**Helicobacter pylori** urease induces pro-inflammatory effects and differentiation of human endothelial cells: Cellular and molecular mechanism

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**Abstract**

**Background:** *Helicobacter pylori* urease (HPU) is a key virulence factor that enables bacteria to colonize and survive in the stomach. We early demonstrated that HPU, independent of its catalytic activity, induced inflammatory and angiogenic responses in vivo and directly activated human neutrophils to produce reactive oxygen species (ROS). We have investigated the effects of HPU on endothelial cells, focusing on the signaling mechanism involved.

**Methods:** Monolayers of human microvascular endothelial cells (HMEC-1) were stimulated with HPU (up to 10 nmol/L): Paracellular permeability was accessed through dextran-FITC passage. NO and ROS production was evaluated using intracellular probes. Proteins or mRNA expressions were detected by Western blotting and fluorescence microscopy or qPCR assays, respectively.

**Results:** Treatment with HPU enhanced paracellular permeability of HMEC-1, preceded by VE-cadherin phosphorylation and its dissociation from cell-cell junctions. This caused profound alterations in actin cytoskeleton dynamics and focal adhesion kinase (FAK) phosphorylation. HPU triggered ROS and nitric oxide (NO) production by endothelial cells. Increased intracellular ROS resulted in nuclear factor kappa B (NF-κB) activation and upregulated expression of cyclooxygenase-2 (COX-2), hemeoxygenase-1 (HO-1), interleukin-1β (IL-1β), and intercellular adhesion molecule-1 (ICAM-1). Higher ICAM-1 and E-selectin expression was associated with increased neutrophil adhesion on HPU-stimulated HMEC monolayers. The effects of HPU on endothelial cells were dependent on ROS production and lipoxygenase pathway activation, being inhibited by esculetin. Additionally, HPU improved vascular endothelial growth factor receptor 2 (VEGFR-2) expression.

**Conclusion:** The data suggest that the pro-inflammatory properties of HPU drive endothelial cell to a ROS-dependent program of differentiation that contributes to the progression of *H pylori* infection.

**KEYWORDS**
endothelial cells, *Helicobacter pylori* urease (HPU), inflammation, reactive oxygen species (ROS)
1 | INTRODUCTION

*Helicobacter pylori* (*H. pylori*) is a bacterial group-I carcinogen that specifically colonizes the human gastric epithelium, inducing disorders such as ulceration, chronic gastritis, and malignant neoplastic diseases. Virulence factors produced by *H. pylori* help bacteria to evade host defense mechanisms and enable colonization and disruption of epithelial barrier functions, inducing damage to gastric epithelium. One of the main factors responsible for a successful infection is the *H. pylori* urease (HPU), whose ureolytic activity results in the production of ammonia, which in turn leads to the neutralization of the gastric acidic medium, allowing the bacteria survival in the stomach. Accordingly, several pieces of evidence have demonstrated that urease-deficient *H. pylori* are unable to colonize gastric epithelium.

We have previously reported that purified HPU, independent of its catalytic activity, is a potent pro-inflammatory agent, able to trigger edema formation and leukocyte accumulation in vivo. HPU also induced in vitro platelet aggregation, human neutrophil chemotaxis, and production of reactive oxygen species (ROS). Platelets activated by HPU display a pro-inflammatory phenotype with modifications in the pre-mRNA processing of pro-inflammatory proteins, and increased levels of mRNAs encoding IL-1β and CD14. These results suggest that the inflammatory response triggered by HPU may contribute to amplify local inflammation, worsening mucosal damage during bacterial infection.

HPU activity was shown to be required for triggering gastric epithelial barrier dysfunction during bacterial infection. More recently, our group demonstrated that HPU can be internalized by gastric epithelial cells inducing the expression of pro-angiogenic mediators.

The interaction of *H. pylori* with endothelial cells was shown to up-regulate the expression of adhesion molecules and the secretion of neutrophil-recruiting chemokines. Thus, a persistent endothelial cell activation induced by bacterial products could be involved in the continuous recruitment of neutrophils to *H. pylori*-infected gastric mucosa and may therefore contribute to tissue damage and ulcer generation. This chronic inflammatory status caused by *H. pylori* infection represents the most common risk factor underlying gastric cancer.

Although evidences have shown that, besides its role as a key enzymatic factor for *H. pylori* colonization, HPU can also act as a pro-inflammatory agent contributing to angiogenesis and the mechanisms involved in HPU-induced endothelial activation remain unclear. To gain insight on this, we have investigated the molecular mechanisms and correspondent signaling pathways involved in the HPU effects on human endothelial cells.

