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Disruption of Nitric Oxide Signaling by Helicobacter pylori Results in Enhanced Inflammation by Inhibition of Heme Oxygenase-1


A strong cellular cross-talk exists between the pathogen Helicobacter pylori and high-output NO production. However, how NO and H. pylori interact to signal in gastric epithelial cells and modulate the innate immune response is unknown. We show that chemical or cellular sources of NO induce the anti-inflammatory effector heme oxygenase-1 (HO-1) in gastric epithelial cells through a pathway that requires NF-κB. However, H. pylori decreases NO-induced NF-κB activation, thereby inhibiting HO-1 expression. This inhibitory effect of H. pylori results from activation of the transcription factor heat shock factor-1 by the H. pylori virulence factor CagA and by the host signaling molecules ERK1/2 and JNK. Consistent with these findings, HO-1 is downregulated in gastric epithelial cells of patients infected with cagA+ H. pylori but not in gastric epithelial cells of patients infected with cagA− H. pylori. Enhancement of HO-1 activity in infected cells or in H. pylori-infected mice inhibits chemokine generation and reduces inflammation. These data define a mechanism by which H. pylori favors its own pathogenesis by inhibiting HO-1 induction through the action of CagA.


Helicobacter pylori exclusively colonizes the human stomach and causes chronic gastritis, peptic ulcers, MALT lymphoma, and gastric adenocarcinoma (1). H. pylori expresses virulence factors implicated in colonization, resistance to the acidic conditions of the stomach, and pathogenesis.

H. pylori associated gastric carcinogenesis.
NO is a signaling molecule with potent immunomodulatory capabilities, and we have directly implicated it as an important host defense component in H. pylori infection. Despite the induction of expression of the enzyme inducible NO synthase (iNOS), H. pylori inhibits NO synthesis by limited arginine availability and by suppressing iNOS translation through the formation of polyamines (16–19). Restoration of high-output NO production by gastric macrophages results in attenuation of gastritis in H. pylori-infected mice (17). However, the cellular mechanism of this anti-inflammatory effect induced by NO remains unknown. It has been described that NO is a potent inducer of heme oxygenase-1 (HO-1), an enzyme that possesses numerous anti-inflammatory properties (20). We thus reasoned that NO and H. pylori may modulate HO-1 in gastric epithelial cells.

We demonstrate in this study that H. pylori inhibits the NO-dependent induction of HO-1 in gastric epithelial cells by a process mediated by CagA. This occurs through activation of the transcription factor heat shock factor-1 (HSF1), which suppresses the NO-induced NF-κB activation and transcription of hmxox-1, the gene that encodes HO-1. Induction of HO-1 in gastric epithelial cells prior to H. pylori infection markedly attenuates IL-8 synthesis by human gastric epithelial cells in vitro and reduces inflammation in vivo.

Materials and Methods

Reagents

The NO donors NOR4 {([E]-ethyl-2-[(E)-hydroxyiminomino]-5-nitro-3-hexenecarbamoylpyrididine; 100 μM) and DEA-NOONate [diethylaminium (Z)-1-(N,N-diethylamino)diazirin-1-ium-1,2-diolate; 100 μM] and the iNOS inhibitor 1400W [N-(3-aminoethylmethylbenzylacetamide; 5 μM] were purchased from Alexis Biochemicals. The CO donor tricarbonyldichlororuthenium(II)-dimer (CORM2) and the HO-1 inhibitor zinc protoporphyrin IX (ZnP) were obtained from Sigma. The second HO-1 inhibitor chromium mesoporphyrin (CrMP) that we used in vivo was purchased from Frontier Scientific. The following pharmacological compounds were obtained from Calbiochem: the NF-κB inhibitor Bay 11-7082 [cr(4-methylthiophenyl)sulfonfyl]-2-propenenitrile; 10 μM]; the MEK inhibitor PD98059 [2′-amino-3′-methoxyflavone; 10 μM]; the ERK1/2 inhibitor ERKI [3(2-aminoethyl)-5-[(4-ethoxyphenyl)methylene]-2,4-thiazolidine-dione, HCl; 20 μM]; the JNK inhibitor SP600125 [anortha[1,9-cd]pyrazolo-[6,2-h]one; 1.9-μg/ml pyrazolone; 1 μM]; the p38 inhibitor SB203580 [4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl)1H-imidazole; 2 μM]; the PK3 inhibitor LYS294022 [4-(4-naphthalenyl)-8-phenyl-4H-1-benzopyran-4-one; 10 μM]; the hypoxia-inducible factor-1 (HIF1) inhibitor HIFI [3(2-[4-(adaman-1-yl)-phenyl]-acetyl)-aminomethylbenzene acid methyl ester; 10 μM]; and the Src inhibitor PP1 [4-amino-5-(4-methylphenyl)-7-(t-buty)pyrazolo-d-3,4-pyrimidine].

