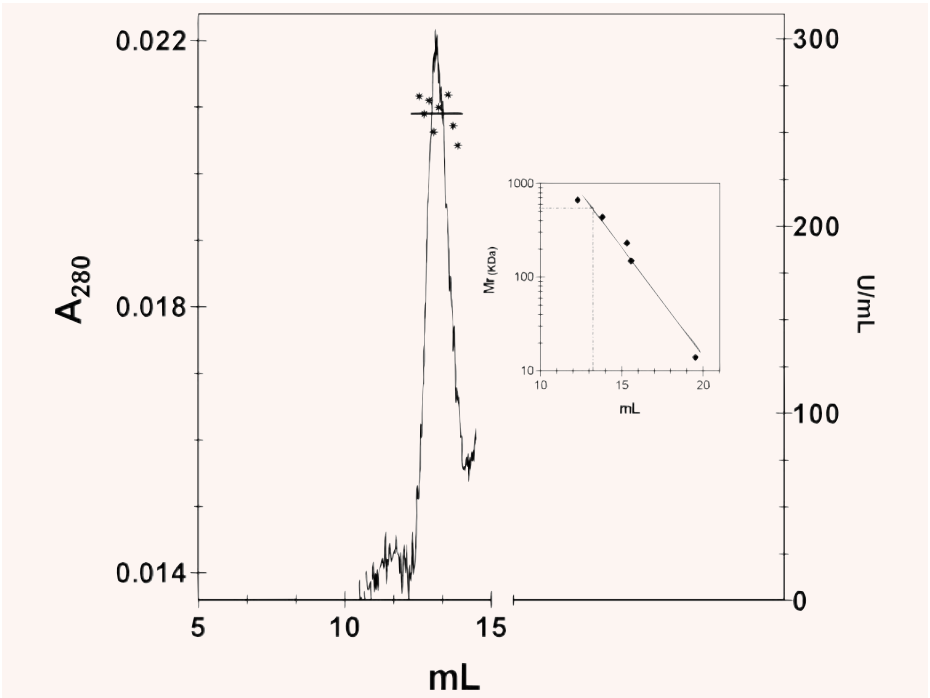

Fig. 1 Purification of HPU. The figure shows the chromatographic pattern of HPU obtained in the last purification step consisting of a gel-filtration in a Superose 6 HR column. Fractions within the peak eluted at 13.2 ml all showed similar values of urease activity (asterisks), mean value of 264.4 ± 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 μmol 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 × *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 μl with saline. Aggregation was triggered by the addition of 100 μl 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 μm polycarbonate membranes (Millipore). The fixed platelets were sequentially dehydrated in 30%, 50%, 70% and 90% (v/v) acetone, for 5 min. each,

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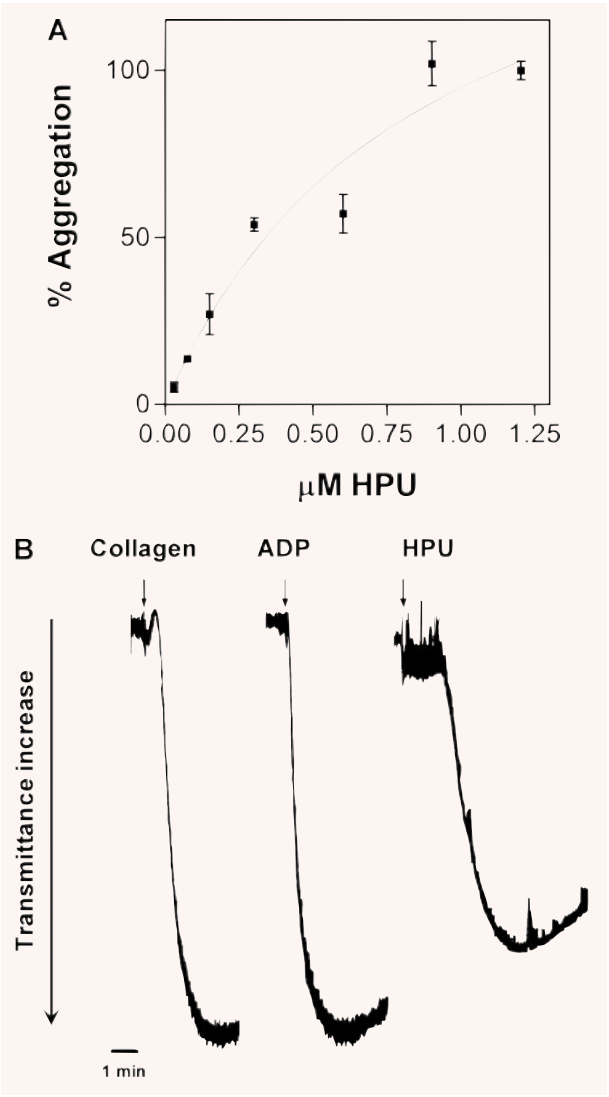

Fig. 2 Aggregation of rabbit PRP suspensions induced by purified HPU. (**A**) Rabbit platetet-rich plasma suspension in microwell plates were exposed to increasing concentrations of HPU or 5 μM ADP (100% aggregation). Aggregation of platelets was monitored every 11 sec. during 20 min. in a SpectraMax plate reader. Results (means ± S.D.) are expressed as percentage of maximal aggregation for four replicates. (**B**) Comparison of aggregation tracings of platelets stimulated by collagen (30 μg/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-lipoxigenase 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 × *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 × *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-hydroxide-eicosatetraenoic 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 ED_{50} of *ca.* 150 μg/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 (ED_{50} 15.8 μg/ml) [13, 17, 18]. Scanning electron microscopy of platelets exposed to HPU

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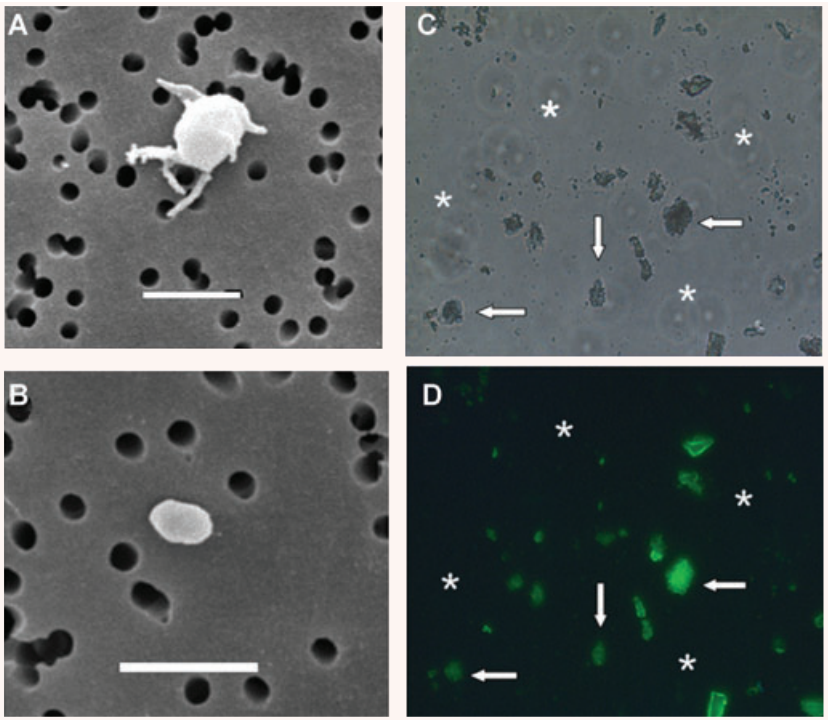

Fig. 3 Scanning electronic, light and fluorescence microscopy of platelets treated with HPU. (**A**) Scanning electronic microscopy of a single rabbit platelet after exposition to 0.3 μM HPU for 2 min. under low stirring (to avoid aggregation). The morphology is typical of an activated platelet showing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

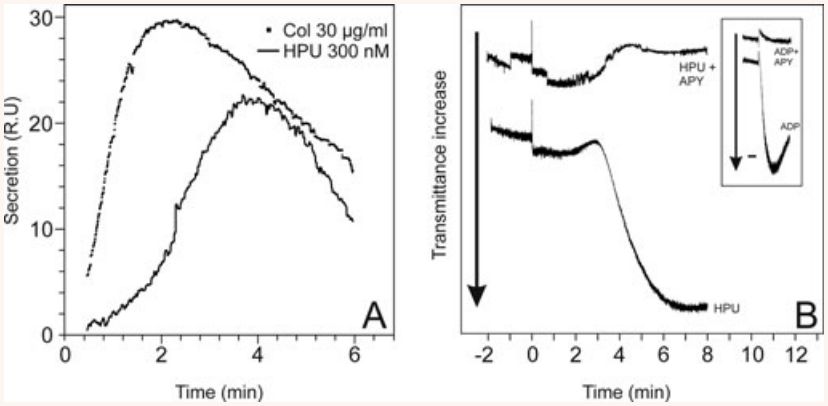

Fig. 4 HPU induces release reaction and ADP-induced aggregation of rabbit platelets. (**A**) Time course of release reaction of platelets activated by HPU (300 nM) or collagen (30 μg/ml) was detected following ATP secretion as light emitted in the presence of a luciferin-luciferase mixture. L.R.U. – light relative units. A typical result out of three replicates is shown. (**B**) Platelet aggregation induced by HPU (300 nM) or 5 μM 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 μM 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).

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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 ± S.D. (*N*)
None		100.00 ± 10.24 (4)
Dexamethasone	(50 μM)	62.64* ± 6.06 (4)
Esculetin	(500 μM)	55.00** ± 6.06 (4)
Indomethacin	(150 μM)	160.74*** ± 12.74 (4)
	(300 μM)	313.26*** ± 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 μM). 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 A_2 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 A_2, 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 μM Web2170 inhibited only 16% the aggregation induced by HPU, while it blocked almost 90% of platelets response to a very high 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).

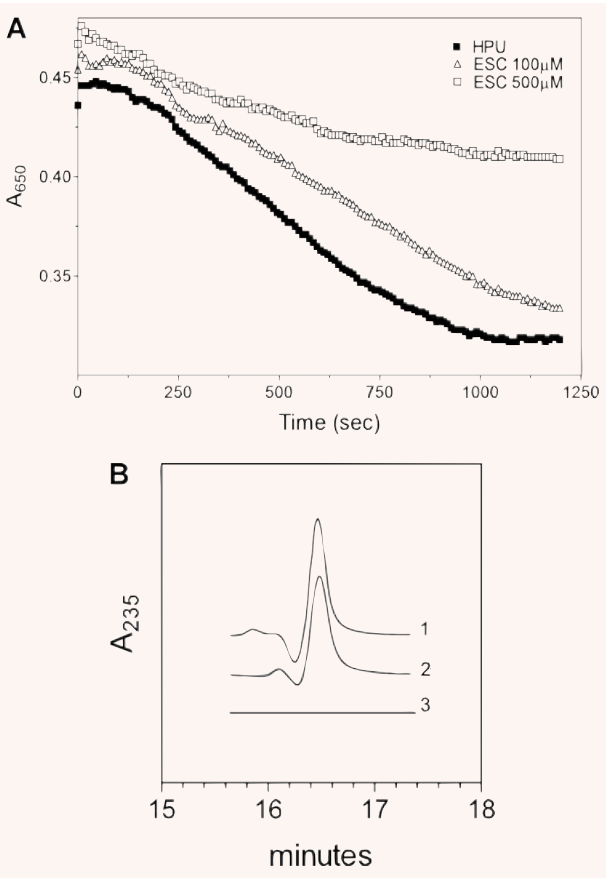

Fig. 5 Involvement of lipoxygenase-derived metabolite(s) in HPU-induced platelet aggregation. (**A**) Rabbit platetet-rich plasma suspensions in microwell plates were incubated for 2 min. at room temperature in the absence or in the presence of the indicated concentrations of esculetin and aggregation was triggered by addition of HPU (0.3 μM). 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 μM 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.

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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 ± S.D. (*N*)
0.3 μM HPU	100.00 ± 7.43 (4)
50 μM Verapamil + HPU	80.83* ± 1.58 (4)
75 μM Verapamil + HPU	63.35*** ± 1.19 (4)
2 μM Web2170 + HPU	83.94* ± 6.45 (4)
50 nM PAF-acether	100.00 ± 3.21 (4)
2 μM Web2170 + PAF acether	13.1*** ± 1.62 (4)

Rabbit platetet-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 μM) or PAF-acether (50 nM) and monitored ever 11 sec. during 20 min. in a SpectraMax plate reader. Results are means ± 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		
D. peruvianus, LD50	0.01% (w/w)	Not toxic	ND
Treated with pHMB	100% active		
Platelet aggregation, EC_{50} (rabbit)	22.2 μg/ml	400 μg/ml	150 μg/ml
Treated with pHMB	100% active	ND	ND
Platelet secretion	Yes (serotonine)	ND	Yes (ATP)
Lipoxygenase inhibitors	Inhibition	Inhibition	Inhibition
Cyclooxygenase inhibitors	Potentiation	Potentiation	Potentiation
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.