2 | MATERIAL AND METHODS

2.1 | Recombinant *H pylori* urease

Recombinant *Helicobacter pylori* urease (HPU) was purified as previously described and stored at 4°C. The purified protein was concentrated using Centriprep cartridges (30 kDa cut-off) to give a 0.5 mg protein/mL solution and dialyzed against 20 mmol/L sodium phosphate, pH 7.5. The buffer from the last dialysis change was used as a negative control in all bioassays (controls) (See Appendix S1).

2.2 | Cell Cultures

Human microvascular endothelial cell (ATCC®) and primary human umbilical vein endothelial cell (HUVEC) cultures were used throughout the experiments (See Appendix S1).

2.3 | Purification of human neutrophils

Neutrophils were isolated from human peripheral blood using a Percoll density gradient as previously described (See Appendix S1).

2.4 | Endothelial paracellular permeability assay

HMEC-1 (5 x 10⁴ cells/well) were cultured onto Transwell inserts (0.4 μm), pretreated with esculentin (10 μmol/L) or polymyxin B (25 μg/mL), and stimulated with HPU (10, 20 and 30 nmol/L) or buffer (control), for 30 minutes. Alterations in endothelial monolayer permeability were evaluated by monitoring the passage of dextran-FITC through the endothelial confluent monolayers, as early described (See Appendix S1).

2.5 | Intracellular ROS and nitric oxide production

HMEC-1 (3 x 10⁴ cells/well) cultured in 96-well black plates were loaded, for 1 hour, with CM-H₂DCFDA (5 μmol/L) for analyzing intracellular ROS, or with DAF-FM (5 μmol/L) for NO detection.

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**FIGURE 1** HPU increases endothelial cells permeability and induces VE-cadherin phosphorylation and dispersion from cell surface.

HMEC-1 (5 x 10⁴) cultured onto Transwell chambers (0.4 μm) were (A) treated or not with HPU (10, 20 and 30 nmol/L), or (B) pre-incubated with esculentin (10 μmol/L) for 15 min, before treatment with HPU (10 nmol/L), in medium containing FITC-labeled dextran (1 mg/mL). The leakage of fluorescent dextran-FITC through HMEC-1 monolayer was quantified in the bottom well after 30 min. C, HMEC-1 (1 x 10⁶) were stimulated or not with HPU (10 nmol/L) at different times (1, 3, 5, 15, and 30 min), and the protein contents of VE-cadherin and pVE-cadherin Tyr658 were evaluated by Western blotting. The blots were analyzed by densitometry. Figure depicts representative blots. D, HUVEC (2 x 10⁵) monolayers were treated or not with HPU for 5 or 15 min and were immunostained for VE-cadherin. Nuclei were labeled with DAPI. Images were analyzed by fluorescence microscopy (at 600x Magnification). Arrow heads show small gaps between adjacent cells (yellow). The results are representative of at least three independent experiments. Data are expressed as mean ± SD. One-way ANOVA followed by Bonferroni post hoc test displayed *P < 0.05 compared to controls; #P < 0.05 compared to group treated with HPU.
After that, cells were pretreated or not with esculetin (10 μmol/L) or polymyxin B (25 μg/mL) for 15 minutes, and then treated with HPU (10 nmol/L) or buffer (control) for 30 minutes at 37°C in a CO₂ air atmosphere. The fluorescence intensity was monitored and quantified using an EnVision® multilabel plate reader. (See Appendix S1 for details).
2.6 | Fluorescence microscopy assays

VE-cadherin expression in HUVECs and E-selectin expression in HMEC-1 were evaluated by immunocytochemistry microscopy assays as previously described.18 For actin cytoskeleton dynamic analysis, cells were labeled with TRITC-phalloidin (binding to polymerized actin)20 and observed under a fluorescence microscope. The images obtained in all experiments were analyzed using Adobe Photoshop software. (See Appendix S1 for details).

2.7 | Neutrophils adhesion to HMEC-1

HMEC-1 (3 x 10⁴ cells/well) cultured on 96 wells black plates were pretreated or not with esculetin (10 µmol/L) or polymyxin B (25 µg/mL) for 15 minutes, and then incubated with HPU (10nmol/L) or buffer (control) for 30 minutes. For the adhesion assays, human neutrophils (1 x 10⁶ cells), previously loaded with 1µM CMFDA (Invitrogen) for 1 hour, were seeded on HMEC-1 monolayers. After 30 minutes of co-incubation at 37°C in a CO₂ air atmosphere, non-adherent neutrophils were removed, and the CMFDA fluorescence intensity was quantified in each well using a multilabel plate reader (See Appendix S1 for details).