Bacteria

We used the cagA H. pylori strains 60190, 7.13, and PMSS1. The following isogenic mutants were also used as described: cagE, cagA, vacA, and flaA of the strain 60190 (21), cagA and vacA in the strain 7.13 (22), and cagE in the strain PMSS1 (23). The ureA mutant was constructed in H. pylori 60190 by deletion of the gene and insertion of a chloramphenicol resistance cassette as described (24). Bacteria grown on blood agar plates overnight were used to infect cells.

Human subjects

Biopsies from gastric tissues were obtained as described (22, 25) under two protocols approved by the Institutional Review Board at Vanderbilt University. The cagA status of H. pylori was determined from these tissues by PCR analysis performed on isolated colonies (26, 27). Histologic grading of gastritis was performed by a gastrointestinal pathologist (M.B.P.) who was blinded to the clinical status of the subjects. For all the figures, *p < 0.05, **p < 0.01, and was induced and inhibited in the stomach of C57BL/6 male mice (The Jackson Laboratory) by i.p. injection of hemin and CrMP, respectively (28). Mice were treated with hemin 4 and 2 d before infection with H. pylori PMSS1 (5 × 10^8 CFU/mouse), a strain that retains its ability to translocate CagA in vivo (23). CrMP was injected the day of the infection and every other day postinfection. Mice were sacrificed 2 d postinoculation with H. pylori.

Cells and transfections

The human gastric epithelial cell line AGS, the mouse conditionally immortalized stomach cells ImSt, and the murine macrophage cell line RAW 264.7 were maintained and used as described (29, 30). Cells were stimulated with H. pylori at a multiplicity of infection of 10. All pharmacological inhibitors of signaling pathways were added 30 min prior to activation.

AGS cells in Opti-MEM I Reduced Serum Media (Invitrogen) were transfected with 1) 100 nM ON-TARGETplus small interfering RNAs (siRNAs) (Dharmacon) directed against hsf1, hmxox-1, or hmox1 (the gene encoding lamin A, used as a control) using Lipofectamine 2000 (Invitrogen); 2) 100 nM SignalSilence siRNAs (Cell Signaling) directed against p38, SAPK/JNK, or ERK1/2 using Lipofectamine 2000; 3) 0.5 μg pNF-κB-Luc plasmid, an inducible reporter plasmid containing the luciferase gene driven by an NF-κB promoter element, or the pCIS-CK negative control plasmid (Agilent Technologies) using Lipofectamine 2000; and/or 4) 2 μg pSp65SRO plasmid vector expressing or not pSp65SRe-wild-type full-length cagA (pWT-cagA) or pSp65SRe-phospho-resistant cagA (pPR-cagA) using Lipofectamine LTX and Plus Reagent. After 6 h, cells were washed, maintained 24–36 h in fresh medium, and then stimulated.

mRNA analysis

RNA purification and real-time PCR were performed as described (31). Primers are listed in Supplemental Table I.

Western blots

For the p-CagA immunoblots, AGS cells were lysed in Tris-HCl 50 mM, pH 8, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, the Protease Inhibitor Cocktail (Set III; Calbiochem), and the Phosphatase Inhibitor Cocktail (Set I; Calbiochem); lysates were then sonicated. For all the other Western blots, nuclear and cytoplasmic proteins were extracted/separated using the NE-PER Nuclear Protein Extraction Kit ( Pierce). Protein concentrations were determined using the BCA Protein Assay (Pierce). Western blots were performed using 30 μg protein per lane for the p-CagA blots and 10 μg for all the others. Primary and secondary Abs are listed in Supplemental Table II.