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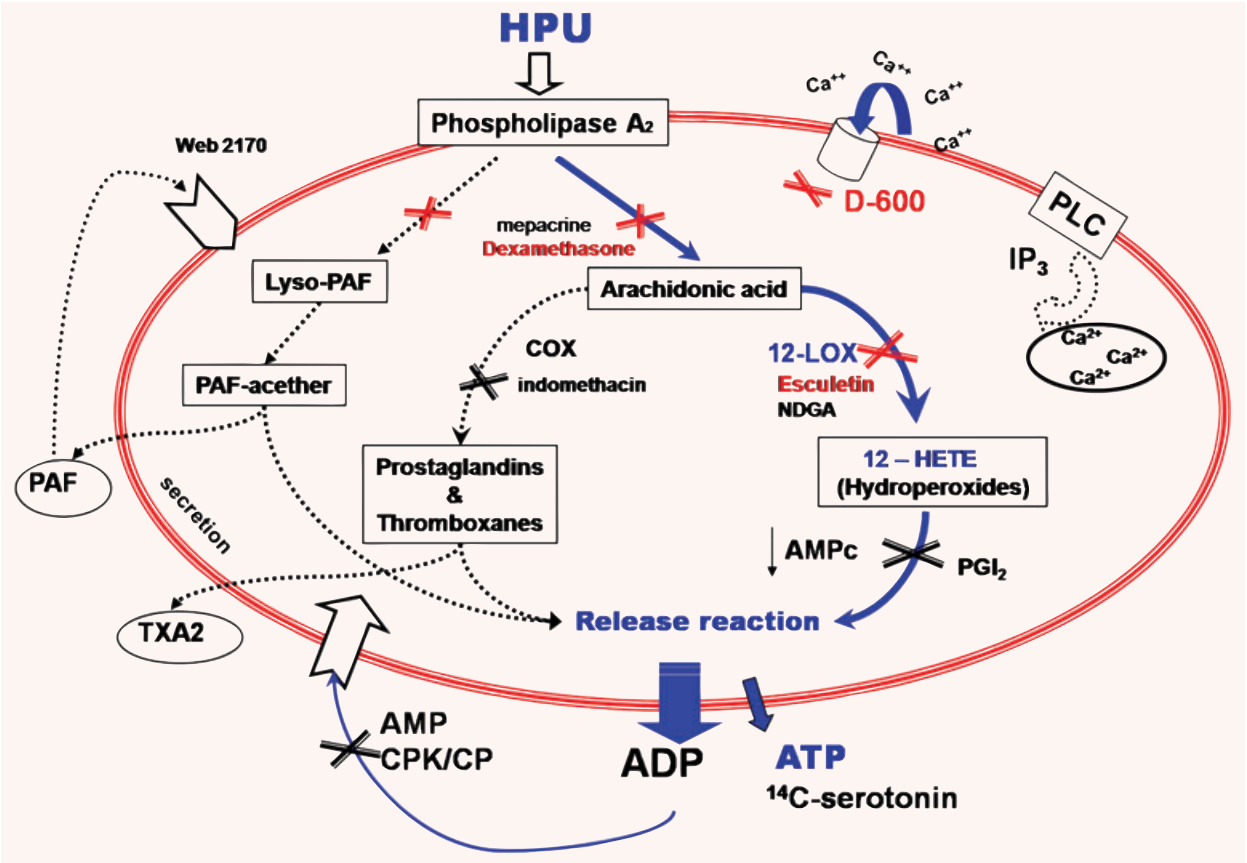

Fig. 6 Proposed mechanism of platelet aggregation induced by *Helicobacter pylori* urease. The biochemical pathways that underlie the platelet-aggregating activity of HPU as well as all ureases studied so far [13, 17–20, 25] are indicated as continuous lines. As depicted in blue, HPU activates platelets 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 Ca^{2+} channels inhibited by verapamil [18] (this work), and occurs without activation of phospholipase C or production of IP_3 [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.

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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 glycogonjugates [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 lipoxygenase-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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Capítulo 2. Estudos com a HPU em Neutrófilos Humanos

- **Ensaio de migração de neutrófilos humanos**

A HPU apresenta efeito quimiotático para neutrófilos. Na figura 5, podemos ver que HPU induziu, de modo dose-dependente, a migração de neutrófilos humanos. Na dose de 100 nM, o efeito quimiotático da HPU foi semelhante ao do controle positivo, fMLP, na mesma concentração.

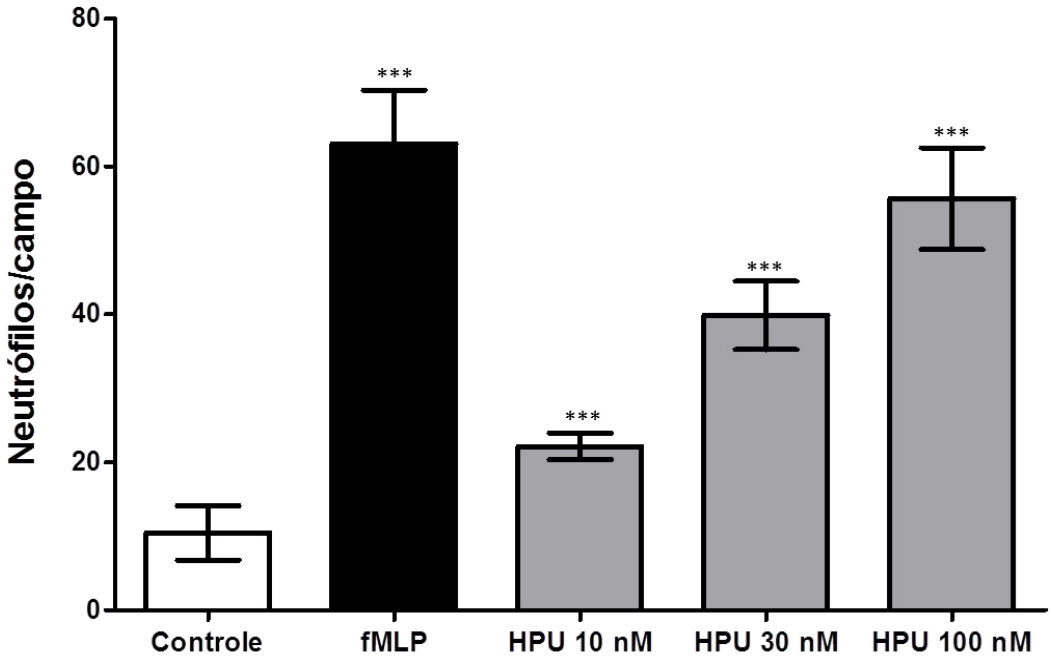

Figura 5. Efeito de HPU na quimiotaxia de neutrófilos humanos. A quimiotaxia foi analisada em Câmara de Boyden de 48 poços. Neutrófilos (10^6 cells/mL em RPMI-0.01% BSA) foram testados com HPU (10 nM, 30 nM e 100 nM) e FMLP (100 nM), ou apenas meio de cultura (migração aleatória; 37°C, 5% CO_2).

- **Efeito de HPU na apoptose de neutrófilos**

A regulação da apoptose em neutrófilos é crucial para a instalação de uma inflamação aguda, sendo retardada em neutrófilos humanos ativados por IL-8, GM-CSF, LPS, ou leucotrieno B4 (Lee *et al*., 1993; Savill *et al*., 2002). Nossos dados mostram que a exposição a 100 nM de HPU protege neutrófilos contra a apoptose, de uma maneira ainda mais intensa que IL-8, na mesma dose. O gráfico da figura 6 mostra uma análise morfológica, através da contagem de núcleos picnóticos, característicos de células apoptóticas.

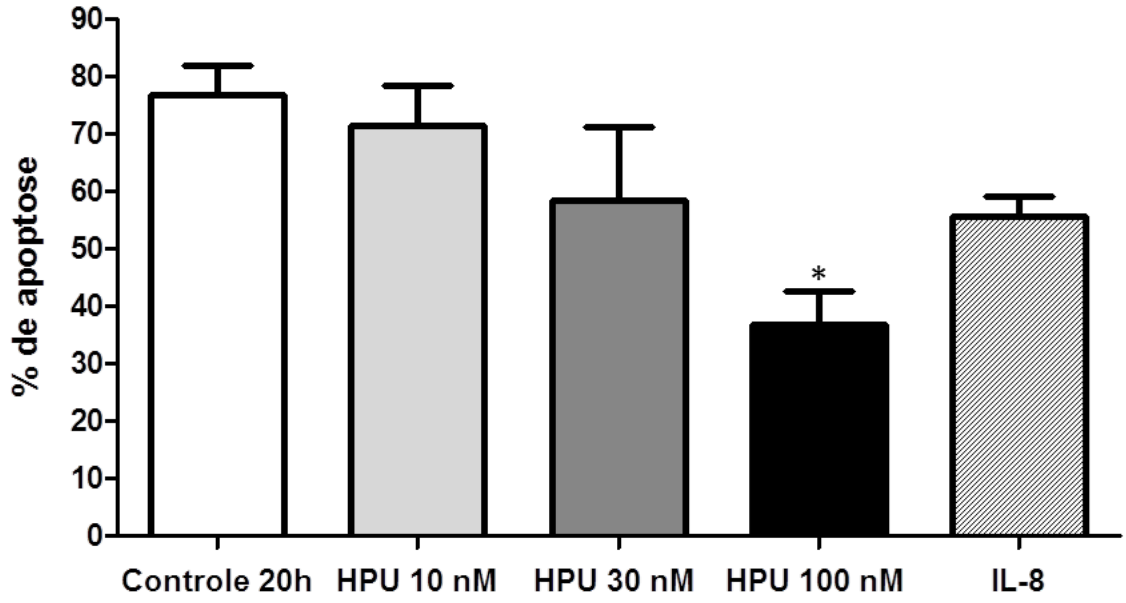

Figure 6. HPU inibe a apoptose de neutrófilos humanos. Neutrófilos (5 x 10^6/ml) foram incubados na presença ou ausência de variadas concentrações de HPU (10 nM, 30 nM e 100 nM) ou IL-8 (100 nM). *P < 0.05 comparado ao controle.

Para confirmar o achado de redução de apoptose, os níveis de proteínas pró- e antiapoptóticas foram medidos em neutrófilos ativados por HPU. Os dados das figuras 7 e 8 corroboram o dado morfológico. Neutrófilos ativados por HPU mostraram taxas

aumentadas de degradação de Bad, uma proteína pró-apoptótica, e níveis diminuídos de Bcl-X_L, uma proteína antiapoptótica.