2.8 | Luciferase Assay

HMEC-1 (2 x 10⁵) were transfected with NF-κB-responsive luciferase reporter construct (NF-kb pMetLuc 2) or control plasmid (pMetLuc 2). They were then stimulated with HPU (10 nmol/L) or buffer (control) for 3 hours. Medium containing luciferase was collected and incubated with luciferin. The luminescence emitted from luciferase-induced luciferin cleavage was quantified as described in the Appendix S1.

2.9 | MTT assay

HMEC-1 (1 x 10⁴ cells/well) seeded onto 96-well plates were incubated overnight in MCDB-131 medium containing 10% FBS, serum starved for 1 hour, and treated with HPU (10 and 50 nmol/L) or buffer (control) for 24 hours. MTT assay22 was performed as described in the Appendix S1.

2.10 | Western blotting analysis

HMEC-1 (1 x 10⁴ cells/well) seeded in 6-well plate were stimulated with HPU (10 nmol/L) or buffer (control) for different time intervals. Alterations in the expression of phospho-VE-cadherin-Tyr658, VE-cadherin, phospho-focal adhesion kinase (FAK) Tyr397, FAK, cyclooxygenase-2 (COX-2), and hemoxygenase-1 (HO-1) were analyzed by Western blotting assay20 as described in the Appendix S1.
FIGURE 3  HPU increases neutrophils adhesion to endothelial cells. A, HMEC-1 (5 x 10^4) were treated with HPU (10 nmol/L) for 15 min. Cells were immunostained for E-selectin and nuclei were labeled with DAPI. Images were analyzed under a fluorescence microscope (600 x). B, HMEC-1 (2 x 10^6) were treated with HPU (10 nmol/L) for 2 h, and the mRNA expression of ICAM-1 was analyzed. GAPDH was chosen as housekeeping gene. C, HMEC-1 (3 x 10^4) were stimulated, with HPU (10 nmol/L) for 15, 30, or 45 min, or (D) were preincubated with esculetin (10 µmol/L) for 15 min, before adding HPU (10 nmol/L) for 30 min. Controls cells were exposed to medium only. Neutrophils, previously loaded with CMFDA (5 µmol/L), were then plated onto endothelial cells monolayers, and after 30 min, fluorescence of adherent neutrophils was monitored. The results are representative of three independent experiments. Data are expressed as mean ± SD. Student’s t test (Figure B) and one-way ANOVA followed by Bonferroni post hoc test (Figure C-D) displayed *P < 0.05 compared to untreated control. #P < 0.05 compared to group treated with HPU.
oxidative metabolism, with increased production of ROS. Inflammatory mediators is often associated with alteration in the redox metabolism. To rule out a possible effect of LPS contamination on signaling pathways modulated by ROS, probably inducing alterations in redox metabolism. To rule out a possible effect of LPS contamination in HPU preparations, these assays were performed in the presence of polymyxin B (25 µg/mL), a condition found not to interfere on HPU effects in endothelial permeability (Figure S2A).

The integrity of endothelial barrier is controlled by VE-cadherin, and phosphorylation of this endothelial adhesion molecule modulates the disruption of endothelial junctions. Treatment of HMEC-1 with HPU induced VE-cadherin phosphorylation at Tyr658 (Figure 1C) and promoted breakdown of junctional contacts, resulting in small gaps between adjacent cells (Figure 1D, arrows). Furthermore, HPU induced profound alterations in the actin cytoskeleton dynamics (Figure 2A). After labeling endothelial cells with TRITC-labeled phalloidin, we observed that, while in non-stimulated HMEC-1 (controls) the filamentous actin assumed a cortical arrangement (Figure 2A, open arrows), the treatment with HPU induced cell spreading and reduced the number of actin cortical rings, promoting a rearrangement of the actin cytoskeleton, with the formation of stress fibers (Figure 2A, solid arrows). Noteworthy, actin cytoskeleton rearrangement in endothelial cells treated with HPU was accompanied by increased phosphorylation (Tyr397) of the focal adhesion kinase (FAK), which peaked at early times (3-5 minutes) (Figure 2B).