The purity of the nuclear and cytoplasmic extracts was verified by Western blotting. Lamin A, a specific nuclear protein, was detected only in the nuclear fraction and was absent in the cytoplasmatic fraction, and inversely, the cytoplasmatic enzyme GAPDH was present only in the cytoplasmatic fraction (data not shown).

Determination of chemokine concentration

IL-8 and KC concentrations were determined in culture supernatants using DuoSet ELISA kits (R&D Systems).

Luciferase activity assay

AGS cells were lysed in RIPA buffer containing protease inhibitors, and luciferase activity was determined using the Luciferase Assay System (Promega). Luminosity was measured in a Synergy 4 microplate reader (BioTek).

HO-1 immunostaining and scoring system

Immunostaining was performed on human and murine gastric tissues as described (17) using a rabbit polyclonal anti-human/mouse HO-1 Ab (1:1000; StressGen). Slides were reviewed and scored by a gastrointestinal pathologist (M.B.P.) who was blinded to the clinical status of the subjects or the treatment conditions of the mice. HO-1 immunostaining intensity was graded on a scale of 0 (absent), 1 (weak), 2 (moderate), or 3 (strong) as described (32). The percentages of epithelial cells staining at each intensity level was multiplied by the intensity score, resulting in a scoring range of 0–300.

Statistical analysis

All the data represent the mean ± SEM. Student t test or ANOVA with the Newman–Keuls test were used to determine significant differences between two groups or to analyze significant differences among multiple test groups, respectively. For all the figures, *p < 0.05, **p < 0.01, and...
**Results**

**H. pylori inhibits NO-stimulated HO-1 expression in gastric epithelial cells through a mechanism that requires CagA**

The expression of *hmox-1*, the gene that encodes HO-1, was first analyzed in AGS cells treated with the NO donor NOR4 and/or infected with *H. pylori* strain 60190. The levels of *hmox-1* mRNA were significantly increased in human AGS cells treated with NOR4, whereas *H. pylori* alone had no effect (Fig. 1A). Further, when *H. pylori* and NO were added simultaneously to the cells, *hmox-1* transcripts were decreased in comparison with cells treated with NOR4 alone (Fig. 1A). Induction of *hmox-1* mRNA by NO and inhibition by *H. pylori* also occurred with ImSt cells infected with strain 7.13 (Fig. 1B), a coculture system that we have characterized well. Similar results also occurred when another NO donor, DEA-NONOate, was used in AGS cells (Supplemental Fig. 1). HO-1 protein levels increased in a time-dependent manner in AGS cells after NO stimulation (Fig. 1C). At each time point tested, there was less HO-1 protein expression when cells treated with NOR4 were also infected with *H. pylori* (Fig. 1C, 1D). Further, when compared with uninfected cells, a decrease in HO-1 protein level was observed in cells exposed to *H. pylori* alone for 18 h (Fig. 1C, 1D). In contrast, the mRNA and protein levels of heme oxygenase-2 did not change with exposure of cells to NOR4 or *H. pylori* (data not shown).

We then sought to identify the *H. pylori* factor(s) involved in the inhibition of *hmox-1* mRNA expression in NO-treated AGS cells. Wild-type (WT) *H. pylori* or vacA, ureA, or flaA mutant strains inhibited *hmox-1* mRNA expression by 70–90% (Fig. 2A); however, this inhibitory effect was substantially lost when either a cagA mutant or a cagE mutant, which fails to translocate CagA, was used (Fig. 2A). Furthermore, the NO-induced expression of *hmox-1* mRNA was effectively inhibited by either pWT-cagA or pPR-cagA compared with the pSP65SRex plasmid vector (Fig. 2B). Accordingly, we found that the Src kinase inhibitor PP1, which effectively decreased CagA phosphorylation in AGS cells (Fig. 2C), had no effect on the inhibition of *hmox-1* expression by *H. pylori* (Fig. 2D). Moreover, the concentrations of PP1 that we used were not toxic for the cells (data not shown). These data indicate that a functional T4SS and the native effector CagA are required to inhibit NO-induced *hmox-1* gene transcription.

The role of CagA was then confirmed in ImSt cells. The inhibition of NO-induced *hmox-1* expression by *H. pylori* 7.13 or by the vacA mutant was lost when the cagA mutant was used (Fig. 2E). Similarly, the strain PMSS1, but not the cagE mutant, inhibited the NO-induced *hmox-1* expression (Fig. 2E).

