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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. *The Journal of Immunology*, 2011, 187: 000–000.

*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, among them the protein cytotoxin associated gene A (CagA) plays a pivotal role in the cause of *H. pylori*-associated gastric diseases (2, 3). Upon attachment to gastric epithelial cells, *H. pylori* uses a type IV secretion system (T4SS) to inject CagA into the cytoplasm of host cells (4). CagA is then phosphorylated on tyrosine residues by the host Src and c-Ab1 kinase families (5). p-CagA induces morphological transformation in gastric epithelial cells characterized by elongated cell shape with cytoskeletal rearrangements and increased motility (6) and also participates in the activation of the innate immune response of these cells (7–9). In addition, unphosphorylated CagA can also induce signaling in host cells and thus modulates cellular functions that include epithelial proliferation (9) and apoptosis (10). The combination of these cellular events mediated by CagA may increase the risk for carcinogenesis.

Innate immune function during *H. pylori* infection is mainly mediated by the MAPK enzyme family and the transcription factor NF-κB (7, 11). The activation of these signaling pathways leads to transcription of inducible genes encoding effectors of innate immunity, including cytokines and chemokines; among them, IL-8 is one of the most effective mediators of innate immunity, including cytokines and chemokines; among them, IL-8 is one of the most effective mediators of *H. pylori* immunopathogenesis as it recruits neutrophils to the mucosa of infected tissues. *H. pylori* infection also results in a mixed Th1/Th17-dominant T cell response (12, 13). These activated immune cells contribute to the establishment of chronic inflammation of the stomach (13, 14). However, these vigorous innate and adaptive responses fail to eradicate *H. pylori*; the persistence of the bacterium and the associated inflammation in the human stomach are major causal factors in gastric carcinogenesis. The failure of specific immunity can be in part due to increased regulatory T cell response during *H. pylori* infection (15), but other pathways are also involved (1). Because the innate immune response is linked to the development of adaptive responses, it is crucial to identify mechanisms by which *H. pylori* interacts with gastric epithelial cells and thus modulates their immune function.
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 limiting i-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 NO-induced NF-κB activation and transcription of hmox-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)-hydroxyimino]-5-nitro-3-hexanecarboxamidopyridine; 100 μM] and DEA-NONOate [diethyleniammonium (Z)-1-(N,N-dimethylamino)diacen-1-ium-1,2-diolate; 100 μM] and the iNOS inhibitor J-1400W [N-(3-aminoethyl)benzylacetamide; 5 μM] were purchased from Alexis Biochemicals. The CO donor tricarbonyldichloro- ruthenium(II)-dimer (CORM2) and the HO-1 inhibitor zinc protoporphyrin IX (ZnPP) were obtained from Sigma. The second HO-1 inhibitor chromium meso(porphyrin) (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 [1:600; StressGen]. Slides were reviewed and scored by a gastrointestinal pathologist (M.B.P.) who was blinded to the clinical status of the subjects.

**Animals and experimental design**

Mice in this study were used under protocol M05/176 approved by the Institutional Animal Care and Use Committee at Vanderbilt University. HO-1 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 × 10^6 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 100 nM ON-TARGETplus small interfering RNAs (siRNAs) (Dharmacon) directed against hsf1, hmox-1, or lnmA (the gene encoding lamin A, used as a control) using Lipofectamine 2000 (Invitrogen); 200 nM SignalSilence siRNAs (Cell Signaling) directed against p38, SAPKJNK, or ERK1/2 using Lipofectamine 2000; 3) 0.5 μg pNFκB-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 pS655Rα plasmid vector expressing or not pS655Rα-wild-type full-length cagA (pWT-cagA) or pS655Rα-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-HCI 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 completely absent in the cytoplasmic fraction, and inversely, the cytoplasmic enzyme GAPDH was present only in the cytoplasmic 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 kit (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 (1:600; 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

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**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.

**Human infection with cagA**

*H. pylori* results in decreased HO-1 levels in gastric epithelial cells

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 ERKi/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 2. Inhibition of NO-induced hmox-1 expression by H. pylori requires CagA. A. Expression of hmox-1 in AGS cells stimulated with NOR4 and infected or not with WT H. pylori or various mutant strains. Values obtained for infected cells treated with NOR4 were compared with those of cells treated with NOR4 alone; n = 3. B. Expression of hmox-1 in AGS cells transfected with pSP65SRO plasmid vector, pWT-cagA, or pPR-cagA and treated with NOR4. Values obtained for transfected cells treated with NOR4 were compared with those of cells not transfected and treated with NOR4; n = 3. C and D. Effect of the Src inhibitor PP1. AGS cells were treated with PP1 for 30 min prior to stimulation with NOR4 with or without H. pylori for 6 h. CagA phosphorylation was assessed by Western blot using the PY99 Ab (C). 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.