3.2 | HPU increases adhesion molecules expression and neutrophils adhesion to endothelial cells

H. pylori infection upregulates the expression of endothelial adhesion molecules, in vivo and in vitro, prompting leukocyte adhesion to endothelium, therefore contributing to neutrophil recruitment to the infected gastric mucosa. We observed that treatment of endothelial cells with HPU increased E-selectin expression, after 15 minutes (Figure 3A, yellow arrows), and augmented ICAM-1 mRNA expression, after 2 hours of treatment (Figure 3B).

Corroborating with these results, stimulation with HPU increased neutrophils adhesion to endothelial monolayers in all time points (Figure 3C). This effect was inhibited by esculetin (Figure 3D). Polymyxin B did not affect neutrophil adhesion (Figure S2B), excluding a possible interference of LPS contamination in HPU preparation.

3.3 | HPU induces production of oxidative mediators by endothelial cells

Endogenous production of ROS can trigger the modulation of signaling pathways that in turn control various biologic responses in endothelial cells. Treatment of HMEC-1 with HPU increased intracellular ROS generation as monitored by CM-H$_2$DCFDA oxidation for 30 minutes (Figure 4A). Moreover, HPU also increased nitric oxide (NO) production by these cells, as detected through DAF-FM oxidation (Figure 4B). Both effects were inhibited by HMEC-1 pretreatment with esculetin (Figure 4A-B). Ruling out a possible contamination with LPS, intracellular production of ROS or NO induced by HPU was not affected by polymyxin B (Figure S2C,D).

3.4 | HPU induces NF-κB pathway activation and increases COX-2, HO-1, and VEGFR-2 expression, contributing to endothelial cells differentiation

Activation of NF-κB pathway has been reported to be modulated by the cell redox status. The treatment of endothelial cells with HPU
induced NF-κB activation as detected by luciferase assay (Figure 5A). In parallel with NF-κB activation, we also observed that stimulation with HPU increased expression of COX-2 (Figure 5B), HO-1 (Figure 5C), and pro-IL-1β (Figure 5D) in endothelial cells. Additionally, we also observed that cells stimulated with HPU displayed higher VEGFR-2 expression (Figure 5E), the main vascular receptor involved in angiogenesis.
4 | DISCUSSION

The ureolytic activity is often detected in several pathogenic bacteria, such as pathogenic *Staphylococcus* strains, *Proteus mirabilis*, and *H. pylori*. Urease has a positive role in microbial physiology, representing a key factor for the infectivity or persistence of microorganisms. HPU enzymatic activity is essential for these bacteria to overcome the lethal effect of the gastric acidic pH and to establish a distinct site of persistent infection. Nevertheless, recent evidences have highlighted pathophysiologic properties of HPU unrelated to its ureolytic activity. We have previously reported that non-catalytic HPU has pro-inflammatory activity in mice and activates host cells, inducing blood platelet aggregation, chemotaxis, and ROS production by human neutrophils. More recently, it was demonstrated that HPU was internalized by epithelial gastric cells, triggering the expression of pro-angiogenic mediators, and induced angiogenesis in vivo in the chicken chorioallantoic membrane model. We are now showing that HPU directly activates human microvasculature endothelial cells, modulating the molecular mechanisms that lead to their differentiation toward a pro-inflammatory profile. The severe local inflammation that occurs during *H. pylori* infection is associated with peptic ulcers and gastric cancers, and its outcome is mostly determined by the expression of specific virulence factors. In this context, HPU emerges as an important pro-inflammatory factor, able to induce significant changes in the oxidative profile of endothelial cells, leading to increased paracellular permeability and neutrophils adhesiveness to endothelium, two main features of inflammatory response.

The integrity of the endothelial barrier is dependent on the presence of VE-cadherin-regulated cell-cell adhesive complexes. The structural and functional maintenance of adherent junctions determines cell permeability. Various stimuli are able to induce VE-cadherin hyper-phosphorylation, disrupting cadherin-catenin complex, dispersing the membrane-associated protein, and consequent dissolution of adherens junctions. We demonstrated that HPU, at concentrations as low as 10 nmol/L, increased endothelial paracellular permeability, which was associated with VE-cadherin phosphorylation and dispersion from cell membrane. The breakdown of endothelial barrier mediated by adherens junction is strongly associated with the reorganization of the actin network. As expected, HPU induced profound modifications in actin cytoskeleton dynamics in endothelial cells, increasing stress fibers formation and reducing actin cortical rings. The actin cytoskeleton rearrangement in HPU-stimulated cells was accompanied by an increased phosphorylation of FAK. This event has a central role in initiating and integrating signaling pathways that control endothelial barrier function. FAK works as an intermediary in the crosstalk between integrins and VE-cadherin, regulating signaling at endothelial intercellular adherens junction, and the cytoskeleton dynamics. The alterations induced by HPU on the activation profile of endothelial cells also included an increasing expression of surface adhesion molecules. HPU increased E-selectin and ICAM-1 expression, contributing to neutrophil-endothelial cells adhesion. The direct effects of HPU on endothelial cells support previous data showing its pro-inflammatory activity in vivo. HPU induced mice paw edema, accompanied by significant leukocyte accumulation in the inflamed tissue, and was a potent chemotactic for human neutrophils in vitro.