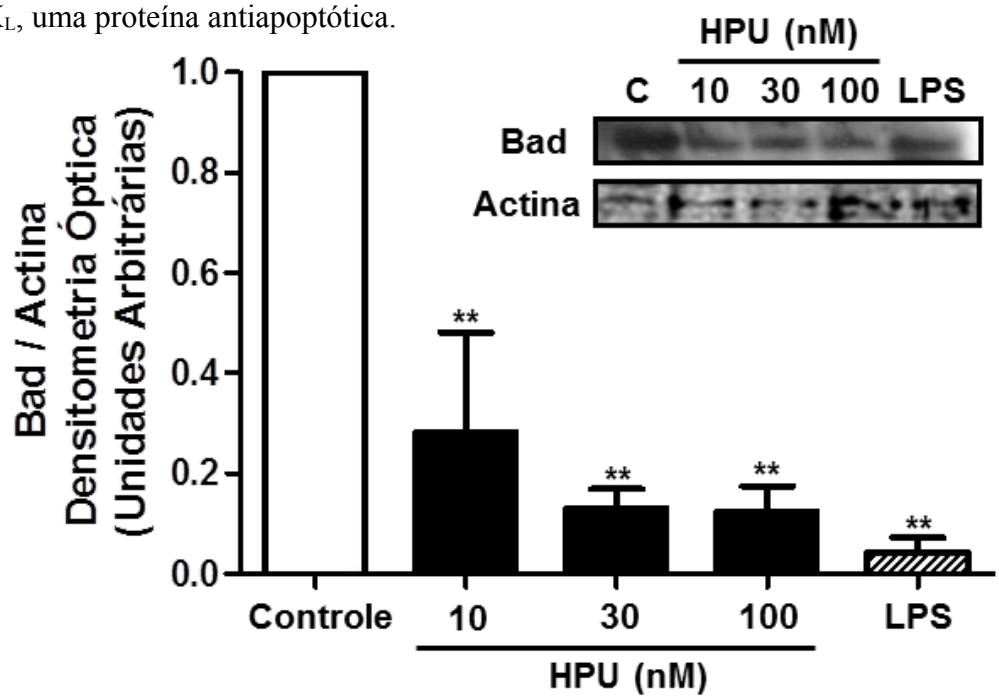

Figura 7. Degradação de Bad induzida por HPU. Neutrófilos humanos (5 x 10^6 células) foram incubados com HPU (10 nM, 30 nM e 100 nM) e LPS (1 µg/mL). Após 4 h, a expressão de Bad foi medida através de análise por Western Blot. Asteriscos = $p < 0,01$ ANOVA

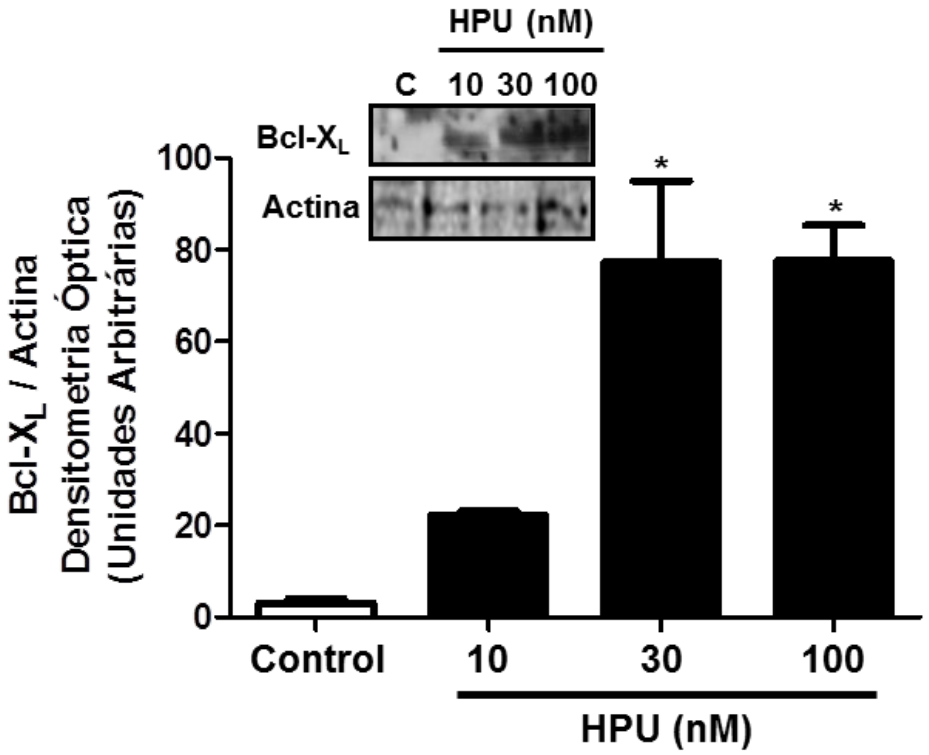

Figura 8. Indução da expressão de Bcl-X_L por neutrófilos ativados por HPU. Neutrófilos (5 x 10^6/ mL) foram incubados em DMEM 10% e estimulados ou não por HPUpor 4 horas. A expressão de Bcl-XL foi analisada por Western Blot. O asterisco significa $p < 0,05$ ANOVA.

- **Neutrófilos ativados por HPU produzem espécies reativas de oxigênio**

Para medir a produção total de ROS, foi utilizada a sonda luminescente luminol. Como a produção de ROS compreende uma descarga intracelular e outra extracelular, foram realizadas medidas usando as sondas lucigenina (luminescente; detecta ROS extracelular) e CM-H_2DCFDA (fluorescente [λ_{ex}470nm/λ_{em}529nm]; detecta ROS intracelular).

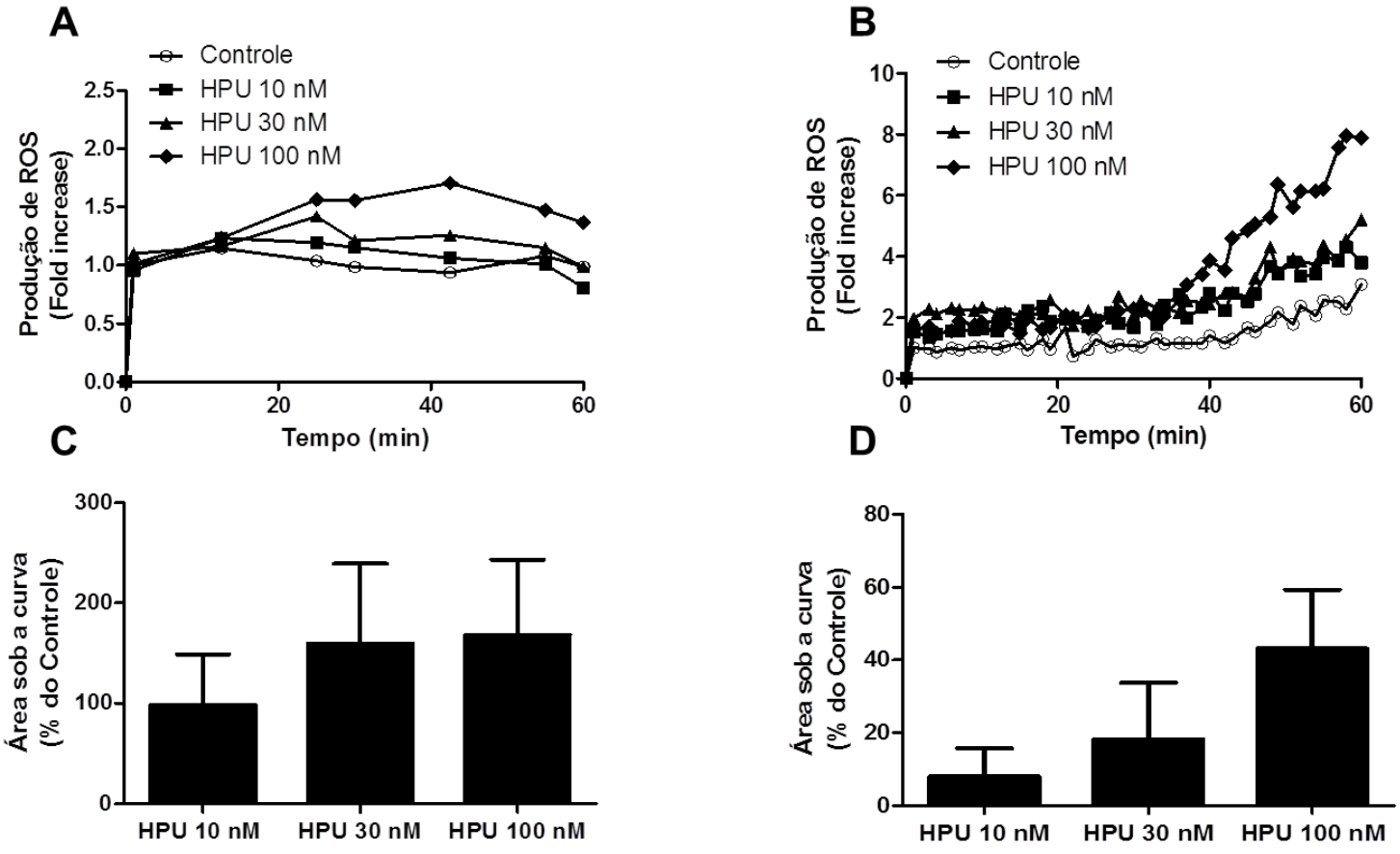

Figura 9. Produção de ROS em neutrófilos ativados por HPU. Neutrófilos (6 x 10^5 células/poço) foram colocados em uma placa branca de 96 poços para o ensaio de luminescência com lucigenina. (A) Células não estimuladas (controle negativo) ou estimuladas com HPU (10, 30 e 100 nM) foram avaliadas na presença de lucigenina (25 μM) durante 60 minutos. (B) O mesmo experimento foi realizado usando

luminol como sonda quimioluminescente. O nível de ROS acumulado foi avaliado como a área sob a curva de emissão da luminescência de lucigenina (C) e luminol (D) por 60 minutos de tratamento.

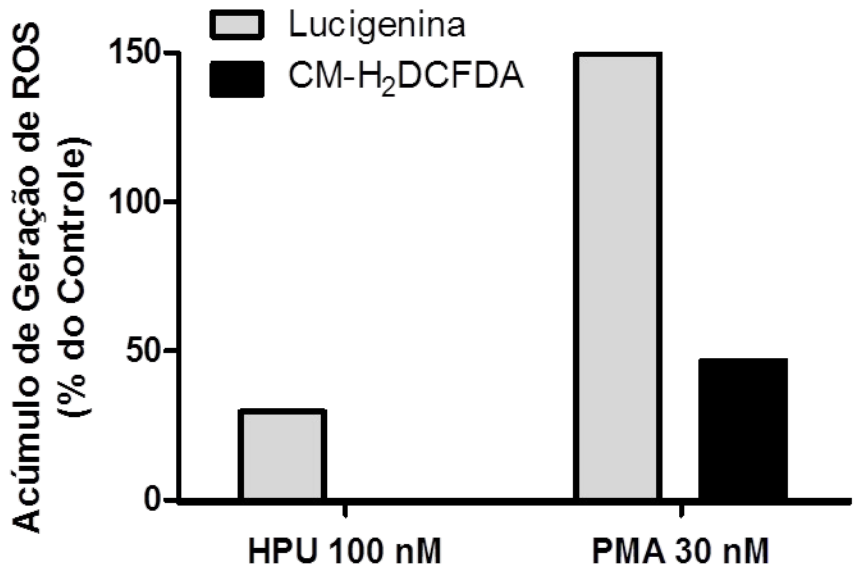

Figura 10. HPU promove a produção de ROS extracelular. Neutrófilos foram colocados em uma placa branca de 96 poços para o ensaio quimioluminescente com lucigenina, e em uma placa preta para o ensaio fluorescente com a sonda CM-H_2DCFDA. Células não estimuladas (controle negativo) ou estimuladas com HPU (10, 30 e 100 nM) foram avaliadas na presença de lucigenina (25 μM) ou CM-H_2DCFDA (5 μM). O acúmulo de ROS foi calculado como a área sob a curva da luminescência de lucigenina e pela fluorescência emitida por CM-H_2DCFDA durante 120 minutos.

Como pode ser visto na figura 10, a produção de ROS em neutrófilos ativados por HPU é exclusivamente extracelular. Para controle positivo, as células foram estimuladas com 30 nM miristato de forbol ester (PMA) (dados não mostrados).

- **Indução da expressão de 5-LO**

Assim como em plaquetas, verificamos que a ativação de HPU provavelmente induz a expressão de lipoxigenase em neutrófilos.

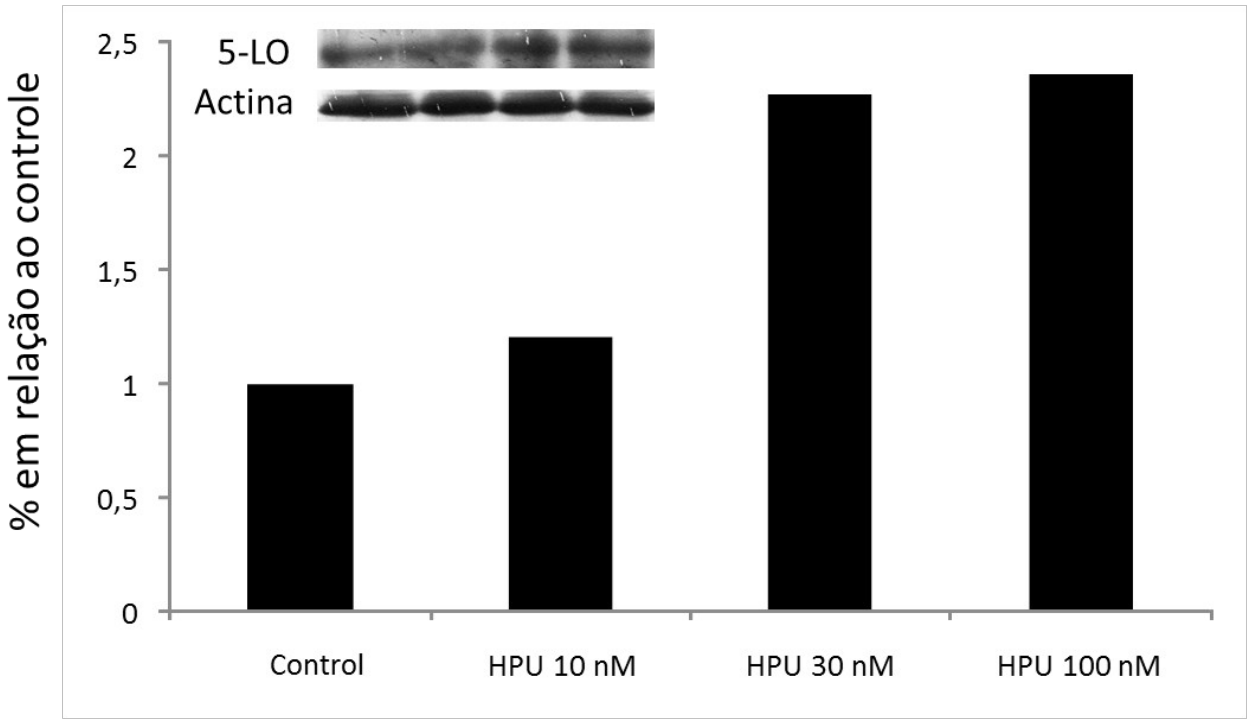

Figura 11. HPU induz a expressão de 5-LO em neutrófilos. Neutrófilos humanos (5 x 106 células) foram incubados com HPU (10 nM, 30 nM e 100 nM). Após 4 h, a expressão de 5-LO foi medida por Western Blot. Dados mostrados são resultado de um único experimento.