FIGURE 3. HO-1 levels are decreased in patients infected with cagA+ H. pylori. A. Expression of hmox-1 in gastric tissues with histologically normal mucosa and from subjects infected with cagA+ or cagA− strains of H. pylori; n = 8 cases per group. B. Representative HO-1 immunoperoxidase staining in gastric tissues. C. Quantification of staining score for HO-1 in gastric epithelium on a 0–300 scale; n = 10 cases per group. D. HO-1 staining and histologic gastritis scores were plotted for each patient. The linear regression line, the correlation coefficient (r), and the p value are shown. *p < 0.05, **p < 0.01.

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 Ser126 (33). Consistent with our findings that implicate HSF1 in the negative regulation of hmox-1 by H. pylori, HSFP1 phosphorylation on Ser126 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 Ser303 in AGS cells infected with H. pylori and/or treated with NOR4 (data not shown).

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. HSF1 was significantly decreased of p-HSF1-Ser 326 in AGS cells infected with H. pylori. HSF1 phosphorylation on Ser 326 was observed in AGS cells 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 Ser303 in AGS cells infected with H. pylori and/or treated with NOR4 (data not shown).

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 Ser126. As shown in Fig. 6B, there was a marked decrease of p-HSF1-Ser126 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
cumulation of p-p65-Ser 536 in the nucleus and the phosphorylation of HSF1 or without NOR4; at this time point, knockdown of lmnA increased phosphorylation of p65 and IκB, thus determined whether HSF1 effectively inhibits NF-kB activation by NOR4. Importantly, there was a marked reduction of NO-induced HSF1 leads to a reduction of NO-induced NF-kB activation.

We found that NO induces hmox-1 through an NF-κB–dependent pathway and that HSF1 downregulates hmox-1 expression. We thus determined whether HSF1 effectively inhibits NO-κB activation. We first analyzed by Western blotting (Fig. 7A) the phosphorylation of p65/Sez36 in the nucleus and pIkB accumulation in the cytoplasm of AGS cells. There was increased phosphorylation of p65 and IkB in cells with knockdown of lmnA followed by infection with H. pylori for 0.5 h, with or without NOR4; at this time point, knockdown of hsf1 had no additional effect. At a later time point (3 h), in cells transfected with lmnA, NF-κB activation by H. pylori was attenuated, but was activated by NOR4. Importantly, there was a marked reduction of p65 and p-IκB accumulation in cells treated with NOR4 when they were also infected with H. pylori. In contrast, knockdown of HSF1 resulted in sustained NF-κB activation at the 3-h time point in H. pylori-infected cells, as well as in cells treated with NOR4 and infected with H. pylori. Lastly, hsf1 siRNA had no effect on NF-κB activation by NOR4 alone. At both time points, similar results were obtained in cells transfected with lmnA siRNA and in non-transfected cells (data not shown).

We then assessed NF-κB activity directly in AGS cells using a reporter plasmid. NF-κB activity was rapidly and transiently increased after H. pylori infection in AGS cells transfected with lmnA siRNA (Fig. 7B). In the lmnA siRNA-transfected cells, NO-induced NF-κB activation was observed after 1 h and was then enhanced at 2 h; this increase was eliminated in NO-treated cells infected with H. pylori. However, knockdown of HSF1 resulted in sustained NF-κB activation in H. pylori-infected cells at 1 and 2 h postinfection, and the inhibition of NF-κB activity by H. pylori in NO-treated cells was no longer observed.

Taken together, the latter results indicate that the induction of NF-κB by H. pylori in gastric epithelial cells is actually suboptimal and is negatively regulated by HSF1. Moreover, the inhibition of NO-induced NF-κB activation by H. pylori is mediated by HSF1.

**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 hmox-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 hmox-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.

**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.
could downregulate H. pylori. We hypothesized that increasing HO-1 expression in the gastric mucosa decreases IL-8/KC release by infected epithelial cells, we have demonstrated that NO signals through NF-

\[ \text{H. pylori} \rightarrow \text{inhibition of IL-8/KC} \]

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 Ser\(^{326}\) 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 Ser\(^{326}\) was not completely inhibited by the ERK1/2 or JNK inhibitors supports the concept that both pathways are required. Further, our findings that \(hmoX\)-I inhibition by \(H.\) pylori in gastric epithelial cells was not completely abrogated when a \(cagA\)-deficient strain was used, whereas the \(cagE\) mutant did not inhibit the transcription of \(hmoX\)-I, suggest that other bacterial effectors injected by the T4SS are involved in the downregulation of HO-1. Moreover, HSF1 was not phosphorylated on Ser\(^{303}\), a residue implicated in the inactivation of HSF1; this result is consistent with the fact that \(H.\) pylori inhibits GSK3\(\alpha\) (50), the kinase responsible for Ser\(^{303}\) phosphorylation (34).

We then demonstrated that \(H.\) pylori-activated HSF1 inhibited the transcription of \(hmoX\)-I. Although two potential heat shock elements, heat shock element-1 and heat shock element-2, which are responsible for Ser\(^{303}\) phosphorylation (34).

\[\text{References} \]


