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Spermine Causes Loss of Innate Immune Response to *Helicobacter pylori* by Inhibition of Inducible Nitric-oxide Synthase Translation*

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***Helicobacter pylori* infection of the stomach elicits a vigorous but ineffective host immune and inflammatory response, resulting in persistence of the bacterium for the life of the host. We have reported that in macrophages, *H. pylori* up-regulates inducible NO synthase (iNOS) and antimicrobial NO production, but in parallel there is induction of arginase II, generating ornithine, and of ornithine decarboxylase (ODC), generating polyamines. Spermine, in particular, has been shown to restrain immune response in activated macrophages by inhibiting proinflammatory gene expression. We hypothesized that spermine could prevent the antimicrobial effects of NO by inhibiting iNOS in macrophages activated by *H. pylori*. Spermine did not affect the up-regulation of iNOS mRNA levels but in a concentration-dependent manner significantly attenuated iNOS protein levels and NO production. Reduction in iNOS protein was due to inhibition of iNOS translation and not due to iNOS degradation. ODC knockdown with small interfering (si) RNA resulted in increased *H. pylori*-stimulated iNOS protein expression and NO production without altering iNOS mRNA levels. When macrophages were cocultured with *H. pylori*, killing of bacteria was enhanced by transfection of ODC siRNA and prevented by addition of spermine. These results identify a mechanism of immune dysregulation induced by *H. pylori* in which stimulated spermine synthesis by the arginase-ODC pathway inhibits iNOS translation and NO production, leading to persistence of the bacterium and risk for peptic ulcer disease and gastric cancer.**

Helicobacter pylori is a Gram-negative, microaerophilic bacterium that selectively colonizes the human stomach. Current prevalence of *H. pylori* is ~30–40% of the population in the United States (1) and substantially higher in underdeveloped regions. *H. pylori* infection induces a vigorous mucosal immune response that fails to eradicate the organism and results in chronic gastritis that can lead to peptic ulcers and gastric cancer. In addition to a chronic lymphocytic response, *H. pylori* infection induces activation of an innate immune response in neutrophils, monocytes, and macrophages (2–8). Inducible NO synthase (iNOS)[1]-derived NO is a central effector molecule in the innate immune response to pathogens, with essential antimicrobial functions in host defense. We have reported that *H. pylori* induces iNOS expression and activity in macrophages (4–7). *H. pylori* is considered a noninvasive pathogen, but it can disrupt epithelial integrity, and its antigens are present in the lamina propria (3). *H. pylori* can induce iNOS and other innate immune response genes in macrophages even when separated by filter supports or when water extracts are used (6). Although *H. pylori*-induced NO production can kill the bacterium *in vitro* (7, 9), it survives in the stomach, despite detection of iNOS in infected gastric mucosa (10).

Production of NO by macrophages can be limited by *H. pylori* arginase that competes with iNOS for the same substrate, L-arginine (7) under conditions of low arginine availability. However, this effect is overwhelmed by increased substrate (7) indicating that other mechanisms of iNOS inhibition are likely important. *H. pylori* induces both arginase II and ornithine decarboxylase (ODC) in macrophages (5). Arginase converts L-arginine to L-ornithine, which is metabolized by ODC to produce the polyamine putrescine that is converted to the polyamines spermidine and spermine. Spermine has been shown to inhibit the immune effector function of monocytes and macrophages in response to LPS (11, 12). We now report that spermine inhibits *H. pylori*-stimulated NO production in macrophages by a post-transcriptional effect on iNOS translation. Our studies are the first to show a bacterial survival strategy in which the effectiveness of the host innate immune response is attenuated by inhibition of iNOS due to spermine synthesis.

EXPERIMENTAL PROCEDURES

Reagents—Reagents for cell culture, RNA extraction, and RT-PCR were obtained from Invitrogen. All other chemicals were from Sigma.

Bacteria, Cells, and Culture Conditions—*H. pylori* SS1 was grown and used as described (4). For bactericidal studies, *H. pylori* were separated from macrophages by filter supports (0.4-μm pore size; Transwell; Corning Inc.), and colony-forming units determined by serial dilution and culture (7). The murine macrophage cell line RAW 264.7 was maintained in complete DMEM (13). For coculture experiments, RAW 264.7 cells were plated in medium without antibiotics for 1 h prior

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[1] The abbreviations used are: iNOS, inducible NO synthase; ODC, ornithine decarboxylase; PAO, polyamine oxidase, DFMO, α-difluoromethylornithine; si, small interfering; RT, reverse transcription; DMEM, Dulbecco's modified Eagle's medium; Ab, antibody.

to addition of *H. pylori*. Experiments were also conducted with peritoneal macrophages isolated from male C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME). Cells were harvested 5 days after intraperitoneal injection of 0.5 ml Bio-Gel P-100 polyacrylamide beads (14).