**Human infection with cagA**

To demonstrate the in vivo relevance of our findings, we analyzed the expression of HO-1 in gastric tissues of patients in which the cagA*+* or cagA*−* status of the infecting *H. pylori* strains was known. The levels of *hmox-1* transcripts in patients infected with cagA*+* strains were significantly decreased compared with those of normal gastric tissues or with those of subjects infected with cagA*−* *H. pylori* (Fig. 3A). We then evaluated the cellular localization of HO-1 protein by immunohistochemistry (Fig. 3B). In uninfected patients, HO-1 expression was abundant in gastric epithelial cells of the glandular necks and the deeper regions of the glands. Tissues from subjects infected with cagA*+* *H. pylori* strains exhibited less staining in epithelial cells compared with that of tissues from controls or persons infected with cagA*−* strains (Fig. 3B, 3C).

When gastric inflammation was graded in the same tissues, there was significantly more histologic gastritis in patients infected with the cagA*+* strains (4.9 ± 0.3) than in those with the cagA*−* strains (3.3 ± 0.3; *p* < 0.01). Further, we found that in gastric biopsies of *H. pylori*-infected patients, increased HO-1 levels correlated significantly with decreased gastritis (Fig. 3D).

**Induction of HO-1 by NO requires NF-κB, and the inhibition of HO-1 by *H. pylori* is mediated by ERK1/2, JNK, and HSF1**

We next determined the molecular mechanisms by which NO stimulates *hmox-1* mRNA expression and *H. pylori* suppresses this expression. We first used a pharmacological approach to inhibit various signaling pathways and transcription factors that may modulate transcription of *hmox-1*. As shown in Fig. 4A (left panel), inhibitors of NF-κB (Bay 11-7082) or PI3K (LY294002) significantly reduced *hmox-1* mRNA expression in AGS cells treated with NO. In contrast, the increased levels of *hmox-1* transcripts with NO treatment were maintained when all the other inhibitors were used (Fig. 4A, right panel). Moreover, the down-regulatory effect of *H. pylori* on NO-stimulated *hmox-1* expression was lost when inhibitors of MEK (PD98059), ERK1/2 (ERKi), or JNK (SP600125) were added to the cells, whereas the inhibitors of p38 (SB203580) or HIF1 had no significant effect (Fig. 4A, right panel); cell viability was not affected by the use of these pharmacologic inhibitors (data not shown). Similarly, the inhibitory effect of *H. pylori* on *hmox-1* induction was lost in cells transfected with ERK1/2 or JNK siRNAs but not in cells transfected with siRNAs for p38 or *hmox-1*, as shown in Fig. 4B. These data suggest that *hmox-1* mRNA expression induced by NO

![FIGURE 1](http://www.jimmunol.org/) Effect of NO and *H. pylori* on HO-1 induction in gastric epithelial cells. A and B, *hmox-1* mRNA expression. AGS (A) or ImSt (B) cells were treated with NOR4 and/or infected for 6 h with *H. pylori* (Hp) strain 60190 or 7.13, respectively. RNA was purified, reverse transcribed, and the expression of *hmox-1* was analyzed by real-time PCR; *n* = 5. C. The presence of the HO-1 and β-actin proteins was detected by Western blotting in cellular extract of AGS cells stimulated for 6, 12, or 18 h with NOR4 and/or *H. pylori*. Representative data of three independent experiments. D, Densitometric analysis of C; *n* = 3. *p* < 0.05, **p** < 0.01, ***p** < 0.001.

***p* < 0.001. The relationship between HO-1 levels and histological scores was determined using Pearson’s correlation test.
AGS cells transfected with pSP65SR were treated with PP1 for 30 min prior to stimulation with NOR4 with or without NOR4; values obtained for transfected cells treated with NOR4 were compared with those of cells not transfected and treated with NOR4 alone (real-time PCR; values obtained for infected cells treated with NOR4 were compared with those of cells treated with NOR4 alone; n = 3). CagA phosphorylation was assessed by Western blot using the PY99 Ab. Expression of hmox-1 mRNA was assessed by real-time PCR; values obtained for infected cells treated with NOR4 were compared with those of cells treated with NOR4 alone (D); n = 3. E, Expression of hmox-1 in ImSt cells stimulated with NOR4 and infected or not with H. pylori 7.13 or PMSS1 or with the corresponding mutant strains. Values obtained for infected cells treated with NOR4 were compared with those of cells treated with NOR4 alone; n = 3. **p < 0.01, ***p < 0.001.

requires PI3K and NF-κB and that H. pylori suppresses this induction through ERK1/2 and JNK.