Nas mesmas condições, não observamos qualquer alteração nos níveis de ciclo-oxigenase(s) em neutrófilos humanos ativados por HPU.

4. Discussão

As ureases são proteínas altamente conservadas, independente de sua origem e de sua organização terciária, apresentando estrutura quaternária e mecanismos catalíticos similares. Nesse trabalho mostramos que ureases vegetais e bacterianas também conservam propriedades não-enzimáticas em comum, em particular, a capacidade de ativar exocitose, recrutar a rota dos eicosanóides, e potencial pró-inflamatório. Com base nos resultados aqui apresentados, que caracterizam outras propriedades não enzimáticas da HPU, postulamos que esta proteína possivelmente está envolvida em outros processos da patogênese causada por *H. pylori*, além de promover a alcalinização do ambiente gástrico, formando um microclima que possibilita a colonização da mucosa gástrica pela bactéria.

Os dados aqui apresentados mostram que a urease de *H. pylori* recruta em plaquetas de coelho as mesmas vias de sinalização já descritas para outras ureases, como a canatoxina (isoforma de urease de *Canavalia ensiformis*) (Carlini *et al*., 1985; Ghazaleh *et al*., 1997) e a urease de *Bacillus pasteurii* (Olivera-Severo *et al*., 2006). Complementando os dados obtidos por Wassermann, 2007, nesse trabalho demonstrando que verapamil, um bloqueador de canais de cálcio voltagem-dependente, diminuiu consideravelmente a agregação induzida por HPU, indicando que a resposta das plaquetas é dependente de influxo de cálcio do meio externo. Ghazaleh *et al.,* 1997, mostraram resultados semelhantes de inibição por verapamil para a ativação de plaquetas induzida por canatoxina, demonstrando tanto o influxo de cálcio do meio externo e como o fato de não haver aumento de inositol trifosfato e mobilização do "pool" intracelular de cálcio.

Para elucidar se outras rotas seriam recrutadas por HPU para induzir agregação plaquetária, pré-tratamentos com Web2170 (antagonista de PAF) foram realizados. O resultado da Tabela 2 do artigo indica que a agregação plaquetária induzida por HPU é independente da síntese de PAF, um fosfolipídio conhecido por seu potencial de agregação e secreção em plaquetas. A resposta de plaquetas à canatoxina também foi descrita como sendo independente de PAF-acéter (Carlini *et al.,* 1985).

Por outro lado, pudemos demonstrar que a resposta de agregação das plaquetas à HPU é sustentada exclusivamente pelo ADP endógeno liberado pelas plaquetas ativadas, sem produção de outros agonistas, como PAF-acéter ou tromboxane A2. Tal demonstração foi possível utilizando AMP (como antagonista do receptor de ADP), e apirase (consumindo rapidamente o ADP liberado), que causaram completa inibição da agregação induzida por ADP (figura 4B do artigo). Em Carlini *et al*., 1985, resultados equivalentes indicando a dependência do ADP liberado para a resposta de plaquetas à canatoxina foram obtidos utilizando-se o antagonista AMP, o sistema *scavenger* creatina quinase-creatina fosfato e ensaios com plaquetas degranuladas por trombina.

Os efeitos da canatoxina e da urease de *Bacillus pasteurii* são abolidos em plaquetas pré-tratadas com inibidores da 12-lipoxigenase, como esculetina e ácido nordiidroguairético, indicando o recrutamento da via dos eicosanóides (Carlini *et al*., 1985; Barja-Fidalgo *et al*., 1991; Olivera-Severo *et al*., 2006). Utilizando esses inibidores, Wassermann, 2007, observou que o efeito indutor de agregação plaquetária da urease de *H. pylori* também é mediado por eicosanóides da via de lipoxigenases. Nesses estudos anteriores, a participação da 12-lipoxigenase foi deduzida com base no efeito de inibidores, sem ter havido identificação/quantificação de metabólitos da enzima. No presente trabalho (figura 5B do artigo), pudemos demonstrar que o metabólito 12-HETE (ácido 12-hidróxi-eicosatetraenóico) é produzido por plaquetas agregadas por HPU. O 12-HETE deriva do 12-HPETE (ácido 12-hidroxiperoxi-eicosatetraenóoico) altamente instável, um conhecido sinalizador de respostas

inflamatórias celulares, que leva à expressão de citocinas pró-inflamatórias e indução de apoptose em células do epitélio gástrico. Em plaquetas induzidas por colágeno, um potente ativador plaquetário fisiológico, a mesma indução de metabólitos de lipoxigenases foi observada (Chen *et al*. 2008; Wen *et al.* 2007).

A urease de *H. pylori* parece ligar-se especificamente à membrana em plaquetas (figuras 3C e 3D do artigo), ativando-as para a morfologia típica com pseudópodes (figura 3A do artigo), sem interagir com eritrócitos eventualmente presentes no mesmo meio. Esses corroboram dados prévios do nosso grupo, de que a urease de *H. pylori* realmente induz agregação de plaquetas, e não um efeito lítico, que também resultaria em liberação do conteúdo intracelular e uma diminuição de turbidez da suspensão de plaquetas, como registrado no lummi-agregômetro. Em outros estudos, observamos que, em concentrações até cinco vezes maiores que a utilizada para agregar plaquetas, a HPU não tem efeito lítico sobre células de gliomas em cultura (dados não mostrados). Também para a canatoxina foi demonstrado não haver efeito lítico sobre vários tipos celulares, normais ou tumorais (Campos *et al*., 1991).

Considerando que estudos epidemiológicos (Mendall *et al. 1994;* Jin *et al.* 2007) mostram uma correlação positiva entre doenças cardíacas tromboembólicas, como a doença cardíaca coronariana, e portadores de *H. pylori*, a propriedade da urease de *H. pylori* de agregar plaquetas pode ter papel fundamental no desenvolvimento destas patologias (Pellicano *et al*., 1999). Sabe-se que a HPU está localizada no citoplasma e embora não seja secretada, quando há lise das bactérias, a enzima é liberada no meio. Como o *H. pylori* também danifica as junções oclusivas entre as células epiteliais gástricas Wroblewski *et al*., 2009), a HPU eventualmente tem acesso aos microcapilares e às células sanguíneas.

Anteriormente ao nosso trabalho, somente a canatoxina tinha sido estudada quanto ao potencial pró-inflamatório em ratos, utilizando-se tanto modelos *in vivo* como

ex-vivo (Benjamin *et al*., 1992; Barja-Fidalgo *et al*., 1992). Em 2007, Wassermann, demonstrou que a urease de *Helicobacter pylori* também compartilha essa propriedade, sendo que estes dados fazem parte do 2º manuscrito dessa dissertação, apresentado no anexo. Nosso objetivo aqui foi investigar mais a fundo a participação da HPU, em especial seu efeito em neutrófilos, nos processos inflamatórios desencadeados pela infecção por *H. pylori*.

Sabe-se que a inflamação induzida por *H. pylori* é caracterizada por uma grande infiltração de neutrófilos, e que a densidade de neutrófilos no sítio inflamatório está correlacionada com o dano tecidual (D'Elios *et al*., 2007). Sendo um patógeno não invasivo, o *H. pylori* estimula a resposta inflamatória através da liberação de diferentes compostos pró-inflamatórios, e é provável que HPU esteja intimamente ligada a esse processo (Hatakeyama, 2006; Isomoto *et al*., 2010). Nesse trabalho, demonstramos que a HPU tem um grande potencial quimiotático em neutrófilos, provendo migração das células em doses equivalentes e na mesma intensidade que o fMLP (n-formil-metionil-leucil-fenilalanina) (Niedel *et al*., 1979), um peptídeo sintético que mimetiza peptídeos bacterianos. Barja-Fidalgo *et al*., 1992, mostraram que a canatoxina induz a migração de neutrófilos para cavidades pleurais e *air-pouch*. Também foi observado que a canatoxina induziu a liberação de um fator quimiotático por macrófagos.

A regulação da apoptose de neutrófilos é um processo importante na resolução da inflamação. Neutrófilos agem liberando enzimas proteolíticas e espécies reativas de oxigênio, induzindo dano tecidual, e são removidos do sítio inflamatório através da indução de apoptose. Os níveis de proteínas pró e anti-apoptóticas são críticos no controle da apoptose. Neutrófilos humanos tem uma meia-vida de 12 horas, caracterizada pela expressão constitutiva de proteínas pro-apoptóticas e níveis quase não detectáveis de proteínas anti-apoptóticas (Akgul *et al*., 2001). Nossos dados mostram que HPU protege neutrófilos contra a apoptose, o que os mantêm ativos por mais tempo, desencadeando um processo inflamatório local, como evidenciado no ensaio de

edema de pata em camundongos. A HPU estimula a degradação da proteína pró-apoptótica Bad, e induz a expressão da proteína anti-apoptótica Bcl-X_L, contribuindo para a manutenção e persistência de neutrófilos ativos no local de inflamação.

Estudos recentes mostram que a gastrite associada com a infecção por *H. pylori* estimula a geração de espécies reativas de oxigênio por células inflamatórias presentes na mucosa gástrica (Handa *et al*., 2010). A produção total de ROS compreende a liberação intra e extracelular. O aumento na produção de ROS está associado a um aumento nos níveis de reparo ao DNA em células de epitélio gástrico (Machado *et al*., 2010). Mostramos aqui que a HPU induz um aumento significativo na produção total de ROS por neutrófilos humanos. Analisando a localização das ROS produzidas, podemos verificar que HPU induz uma liberação extracelular, corroborando dados que mostram que *H. pylori* pode interferir na atividade da NADPH oxidase de neutrófilos humanos, induzindo a liberação extracelular de ROS (Allen *et al*., 2005).

Recentemente tem sido proposto um papel importante para a via do ácido araquidônico no desenvolvimento de inflamação crônica e carcinogênese gástrica (Venerito *et al*., 2008; Wang *et al*., 2010). Metabólitos de lipoxigenase, como LTB_4, podem aumentar a proliferação de células epiteliais e induzir oncogenes nestas células (Chen *et al*., 2004). Assim como em plaquetas, nossos dados mostram que a HPU aumenta os níveis de lipoxigenase de neutrófilos, sugerindo o recrutamento da rota do ácido araquidônico (figura 11).

5. Conclusões

Nossos dados podemos concluir que HPU está envolvida nos seguintes processos:

1) **Em plaquetas:**

   a) A HPU ativa plaquetas de coelho de maneira dose-dependente, levando a shape-change, agregação e degranulação;

   b) Parece haver uma interação específica da HPU com membranas de plaquetas;

   c) A resposta de agregação das plaquetas à HPU é sustentada exclusivamente pelo ADP endógeno liberado pelas plaquetas ativadas;

   d) Plaquetas ativadas por HPU produzem 12-HETE, um metabólito de 12-lipoxigenase;

   e) A agregação plaquetária ativada por HPU é dependente da ativação de canais de cálcio voltagem-dependentes, mas é independente de PAF e de tromboxane A2, importantes agonistas plaquetários.