The chronic inflammatory response often present during *H. pylori* infection has been imputed to a persistent oxidative stress that contributes to gastric carcinogenesis. The release of ROS is associated with higher levels of DNA repair in gastric epithelial cells. Neutrophils are considered the main extracellular source of ROS, and HPU is a potent inducer of ROS production by these cells. In endothelial cells, although an excess of ROS can lead to apoptosis, at physiologic concentrations the intracellular ROS may act as signaling molecules mediating various biological responses. This includes the upregulation of adhesion molecules and chemokines, cytoskeleton reorganization, generation of intercellular gaps, and leukocyte transmigration. Our data revealed that HPU induced intracellular production of ROS and NO by endothelial cells, and that esculetin, a potent antioxidant and a non-specific lipoxygenase inhibitor, inhibited these effects. Moreover, we also demonstrated that the impairment of ROS production and lipoxygenase inhibition by esculetin inhibited HPU-induced increase in paracellular permeability and neutrophil adhesion to endothelial cells. The increase in endothelial permeability by ROS was associated with VE-cadherin phosphorylation. Furthermore, lipoxygenase metabolites were shown to regulate vascular permeability and to induce the breakdown of tight junctions, increasing endothelial permeability in dependence of ROS production. Our results are in accordance with previous data showing that HPU pro-inflammatory activity seems to be modulated by lipoxygenase-derived eicosanoids, and the data suggest that the effects of HPU on endothelial cells involve redox modulation signaling.

The effects of intracellular ROS on signaling pathways, interfering on the activity of different signaling proteins such as PI3K-Akt, MAPK, NF-κB, and NR-F2, among others, have been well described. The transcription factor NF-κB is crucial in a series of cellular processes, including immune and inflammatory responses, cellular adhesion, differentiation, proliferation, and apoptosis. The inhibition of ROS production, associated with the inactivation of NF-κB signaling, has been pointed as a therapeutic target for numerous inflammatory diseases. This transcription factor regulates the expression of several inflammatory mediators including enzymes such as COX-2, NOS-2, and HO-1, which have been also associated with gastric cancer. Supporting the role of HPU as a pro-inflammatory factor, we observed an increasing expression of pro-IL-1β, COX-2, and HO-1, as consequences of NF-κB activation. Although HPU has increased NO production by endothelial cells, an effect inhibited by esculetin, we could not detect iNOS expression (data not shown), suggesting that perhaps HPU may be signaling to activate eNOS, through intracellular ROS production. The eNOS upregulation in *H. pylori*-infected gastric mucosa correlates with inflammation and angiogenesis and seems to be secondary to an overexpression of VEGF. Interestingly, besides its ability to stimulate inflammatory pathways related to angiogenesis (IL-1β, COX-2, and HO-1), HPU also
increased VEGFR-2 expression. The upregulation of this receptor is a strong indicator that HPU prompts endothelial cells to a more responsive phenotype that, associated with increased migration and invasion, may result in the differentiation of new blood vessels. The data give further support to previous observations on the pro-angiogenic properties of HPU. Over the past years, the knowledge on the role of HPU in the bacterial infection was restricted to its ureolytic activity, an essential step for *H pylori* colonization of the gastric environment. Nevertheless, in the last decade a consistent body of evidence has identified HPU as a virulence factor that, independently of its catalytic properties, acts as a microbial inflammatory mediator, contributing to the persistence of the infection through a direct interaction with host components. Our data show that the pro-inflammatory properties of HPU can drive endothelial cells to a differentiation program through a ROS-dependent signaling mechanism that probably subsidizes the progresses of *H pylori* infection to gastric carcinogenesis.

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**DISCLOSURES OF INTERESTS**

The authors report no conflict of interest.

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**REFERENCES**


SUPPORTING INFORMATION

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