The transcription factor HSF1 may modulate the transcription of various genes encoding heat shock proteins, such as hmox-1. Because a specific pharmacologic inhibitor of HSF1 does not exist, we transfected AGS cells with siRNAs for hsf1 or lmnA (used as control). The H. pylori-mediated inhibition of NO-induced hmox-1 transcription (Fig. 5A) and HO-1 protein expression (Fig. 5B) was completely abrogated by hsf1 siRNA, whereas the lmnA siRNA had no effect, demonstrating that H. pylori inhibits the induction of HO-1 through HSF1. Additionally, the enhancement of expression of hmox-1 in AGS cells treated with NOR4 and H. pylori that occurred with knockdown of HSF1 was inhibited by 89.7 ± 0.3% with the NF-κB inhibitor Bay 11-7082 (Fig. 5C). These data demonstrate that the HSF1-dependent downregulation of hmox-1 expression occurs through the inhibition of NO-induced NF-κB activation.

Activation of HSF1 by H. pylori is mediated by CagA, ERK1/2, and JNK

It has been reported that the activation of human HSF1 requires the phosphorylation of Ser326 (33). Consistent with our findings that implicate HSF1 in the negative regulation of hmox-1 by H. pylori, HSF1 phosphorylation on Ser326 was observed in AGS cells infected with H. pylori for 30 min, and this persisted for a 2-h period; this was not altered by NOR4 (Fig. 6A). Other studies have revealed that phosphorylation of HSF1 on a serine at position 303 represses its transcriptional activity (34). However, we found that HSF1 was not phosphorylated at Ser 303 in AGS cells infected with H. pylori, whereas the phosphorylation of Ser 326 (33). Consistent with our findings that phosphorylation of HSF1 on a serine at position 303 represses its transcriptional activity (34).

Because we found that ERK1/2, JNK, and HSF1 mediate the inhibition of hmox-1 expression by H. pylori, we reasoned that the signaling kinases ERK1/2 and JNK might favor HSF1 phosphorylation on Ser326. As shown in Fig. 6B, there was a marked decrease of p-HSF1-Ser326 in H. pylori-infected cells pretreated with ERKi or SP600125 compared with that of infected cells without any inhibitor. Consistent with our findings that the p38 inhibitor SB203580 had no effect on hmox-1 mRNA levels (see Fig. 4A), it failed to alter phosphorylation of HSF1 (Fig. 6B).

Because the inhibition of NO-induced hmox-1 expression by H. pylori requires CagA, we determined the involvement of this virulence factor in HSF1 phosphorylation. HSF1 was significantly less phosphorylated at Ser326 when cells were stimulated with the
H. pylori promotes inflammation by HO-1 inhibition

H. pylori-induced HSF1 downregulates hmxo-1 expression. A and B, AGS cells were transfected with siRNAs directed against hsf1 or lmnA and then treated with NOR4 and/or H. pylori. The levels of hmxo-1 mRNA (A; 6 h) and HO-1 protein (B; 18 h) were assessed by real-time PCR and Western blotting, respectively; n = 4 for A, and B is the representative data of three independent experiments. C, AGS cells, transfected with hsf1 or lmnA siRNAs, were treated for 6 h with NOR4 with or without H. pylori, with or without the NF-κB inhibitor Bay 11-7082 (Bay). The level of hmxo-1 transcripts was analyzed by real-time PCR; n = 4. ***p < 0.01, ****p < 0.001.