2) **Em neutrófilos:**

   a) A HPU induz, de maneira dose-dependente, migração de neutrófilos humanos;

   b) Neutrófilos ativados por HPU produzem espécies reativas de oxigênio exclusivamente para o meio extracelular;

   c) A ativação por HPU protege neutrófilos humanos contra apoptose, aumentando sua meia-vida;

   d) Neutrófilos ativados por HPU mostram aumento nos níveis de 5-lipoxigenase (Dados preliminares).

3) As propriedades não-enzimáticas da urease de *Helicobacter pylori* aqui demonstradas são relevantes para o entendimento dos processos inflamatórios desencadeados pela infecção por esta bactéria.

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Mouse Paw Edema and Human Neutrophil Activation by a Recombinant Urease from *Helicobacter pylori*

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Abstract

Ureases (EC 3.5.1.5), nickel-dependent enzymes that hydrolyze urea into ammonia and CO_2, are produced by plants, fungi and bacteria. Previous data of our group showed that ureases display ureolysis-independent effects, promoting exocytosis in several cell types through a lipoxygenase-derived eicosanoid-dependent pathway.

The spirochete *Helicobacter pylori*, an etiological agent of gastric ulcers, is possibly involved in the development of gastric cancer. Urease produced by *H. pylori* is an important virulence factor as its ureolytic activity enables the bacterium to survive in the acidic medium of the stomach. In this work, we used a recombinant *H. pylori* urease (rHPU) produced in *Escherichia coli* to evaluate biological effects independent of its enzyme activity. rHPU induced mouse paw edema in a dose (0.5 to 45 μg) and time-dependent (peak at 6 h) manner. Mouse paw edema induced by rHPU was partially inhibited in mice pretreated with lipoxygenase inhibitors. Moreover rHPU (100 nM) induced chemotaxis of human neutrophil (88% of that observed for fMLP), accompanied by ROS production, and decreased their apoptosis rate (40.5% compared to control). Consistent with the observed decrease in apoptosis, rHPU-treated neutrophils showed an increase in the Bcl-X_L content and a decrease in Bad levels. These effects of rHPU persisted even when its enzyme activity was blocked by acetohydroxamic acid or p-hydroxymercurybenzoate. Treatment of human neutrophils with rHPU (100 nM) led to a 2.4-fold increase in lipoxygenase levels, as determined by immunoblotting, while no alteration of cycloxygenase levels was detected.
These pharmacological properties indicate that HPU could play an important role in the pathogenesis of the gastrointestinal inflammatory disease caused by *H. pylori*.

Keywords: urease, inflammation, neutrophils, apoptosis, chemotaxis

Introduction

Helicobacter pylori infects at least half of the world´s population and is the 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 [1]. Gastric colonization with *H. pylori* is usually accompanied by infiltration of polymorphonuclear leukocytes, macrophages and lymphocytes. The degree of mucosal damage is correlated with the degree of neutrophil infiltration [2]. Neutrophils act 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 [3].

H. pylori causes gastric ulcers and gastric adenocarcinoma by mechanisms not fully understood [4,5].

This bacterium produces factors that damage gastric epithelial cells, such as the vacuolating cytotoxin VacA, the cytotoxin-associated protein CagA, and a urease 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 [5,6].

The urease of *Helicobacter pylori* accounts for about 10% of total protein and is consistently present in all naturally occurring strains [7]. It is known that genetically engineered urease-deficient *H. pylori* is unable to colonize either germfree piglets, ferrets, or mice [8,9,10]. Reports show that *Helicobacter pylori* whole cells or extracts of its water-soluble proteins promote inflammation, activate neutrophils and release cytokines [11,12]. *H. pylori* urease can stimulate macrophages, eliciting the production of reactive species and cytokines, and, thus, mediate tissue inflammation and injury [13]. We have previously reported that *H. pylori* urease activates platelets through a lipoxygenase-mediated pathway, leading to ADP exocytosis and, therefore, platelet aggregation [14].

The aim of this study was to evaluate the participation of *H. pylori* urease (HPU) in the inflammatory process promoted by this bacterium. For that purpose we worked with a purified recombinant HPU (rHPU) produced in *E. coli*. Our results showed that rHPU induces: (i) rat paw edema; (ii) human neutrophil migration; (iii) protection of human neutrophils against apoptosis; and (iv) induction of expression of lipoxygenase(s) in human neutrophils.

Materials and Methods

Recombinant *H. pylori* urease (rHPU)

Helicobacter pylori recombinant urease (rHPU) was produced by heterologous expression in *Escherichia coli* SE5000 transformed with plasmid pHP8080 [15], kindly provided by Dr. Harry T Mobley, University of Michigan Medical School. HPU was purified from bacterial extracts according to Wassermann et al., 2010. Briefly, after

cultivation, cells were harvested by centrifugation, suspended in 20 mM sodium phosphate, pH 7.5 containing 1 mM 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 sulfate precipitation. The precipitate formed between 0.3-0.7 saturation was dissolved in EB and dialyzed to remove the excess of salt. This material was submitted to anion exchange chromatography in Q-Sepharose (GE Healthcare) 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 stepwise eluted and the urease-enriched fraction was recovered with EB containing 200 mM NaCl, pH 7.8. After dialysis and concentration on Centriprep (Millipore) 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 per min. The fractions with urease activity were pooled and freeze-dried (in EB buffer). The specific activity of purified rHPU was typically 252 U/mg of protein. 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.

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 [16]. One unit of urease releases one μmol of ammonia per min, at 37°C, pH 7.5. For studies of urease inhibition, protein solutions were incubated overnight ar 4°C with 1 mM p-hydroxymercurybenzoate or with 10 mM acetohydroxamic acid followed by extensive dialysis to remove excess of inhibitor.

Neutrophil isolation and culture

Neutrophils were isolated from EDTA (0.5%)-treated peripheral venous blood of healthy human volunteers by Percoll gradient [17] and suspended in RPMI medium (97% of viable cells, as assessed by trypan blue exclusion). Residual erythrocytes were removed by hypotonic lysis.

Neutrophil migration assay

Chemotaxis was assayed in 48-well microchemotaxis chambers (NeuroProbe, Gaithersburg, MD) using 5-μm PVP-free polycarbonate filter [17]. Neutrophils (10^6 cells/mL in RPMI-0.01% bovine serum albumin [BSA]) were allowed to migrate toward formyl-methionylleucyl-phenylalanine (fMLP, 700 nM), rHPU (10 nM, 30 nM, 100 nM) and medium alone (random migration; 37°C, 5% CO_2). After 1 hour, 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 [17]. Each treatment was assayed in triplicate. Results are expressed as number of neutrophils per field ± S.D.

Assessment of neutrophil apoptosis

Morphology. Cells were cytocentrifuged, stained with Diff-Quik (Dade Behring, Switzerland), and counted under light microscopy (x 1000) to determine the proportion of cells showing characteristic apoptotic morphology. At least 400 cells were counted per slide. The results were expressed as mean ± SD.

Preparation of cell extracts

For obtaining whole cell lysates, neutrophils (5×10^6cells/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 the following protease inhibitors cocktail: 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).

Western blot analysis

The total protein content in the cell extracts was determined by the Bradford's method [18]. 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 by 12% SDS–PAGE and proteins were transferred to PVDF membranes (Hybond-P, Amersham Pharmacia Biotech). Rainbow markers (Amersham Pharmacia Biotech) were run in parallel to estimate molecular weights. Membranes were blocked with Tween-TBS (20 mM Tris–HCl, pH 7.5, 500 mM NaCl, 0.1% Tween-20) containing 1% bovine serum albumin and probed with polyclonal anti-Bcl-XL (Santa Cruz Biotechnology, 1:500), polyclonal anti-Bad (Santa Cruz Biotechnology, 1:500), polyclonal anti-5-LO (Cayman Chemicals, 1:500), polyclonal anti-COX (Cayman Chemicals, 1:500). After extensive

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. The bands were also quantified by densitometry using Scion Image Software (Scion Co., MD, USA).

Reactive Oxygen Species (ROS) measurement

The luminol-enhanced chemiluminescence of human neutrophils was measured using microplate-reader Spectramax (Molecular Devices, CA, USA), as described previously [19]. Briefly, the cells were stimulated with rHPU (10, 30 or 100 nM) or Phorbol 12-miristate 13-acetate (PMA; 1 mg/ml) 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 we used CM-H_2DCFDA (chloromethyl-2',7'-dichlorodihydrofluorescein diacetate; λ_{ex}470nm/λ_{em}529nm) and lucigenin (bis-N-methylacridiniumnitrate), respectively. For lucigenin the same protocol as luminol was used [20]. For CM-H_2DCFDA, neutrophil were incubated with the dye for 15 min. at 37°C prior to stimulation [21].

Paw Edema

Male Swiss mice (20 - 22 g), housed at 22 ± 3 ^{o}C with a 12/12 h light/dark cycle were used for the experiments. On the day of the experiments, the mice received, under light ether anesthesia, a 0.03 mL intraplantar injection of different doses of rHPU into the right hind paw. The left hind paw was used as control receiving an injection 0.03 mL of PBS. In some experiments the animals were pre-treated with anti-inflammatory drugs given subcutaneously 1 hour (esculetin, 50 mg/kg, Sigma) or 4 hours (dexamethasone, 0.5 mg/kg, Sigma) before rHPU administration.

Increased paw thickness due to edema was measured with a micrometer (Mitutoyo, 0 to 25 mm at 0.002 mm increments) at the indicated time intervals after the injections. Paw edema is expressed as the difference between the thickness in mm of right and left paws of the same animal. Thus the results represent the net edema (mm) induced by HPU.

Animal experimentation

All procedures involving animals were conducted in strict accordance to Brazilian legislation (Law no. 6.638/1979) and are in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (publication no. 85-23, revised in 1985).

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.

Results

rHPU has a pro-inflammatory potential

To investigate whether rHPU possesses pro-inflammatory activity the model of mouse paw edema was used. Figure 1 shows the time course and dose-dependency curves of paw edema induced by recombinant HPU. As low as 0.5 μg of protein injected into the mouse hind paw produced a significant edema after 2 hours with maximal edema seen after 4-6 hours. For higher doses the edema peaked at 4-6 hours and lasted more than 24 hours. In mice pre-treated with dexamethosone or esculetin (Fig. 2), there was a significant reduction in the paw edema indicating that eicosanoids, particularly lipoxygenase metabolites, mediate the pro-inflammatory activity of rHPU.

rHPU induces human neutrophil chemotaxis

H. pylori infection induces an acute neutrophil-dominant inflammation and neutrophil density is correlated with tissue damage [22]. Bacterial extracts stimulate chemotaxis and activation of neutrophils *in vitro* [23]. Here, the ability of purified rHPU to activate human neutrophils and induce chemotaxis was investigated. Figure 3 shows that 100 nM rHPU can stimulate neutrophil migration in a dose-dependent manner. The chemotactic effect of 100 nM rHPU (55.6 ± 6.8 neutrophils/field) has the same extent as that of fMLP (63 ± 7.2 neutrophils/field). This property of HPU is independent of its ureolytic activity, as rHPU treated with inhibitors did not alter the migration profile.

HPU induces the increase of reactive oxygen species production by human neutrophils

It has been reported that *H. pylori* whole cells stimulate the generation of reactive oxygen species by neutrophils [19]. Total ROS production comprises intra- and extracellular release and increase of ROS production is associated with an increased level of DNA repair by epithelial cells [24]. We evaluated the total production of reactive oxygen species, and also measured the intra- and extracellular levels of ROS. Total ROS production was measured using luminol-amplified luminescence. Cells were exposed to rHPU and PMA (positive control, not shown). Figure 4 shows that neutrophil exposed to 100 nM rHPU had a 2.5 fold increase in ROS production as compared to controls. As the total ROS production represents the sum of intra- and extracellular release, we investigated these two parameters separately. Extracellular ROS release was measured using lucigenin, a chemiluminescence probe that is more specific for superoxide anions released extracellularly. CM-H_2DCFDA was used to measure intracellular ROS production. Figures 4 and 5 show that the increased ROS production induced by rHPU is totally directed to the extracellular medium.

HPU protects neutrophils from apoptosis 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. Neutrophils act by releasing proteolytic enzymes and reactive oxygen species, and can also induce tissue damage. Neutrophils are removed from the site of inflammation by the induction of apoptosis [25]. As in the case of H. *pylori*

infection the intensity of tissue damage correlates with neutrophil density [26], we investigated the role of rHPU in neutrophil apoptosis. First we examined neutrophil viability after a 20 h culture in the presence of 10, 30 and 100 nM rHPU or 100 nM interleukine 8. Figure 6 shows that neutrophil apoptosis is delayed when the cells are exposed to rHPU.