HO-1 suppresses chemokine production by H. pylori-infected gastric epithelial cells

We then analyzed the role of HO-1 in the innate immune function elicited by H. pylori in gastric epithelial cells. We first stimulated cells for 18 h with NOR4 to induce HO-1, and then infected them with H. pylori, in the presence or absence of the HO-1 inhibitor ZnPP. As expected, H. pylori induced IL-8 mRNA expression and IL-8 secretion in AGS cells (Fig. 8A, 8B, respectively). When infected cells were pretreated with NOR4, and thus express HO-1, we found a significant decrease in the levels of IL-8 mRNA and protein, and this inhibition was abolished when ZnPP was added to these cells (Fig. 8A, 8B). Pretreatment with NOR4 and/or treatment with ZnPP had no effect on IL-8 induction in uninfected cells. To verify further the ability of HO-1 to inhibit H. pylori-stimulated IL-8 generation, we used an siRNA approach. AGS cells were first transfected with siRNA directed against hmxo-1 or lmnA, pretreated with NO for 18 h, and then infected or not with H. pylori. The inhibitory effect of NOR4 pretreatment on H. pylori-induced IL-8 gene expression was eliminated using hmxo-1 siRNA but not suppressed using lmnA siRNA (Fig. 8C).
Because HO-1 converts heme into CO and bilirubin, we next ascertained the effect of these products on IL-8 expression in AGS cells. The CO donor CORM2 was capable of significantly inhibiting H. pylori-stimulated IL-8 mRNA levels with a concentration as low as 1 μM, whereas bilirubin had no significant effect (Fig. 8D). Both compounds had no significant effect on IL-8 mRNA expression in uninfected cells (data not shown).

We then determined the role of NO generated by cells on HO-1 induction and H. pylori-induced chemokine synthesis by gastric epithelial cells. We used a model in which ImSt cells in 0.2-μm filter supports were cocultured for 18 h with RAW 264.7 macrophages that were pretreated or not with IFN-γ to stimulate NO production. There was an increase in HO-1 protein levels in ImSt cells cultured with activated macrophages compared with those in ImSt cells cultured with unactivated macrophages (Fig. 9A); when the iNOS inhibitor 1400W was added to the coculture of ImSt cells with IFN-γ–treated RAW 264.7 macrophages, HO-1 was no longer induced (Fig. 9A). After coculture, ImSt cells were removed, washed, and infected with H. pylori. There was significantly less production of the neutrophil chemoattractant KC (also known as CXCL1) by H. pylori-infected ImSt cells expressing HO-1, due to being previously cocultured with activated macro-

![Figure 6. H. pylori activates HSF1. A, Immunodetection of p-HSF1 on Ser326 and total HSF1 in AGS cells exposed to NOR4 and/or H. pylori. B, AGS cells pretreated with ERKi, SP600125 (SP), or SB203580 (SB) were then infected with H. pylori for 1 h; HSF1 phosphorylation was analyzed by Western blotting. C, HSF1 phosphorylation in AGS cells infected for 1 h with H. pylori 60190 WT or with each mutant strain. D, Densitometric analysis of C; n = 3. E, Levels of p-HSF1(Ser326) in cells transfected with pSP65SRα, pWT-cagA, or pPR-cagA. *p < 0.05, **p < 0.01.](http://www.jimmunol.org/)

![Figure 7. H. pylori-induced HSF1 decreases NF-κB activation. AGS cells knocked down for lamin A or HSF1 were treated with NOR4 and/or infected with H. pylori. A, Nuclear p-p65 and cytoplasmic p-IκBα (3 h) were analyzed by Western blotting. B, NF-κB activity was determined in cells expressing the pNF-κB-Luc plasmid; n = 3. *p < 0.05.](http://www.jimmunol.org/)
phages, compared with that of ImSt cells cocultured with unactivated RAW 264.7 cells or with that of activated macrophages treated with 1400W (Fig. 9B).

Induction of HO-1 in the stomach leads to a reduction of host immune response to H. pylori

Because H. pylori inhibits HO-1 expression and HO-1 activity decreases IL-8/KC release by infected epithelial cells, we hypothesized that increasing HO-1 expression in the gastric mucosa could downregulate H. pylori-induced innate immune activation. We observed an increase of hmox-1 mRNA (Fig. 10A) and HO-1 protein (Fig. 10B) levels in mice treated with hemin compared with those of untreated animals. The HO-1 staining localized strongly to the gastric epithelium, and this was absent in tissues incubated with an isotype IgG control (Fig. 10B). The induction of hmox-1 mRNA and HO-1 protein by hemin remained in the presence of H. pylori or CrMP (Fig. 10A, 10B). The levels of H. pylori colonization were not significantly modulated by the hemin or CrMP treatments (Supplemental Fig. 2). It has been demonstrated that the infiltration of polymorphonuclear neutrophil (PMN) peaks at 2 d postinoculation with H. pylori in mice (35). Consistent with this, we detected a significant increase in expression of the chemokine KC (Fig. 10C) and in the number of PMN cells (Fig. 10D) in the antral mucosa of H. pylori-infected mice at this time point. These increases were abolished in animals treated with hemin and thus expressing HO-1, but these benefits of hemin treatment were lost in mice receiving CrMP (Fig. 10C, 10D). Treatment of infected mice with CrMP alone had no significant effect on KC expression and PMN recruitment. Taken together, these data suggest that the level of HO-1 in H. pylori-infected mice is not sufficient to regulate inflammation and that under conditions of experimentally enhanced HO-1 expression, acute gastritis is significantly attenuated. The major findings related to these data are summarized in Fig. 11.

Discussion

The molecular cross-talk that occurs between H. pylori and gastric epithelial cells is critical for the pathogenesis of the infection, given that these cells are the first in contact with the bacteria, and plays an important role in the development of the immune and inflammatory responses, as well as in carcinogenesis. In this context, our work has identified a novel mechanism by which H. pylori hijacks the signal transduction of the gastric epithelium and favors its own immunopathogenicity. As shown in Fig. 11, we have demonstrated that NO signals through NF-κB in gastric epithelial cells to stimulate the synthesis of HO-1, which inhibits the H. pylori-elicited production of IL-8; however, H. pylori activates HSF1, which inhibits NO-induced NF-κB activation and hmox-1 expression. Therefore, we propose that H. pylori has developed a strategy to promote its own pathogenesis by inhibiting HO-1 induction.

Our in vitro findings have direct significance in vivo as we have also shown that 1) the levels of HO-1 in gastric epithelial cells are decreased in patients infected with cagA+ H. pylori; 2) the level of
HO-1 expression is inversely correlated with the gastritis in infected patients; and 3) induction of HO-1 in cagA+ H. pylori-infected mice results in decreased acute gastritis as determined by KC mRNA expression and infiltration of PMN cells. It should be noted that there was no inhibition of hmox-1 expression in mice infected for 2 d; however, we have found that in mice infected for 4 mo with H. pylori PMSS1, the levels of hmox-1 mRNA were significantly decreased by 62% (data not shown). Conversely, it has been reported that the amount of HO-1 protein is higher in H. pylori-positive patients than that in controls when assessed by immunohistochemistry (36); however, the cagA status of the strains that infected these patients was not determined and may account for the discrepancies between our data and the former study. Additionally, we have confirmed our findings with mRNA analysis, which was not performed in the previous study.

In the current study, we analyzed the effect of NO on H. pylori-induced innate immune response of gastric epithelial cells. When cells were treated with NO donors, there was a strong induction of HO-1; importantly, this same induction was observed when cells were stimulated with NO released from activated macrophages, supporting the likelihood that this event can occur in the gastric niche where activated macrophages and epithelial cells are in very close proximity (37). We have demonstrated that gastric epithelial cells pretreated with NO and thus expressing HO-1 produce less IL-8 after H. pylori infection than cells not treated with NO, suggesting that NO may play an anti-inflammatory role in gastric tissue. Therefore, NO could be envisioned as a paracrine mediator produced by host gastric macrophages to limit the inflammatory response through the upregulation of HO-1 in epithelial cells. In this context, H. pylori could favor its own pathogenesis by limiting HO-1 induction by two strategies: first, the impairment of NO production by macrophages using its own arginase (16, 18) and the induction of host arginase II (19) and polyamine synthesis (17) that inhibit iNOS translation, and second, the direct inhibition of NO-induced hmox-1 mRNA expression, as shown in this study.

Several transcription factors, including NF-κB and HSF1, have been implicated in the regulation of hmox-1 transcription depending on the type of cells and the nature of stimulus (38). We show in this study that NF-κB is the main factor responsible for hmox-1 mRNA expression in gastric epithelial cells after stimulation with NO. Similar data have been obtained with human periodontal ligament cells (39) and in the cardiac tissue of transgenic mice expressing iNOS (40); in addition, NF-κB–dependent upregulation of the hmox-1 gene has been reported in human gastric carcinoma cells stimulated with heme or cadmium (41). It is notable that two NF-κB binding sites exist in the proximal promoter region of hmox-1 and are involved in the transcription of this gene (42).