The levels of anti- and proapoptotic proteins play a major role in the control of apoptosis. Human neutrophils have a very short half-life, characterized by the constitutively expression of proapoptotic proteins, and almost undetectable levels of anti-apoptotic proteins [27]. Figure 7 shows that all concentrations of rHPU tested resulted in lower levels of Bad, a proapoptotic Bcl-2 member, implying faster degradation rates. On the other hand, rHPU induced the expression of Bcl-x_L (Fig. 8), increasing the survival of neutrophils.

HPU activates the arachidonic acid pathway in neutrophils

Lipids play a major role in chronic inflammation and imbalances of lipid signaling pathways contribute to disease progression. Two metabolites of the 5-LO pathway, leukotriene B_4 and 5-hydroxyeicosatetraenoic acid, have been identified as important mediators of the inflammatory process in the gastrointestinal tract [28]. Considering that platelet activation by rHPU depends on activation of the 12-lipoxygenase [14], here we studied the participation of 5-lipoxygenase in rHPU-activated neutrophil signaling. Figure 9 show that rHPU-activated neutrophils have increased levels of 5-LO expression, suggesting the possible involvement of leukotrienes or 5-HETE in neutrophil's response to rHPU. On the other hand, there was no indication of participation of the cycloxygenase pathway in the responses of neutrophils to rHPU (not shown).

Discussion

Ureases (EC 3.5.1.5) are highly homologous nickel-dependent enzymes widespread among plants, bacteria and fungi, that hydrolyze urea into ammonia and carbon dioxide [29,30]. The physiological role of urease in plants is still largely unknown despite its ubiquity in virtually all plants [31,32]. Jackbean and soybean ureases display fungicidal [33] and insecticidal activity, suggestive of a role in plant defense [34,35]. The insecticidal activity is due to a ~10 kDa internal peptide released from plant ureases upon digestion by insect cathepsins [36, 37]. We have previously reported that canatoxin [38], an isoform of jackbean (*C. ensiformis*) urease [39], presents biological properties that are independent of its enzyme activity, as binding to sialylated glycoconjugates, activation of blood platelets [40-42] and pro-inflammatory effect [43]. Submicromolar concentrations of canatoxin induced exocytosis in a number of cell system *in vitro* including platelets, synaptosomes, pancreatic islets, macrophages, neutrophils and mast cells [41,44]. Canatoxin also induced hypothermia, bradycardia, hypoglycemia, hyperinsulinemia, hypoxia and preceding convulsions and death of the animals, as well as paw edema in rats and mice [45]. Lipoxygenase metabolites were shown to modulate most of canatoxin's pharmacological effects [40,

41, 42, 44, 45] either in vivo or in vitro. Jackbean, soybean and *B. pasteurii* ureases also induce aggregation of platelets in nanomolar concentrations independently of enzyme activity [35, 46] and more recently, we demonstrated that the purified recombinant *H. pylori* urease also promotes degranulation and aggregation of rabbit platelets recruiting the lipoxygenase pathway [14].

Paw edema is a well accepted model of the inflammatory process [47]. We have previously shown [43] that intraplantar injection of canatoxin induced in rats a dose-dependent hind-paw edema which was distinguished by two phases. In the initial 2 hr after canatoxin injection, the increase in paw volume apparently did not involve inflammatory phagocytic cells. The second phase (after 3 hr) was characterized by an intense cellular infiltration and a further increase in paw swelling. CNTX-induced edema was characterized as a multi-mediated phenomenon with histamine, serotonin, PAF and prostaglandins likely involved in the first phase, while lipoxygenase metabolites, probably leukotrienes, may account for the development of the intense cellular infiltration at the inflammatory site during the second phase [43].

The time-course of HPU-induced mouse paw edema is very similar to the rat paw edema induced by intraplantar injections of canatoxin [43]. rHPU is about 10-fold more potent in inducing paw edema, although differences in inflammatory reactions of animal models have also to be considered. As described for canatoxin, eicosanoids derived from lipoxygenase(s) pathways play an important role in rHPU-induced inflammation, as evidenced by the reduction in paw edema in mice pre-treated with esculetin and also on the increased levels of 5-lipoxygenase in rHPU-activated human neutrophils. An increasing amount of evidences are pointing to an important role of the arachidonic acid pathway in the development of chronic inflammation and gastric carcinogenesis [48,49]. Lipoxygenase metabolites such as LTB4 enhance the proliferation of epithelial cells and may induce oncogenes in these cells [50].

Here we showed that purified recombinant *H. pylori* urease directly activates human neutrophils in nanomolar doses. Chemotaxis induced by 100 nM rHPU was similar to that produced by 100 nM fMLP, a synthetic peptide that mimicks bacterial peptides [51]. The chemotactic effect of rHPU did not to require the enzymatic release of ammonia. Barja-Fidalgo *et al*., 1992, reported that canatoxin induced neutrophil migration into pleural cavity and air-pouch of rats and also that canatoxin induced macrophages to release of a neutrophil-chemotactic factor.

Purified *H. pylori* urease was previously reported to directly activate primary human blood monocytes and to stimulate dose-dependent production of inflammatory cytokines [52]. Enarsson et al. 2005, reported that *H. pylori* induced significant T-cell migration in a model system using human umbilical vein endothelial cells and that purified *H. pylori* urease alone induced a migration effect similar to that of whole bacteria. On the other hand, mutant *H. pylori* negative for urease A subunit still promoted significant cell migration which the authors imparted to a contribution of the functional *cag* pathogenicity island to the transendothelial migration [53]. Another interpretation of the data would be that the ability of the bacterial urease to induce this effect relies only on its B chain.

Handa *et al*., 2010, reported that *H. pylori* infection stimulates inflammatory cells within the gastric tissue to release ROS [54]. Increased ROS production is associated to higher levels of DNA repair in gastric epithelial cells [55]. Contributing to damage the gastric tissue, we demonstrate here that rHPU-activated neutrophils release ROS extracellularly. This result corroborates studies by Allen et al., 2005, showing that *H. pylori* infection interferes on the activity of human neutrophil NADPH oxidase leading to extracellular release of ROS [56].

The half-life of human neutrophils is typically of 12 hr, as a result of the constitutive expression of pro-apoptosis proteins and almost non-detectable levels of anti-apoptosis proteins [57]. Neutrophils release proteolytic enzymes and ROS that inflict local tissue damage and are removed from an inflammatory site by induction of apoptosis. Thus fine regulation of pro- and anti-apoptosis proteins that control neutrophil apoptosis is a critical process for definition of inflammation. Our data show that rHPU protect neutrophils against apoptosis, prolonging their life and contributing to the underlying local tissue, as seen in the mouse paw edema assay. Increased half-life 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, that would ultimately lead to a persistent inflammatory status.

In vitro studies showed that *H. pylori* can induce apoptosis in gastric epithelial cell lines [58]. However, Kim et al., 2001, and Cappon et al., 2010, demonstrated that products of H. pylori exert an important role in maintaining inflammation, by suppressing human neutrophil apoptosis [59,60].

The fact that bacterial and plant ureases evolutionarily conserved the property of inducing exocytosis in some cell types independent of ureolytic activitiy may shed new lights into the so far poorly understood biological functions of these proteins. Another important aspect to be investigated is whether or not other biological activities displayed by *H. pylori* urease depend on its ureolytic activity. This finding and the modulation of its pro-inflammatory activity by lipoxygenase-derived eicosanoids could be particularly relevant to the elucidation of mechanisms leading to gastrointestinal disease caused by this bacterium and should be taken into consideration in the development of more efficient therapeutic approaches.

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Legends to figures:

Figure 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 (PBS). 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.

Figure 2. Involvement of phospholipase A2 and lipoxygenase-derived eicosanoids in rHPU-induced mice paw edema. Mice were received subcutaneously esculetin (50 mg/Kg) or dexamethasone (0.5 mg/kg), 1 hour or 4 hours before rHPU administration. 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 (PBS). 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.

Figure 3. Effect of rHPU on human neutrophil chemotaxis: Chemotaxis was assayed in 48-well microchemotaxis chambers (NeuroProbe, Gaithersburg, MD) using 5-µm PVP-free polycarbonate filter. Neutrophils (10^6 cells/mL in RPMI-0.01% bovine serum albumin [BSA]) were allowed to migrate toward rHPU (10 nM, 30 nM or 100nM; treated or untreated with the inhibitor p-hydroxymercury benzoate 50 µM) and fMLP (100 nM), or medium alone (random migration; 37°C, 5% CO2). After 1 hour, 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. Each sample was assayed in triplicate. Results are expressed as number of neutrophils per field. All data show mean ± S.D. from at least three independent experiments done in triplicates. *P < 0.05 compared to control.

Figure 4. rHPU induces neutrophil ROS production. (A) Neutrophils (6 x 105 cells/well) were placed in a white flat-bottom 96-wells plate. Cells were then left unstimulated (circles) or stimulated with 10 nM (squares), 30 nM (triangles) and 100 nM (diamonds) of rHPU in the presence of lucigenin (25 mM). (B) Same experiment was performed using luminol as chemiluminogenic probe (500 mM). Data are representative mean of three independent experiments. ROS accumulation was evaluated calculating the area under curve using lucigenin (C), or luminol (D) over 60 minutes treatment. Results are mean + SEM of three independent experiments.

Figure 5. rHPU promotes extracellular ROS production while maintaining the intracellular redox status. Neutrophils (6 x 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 left unstimulated or stimulated with rHPU 100 nM or PMA 30 nM in the presence of lucigenin (25 mM) or CM-H_2DCFDA (5 mM). Accumulated ROS generation was evaluated calculating the area under curve emitted by lucigenin-dependent chemiluminescence and CM-H_2DCFDA fluorescence accumulation over 120 minutes treatment.

Figure 6. rHPU inhibits human neutrophil apoptosis. Neutrophils (5 x 10^6/ml) were incubated in the absence or in the presence of rHPU (10 nM, 30 nM and 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. *P < 0.05 compared to control.

Figure 7. Bad degradation induced by rHPU in neutrophils. Human neutrophils (5 x 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.D. of triplicates. Data shown in the inset are of a typical experiment. **P < 0.01 compared to control.

Figure 8. rHPU induces the expression of Bcl-XL in neutrophils. Human neutrophils (5 x 10^6 cells) were incubated with rHPU (10 nM, 30 nM and 100 nM) and LPS (1 μg/mL). After 4 h, Bcl-XL protein expression was assessed by Western blot analysis. Results are mean S.D. of triplicates. Data shown in the inset are of a typical experiment. *P < 0.05 compared to control.

Figure 9. rHPU induces the expression of 5-lipoxygenase in neutrophils. Human neutrophils (5 x 10^6 cells) were incubated with rHPU (10 nM, 30 nM and 100 nM). After 4 h, 5-LO protein expression was assessed by Western blot analysis. Data shown are of a typical experiment.

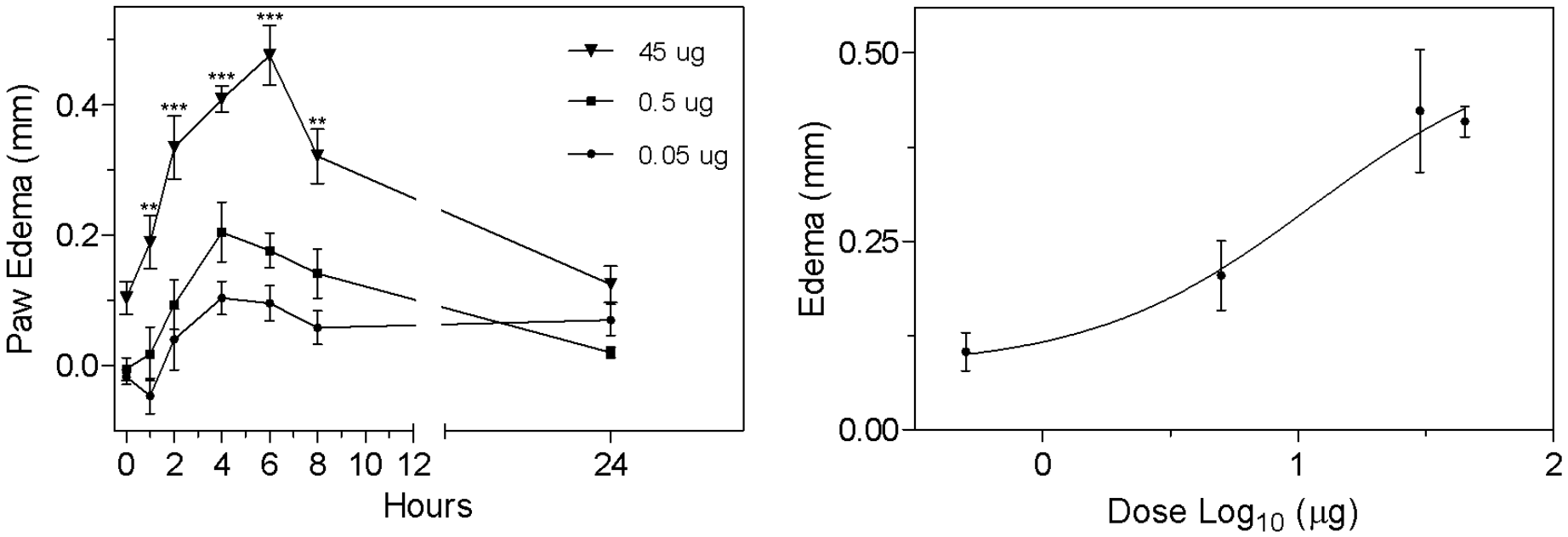

Figure 1. Rat Paw Edema induced by recombinant *H.pylori* urease

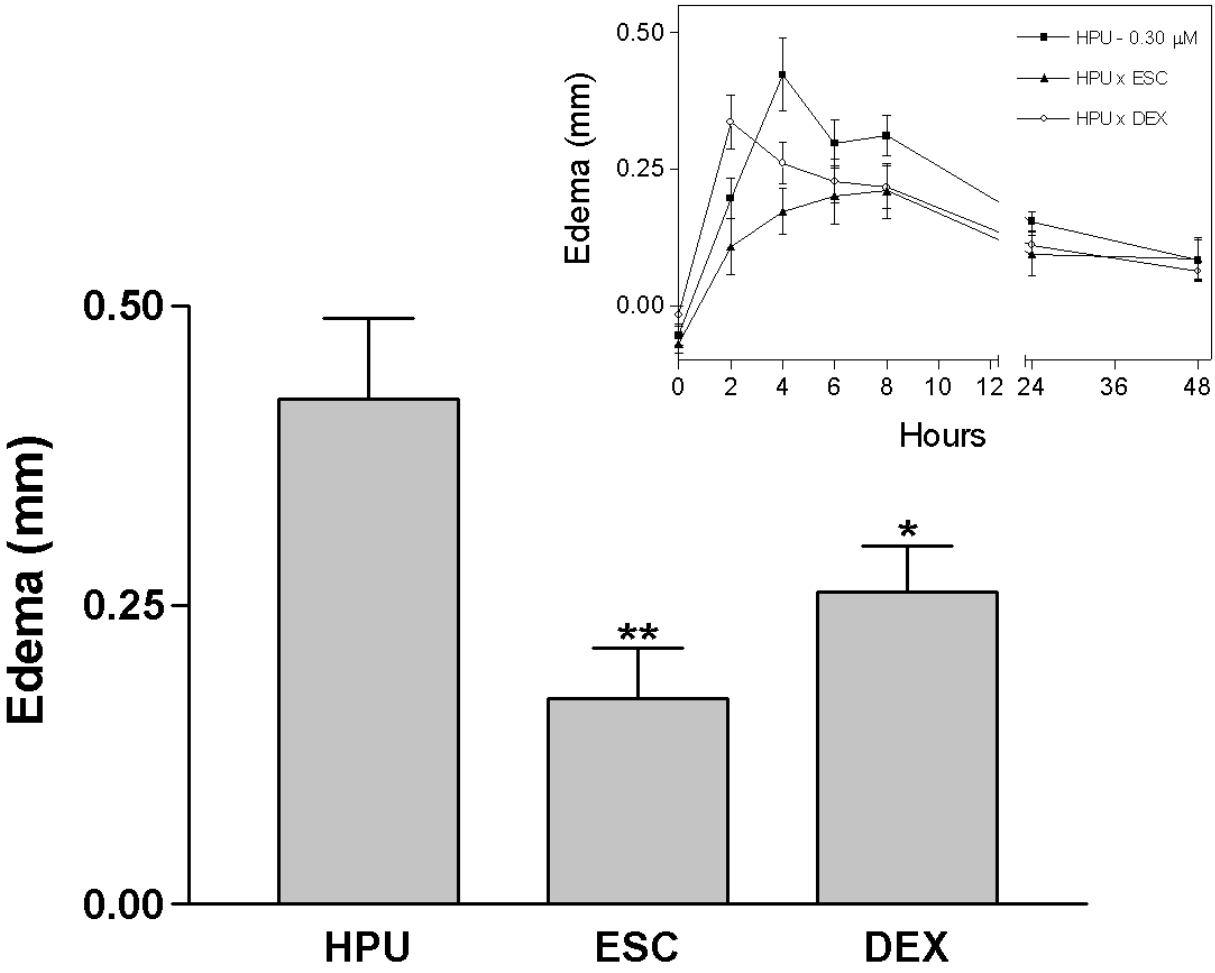

Figure 2. Lipoxygenase-derived eicosanoids in HPU-induced mice paw

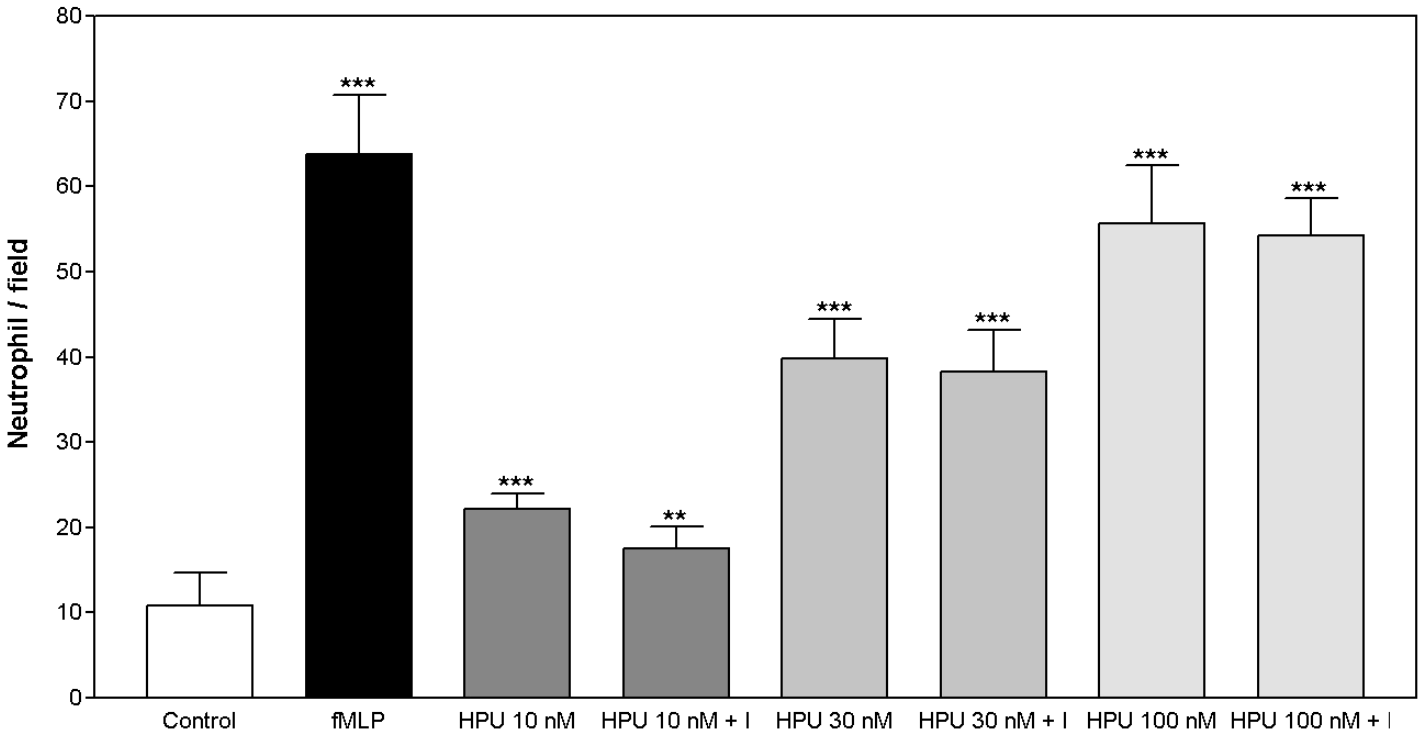

Figure 3. Neutrophil chemotaxis induced by HPU

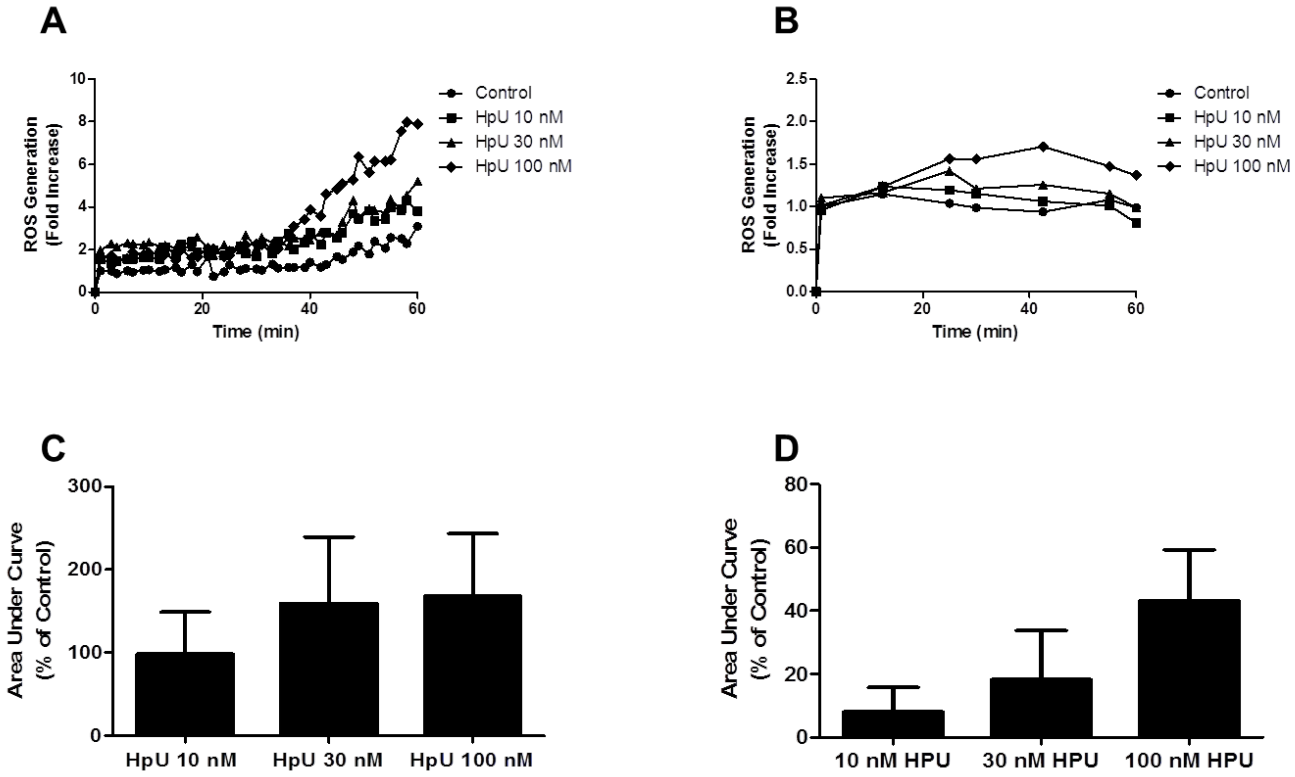

Figure 4. Ros production by HPU-activated neutrophils

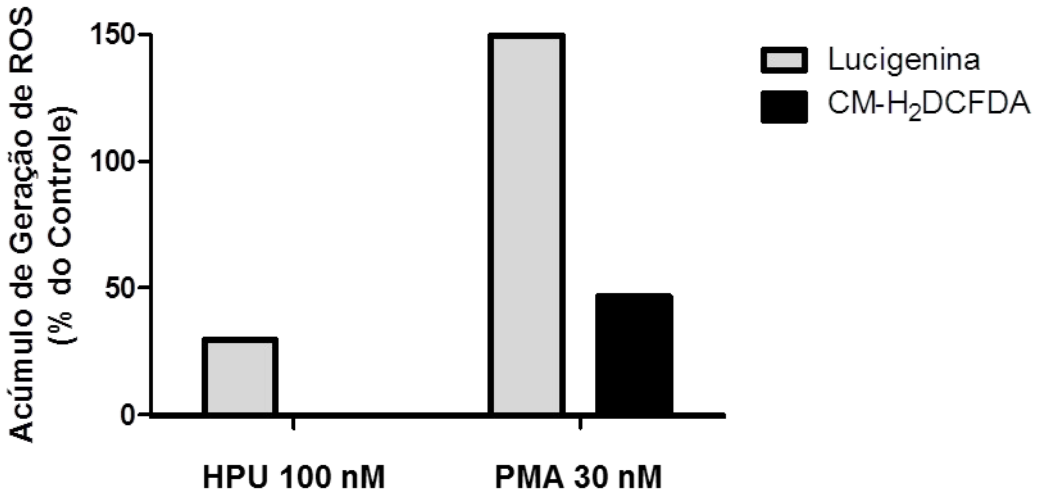