In contrast to the finding that pathogenic Escherichia coli stimulates hmox-1 mRNA expression in enterocytes (31), we have now shown that H. pylori inhibits HO-1 induction in gastric epithelial cells through a mechanism that requires HSF1. We show...
that HSF1 is rapidly phosphorylated on Ser326 through a signaling cascade involving CagA translocation, ERK1/2, and JNK. Accordingly, it has been reported that nonphosphorylated CagA interacts with the growth factor receptor-binding protein-2 (43) or with the c-Met receptor (44) and inhibits epidermal growth factor receptor endocytosis thus favoring its abundance at the cell surface (45); together, these events may mediate the phosphorylation/activation of ERK1/2 (43, 46). However, it is not clear how H. pylori induces JNK activation; although the cag pathogenicity island is required for JNK activation (47, 48), CagA and the classical signaling pathways, including NOD1, Cdc42, Rac1, MKK4, and MKK7, are not involved (48, 49). Therefore, we speculate that HSF1 is activated by a mechanism that requires the T4SS and both CagA-mediated ERK1/2 activation and a JNK-dependent pathway (Fig. 11). The fact that HSF1 phosphorylation on Ser326 was not completely inhibited by the ERK1/2 or JNK inhibitors supports the concept that both pathways are required. Furthermore, HSF1 was not phosphorylated on Ser303, a residue injected by the T4SS are involved in the downregulation of HO-1.

Moreover, HSF1 was not phosphorylated on Ser303, a residue implicated in the inactivation of HSF1; this result is consistent with the fact that H. pylori inhibits GSK3α (50), the kinase responsible for Ser303 phosphorylation (34).

We then demonstrated that H. pylori-activated HSF1 inhibited the transcription of hmxox-1. Although two potential heat shock elements, heat shock element-1 and heat shock element-2, which can bind HSF1, are present in the promoter region of hmxox-1, these regions are not functional in humans (38). This suggests that despite being activated by H. pylori, HSF1 cannot initiate the transcription of hmxox-1. Further, it has been reported that HSF1 binding to heat shock element-1 represses the hmxox-1 expression in human Hep3B hepatoma cells (51). In parallel, we showed that activated HSF1 attenuates NF-κB activation. Similarly, it has been reported that heat-induced HSF1 blocks the DNA binding activity of NF-κB and suppresses the NF-κB–dependent transcription of TNF-α or iNOS in murine macrophages stimulated with LPS (52, 53). Although it is well established that H. pylori stimulates NF-κB activation, we now propose that this activation in gastric epithelial cells is transient and not maximal due to HSF1 activation. Together, these data demonstrate that H. pylori stimulates the activation of HSF1, which in turn inhibits NO-induced NF-κB activation and hmxox-1 mRNA expression. HSF1 may also directly decrease the binding of NF-κB to the hmxox-1 promoter region, as heat shock element-1 overlaps with one of the κB consensus sequences (42).

Aside from its role in heme degradation, the anti-inflammatory role of HO-1 has been well documented in other diseases such as colitis (54). HO-1 is implicated as a major immunomodulatory effector through 1) inhibition of the innate immune response by disrupting MAPK, NF-κB, or STAT-1 signaling (31, 55); 2) limitation of Th1 cytokine synthesis (56); 3) suppression of T cell proliferation (57), including Th17 cells (58); and 4) enhancement of regulatory T cell function (59). Our studies strongly support the concept that the ability of a pathogenic bacterium to limit the expression of an anti-inflammatory enzyme such as HO-1 will enhance its pathogenesis. Accordingly, our results show that inducing HO-1 in the stomach of H. pylori–infected mice results in decreased KC mRNA expression and PMN cell infiltration, indicating less acute inflammation. Moreover, the correlation of the low levels of HO-1 with the high levels of gastritis in patients with cagA1 infection and the higher levels of HO-1 that correlated with lower levels of cagA1 patients suggests that modulation of HO-1 may represent an important link between the cagA status of H. pylori and the development of inflammation. Strategies to induce HO-1 may represent a novel therapeutic approach to limit the severity of gastritis without the need to target H. pylori as a means to overcome the growing problem of antibiotic resistance.

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Disclosures

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References