Figure 5. Intracellular production by HPU-activated neutrophils

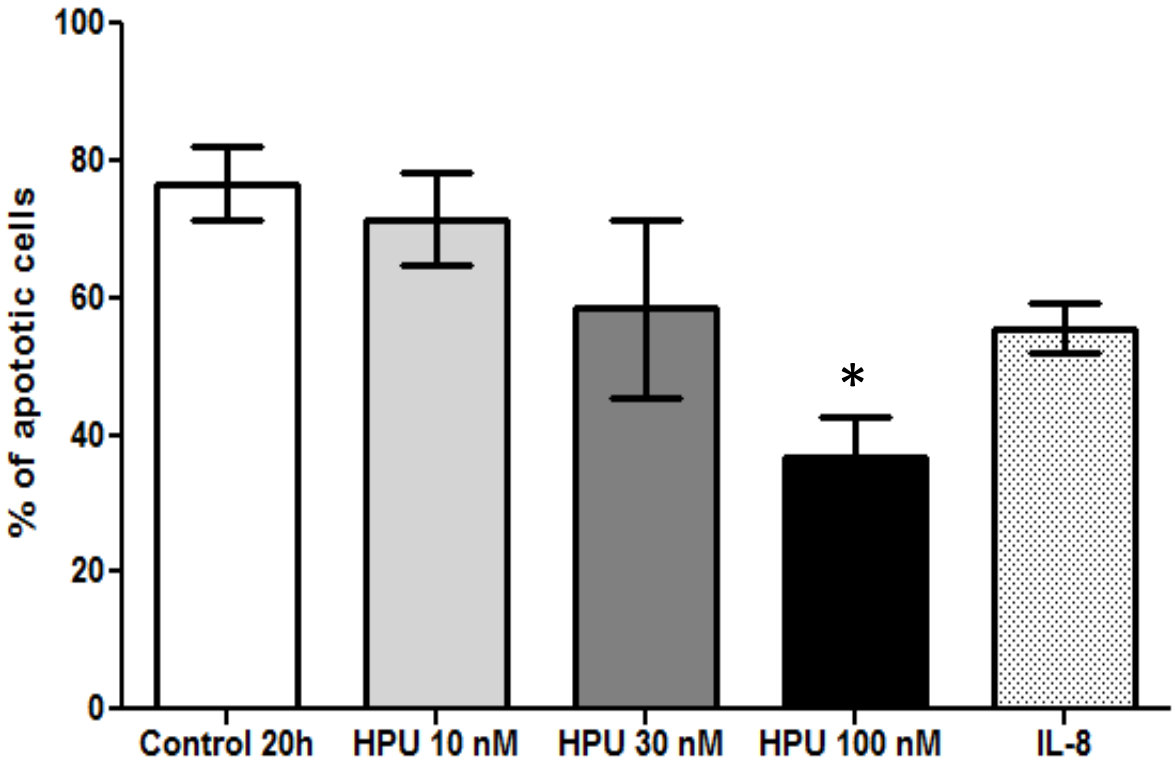

Figure 6. HPU inhibits human neutrophil apoptosis.

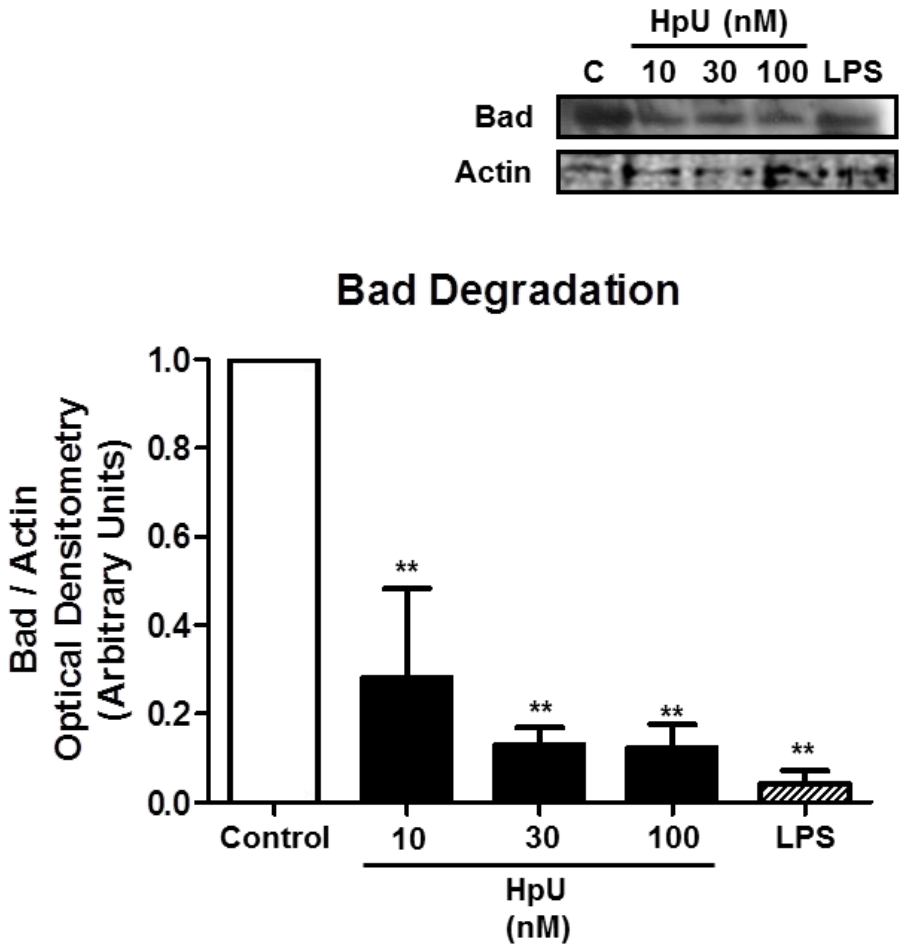

Figure 7. Bad degradation induced by HPU in neutrophils.

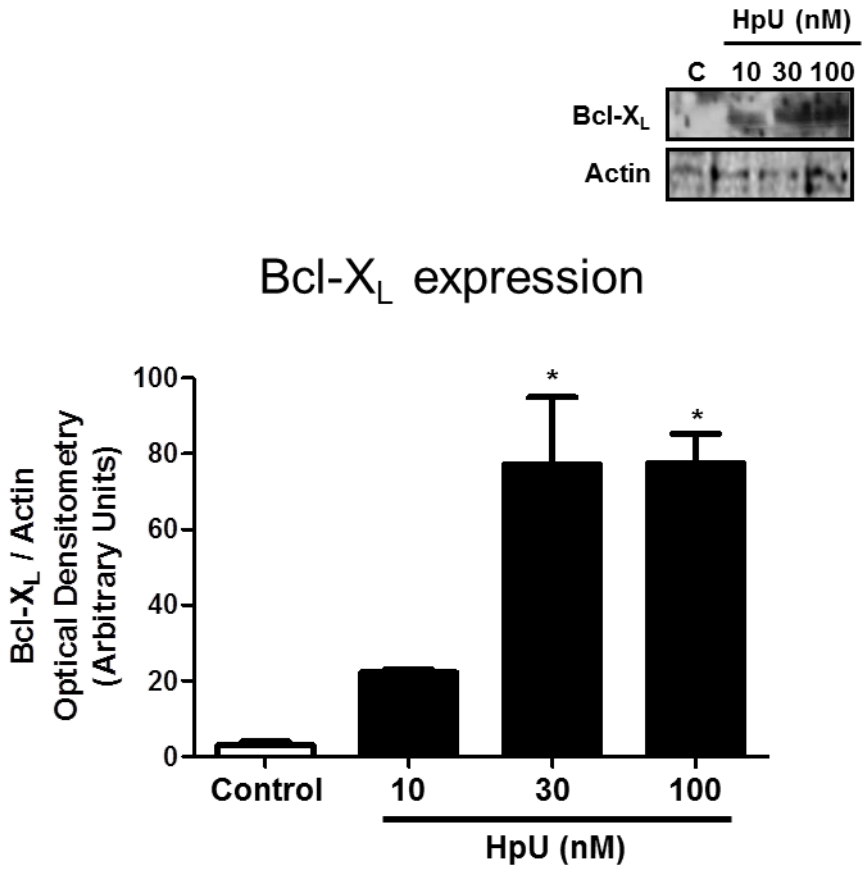

Figure 8. HPU induces the expression of Bcl-XL in neutrophils.

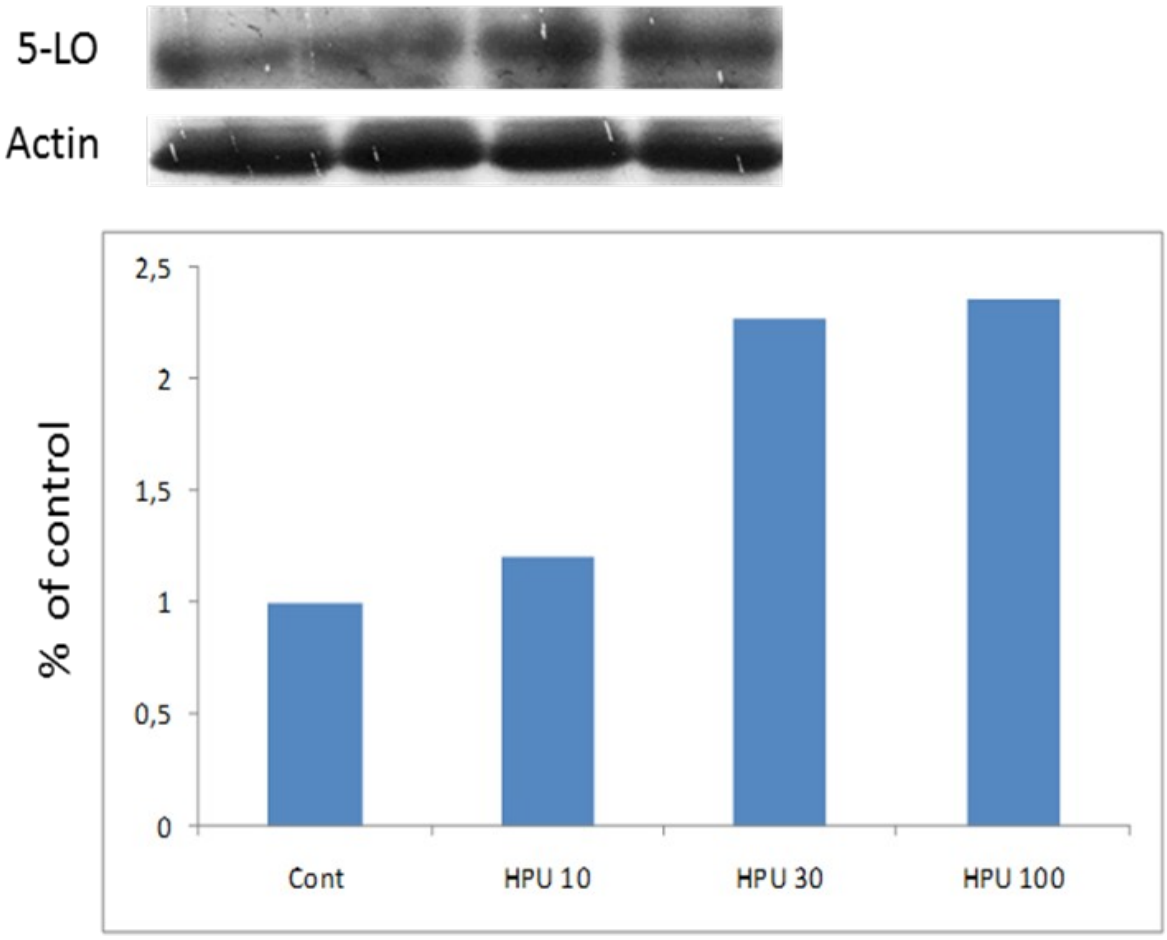

Figure 9. HPU induces the expression of 5-lipoxygenase in neutrophils.