Measurement of NO—Concentration of the oxidized metabolite of NO, nitrite (NO_2^-), was assessed by the Griess reaction (4–7).

RT-PCR—RAW 264.7 macrophages were seeded at 1×10^6/well in 6-well plates. After 6-h stimulation, total RNA was isolated and RT-PCR for iNOS performed exactly as described (5, 6).

Immunoblotting Analysis for iNOS, ODC—After coculture with *H. pylori*, RAW 264.7 macrophages were lysed and 100 μg of protein/lane separated by SDS-PAGE using 12% gels and transferred to Immobilon-P membranes (Millipore, Bedford, MA) by electroblotting. Membranes were blocked overnight at room temperature with 5% nonfat dry milk. iNOS (130 kDa), ODC (53 kDa), and β-actin (42 kDa) proteins were detected with a rabbit polyclonal iNOS Ab (1/1000; Pharmingen), a goat polyclonal ODC Ab (1/1000; Santa Cruz Biotechnology, Santa Cruz, CA), and a mouse polyclonal β-actin Ab (1/5000; Sigma), respectively. Chemiluminescent detection was performed as described (5, 6).

Immunoprecipitation Analysis for iNOS—After 18-h coculture of RAW 264.7 macrophages with or without *H. pylori*, and in the presence or absence of spermine, cells were washed and placed in methionine-depleted medium for 2 h in the presence or absence of spermine. Then, [^{35}S]methionine (0.2 mCi/ml, Amersham Biosciences) was added for 4 h and cells lysed in radioimmune precipitation assay buffer with protease inhibitors. For pulse-chase analysis, RAW 264.7 macrophages were stimulated and proteins labeled with [^{35}S]methionine. Spermine (12.5 μM) was added and cells harvested and lysed as above.

For immunoprecipitation, equivalent counts of trichloroacetic acid-precipitable [^{35}S]methionine-labeled proteins (2×10^8 cpm) were incubated overnight with monoclonal iNOS Ab (Pharmingen) and protein G-Sepharose (Calbiochem). After the immunoprecipitated complexes were washed, pellets were dissolved in gel-loading buffer and separated by SDS-PAGE. Gels were dried and imaged using a PhosphorImager (Storm 840 Phosphor Screen, Amersham Biosciences).

Transient Transfection of ODC siRNA in Macrophages—Four siRNA duplexes targeting ODC were designed and synthesized by Qiagen (Valencia, CA). These were tested for knockdown of ODC. The most effective siRNA duplex targeted nucleotides 1980–1998 (sense, 5′-CUCAUGAAACAGAUCCAGA-3′; antisense, 5′-UCUGGAUCUGUUUCAUGAG-3′). Scrambled control siRNA that had no sequence homology to any known genes was used as a control. Conditions for transfection and activation were exactly as described (13).

Measurement of ODC Activity and Polyamines—Cells were lysed and ODC activity was determined by a radiometric analysis of $^{14}CO_2$ liberated from L-[^{14}C]ornithine as described (5, 13). Polyamine levels were determined by precolumn dansylation reverse phase high-performance liquid chromatography as reported previously (15).

Statistical Analysis—Quantitative data are shown as the mean ± S.E. Comparisons between groups were made by using analysis of variance with the Student-Newman-Keuls multiple comparisons test.

RESULTS

Spermine Inhibits NO Production from H. pylori-stimulated Macrophages by Inhibiting iNOS Protein Translation—Since we have reported that NO production, measured as NO_2^-, peaks at 24 h after *H. pylori* stimulation (4, 5), we determined the effect of spermine on NO_2^- levels at that time point. As shown in Fig. 1*A*, addition of spermine resulted in a concentration-dependent inhibition of NO_2^- production in RAW 264.7 cells (*left panel*) and Bio-Gel-elicited mouse peritoneal macrophages (*right panel*). These data show an IC_{50} for spermine of 9.2 μM in RAW 264.7 cells and 9.0 μM in peritoneal macrophages.

We then determined the effect of spermine on iNOS in RAW 264.7 cells. Because *H. pylori*-stimulated iNOS mRNA expression peaks at 6 h (5), we assessed the effect of spermine at that time point. Addition of spermine (5–100 μM) did not inhibit *H. pylori*-stimulated iNOS mRNA expression (Fig. 1*B*). By Western blot analysis, spermine inhibited iNOS protein expression in a concentration-dependent manner (Fig. 1*C*) that paralleled the decrease in NO levels. This effect was not attributable to toxicity of spermine or its serum-mediated oxidation, since we measured cell viability by XTT assay and found that at a spermine concentration of 25 μM where cells had maximal

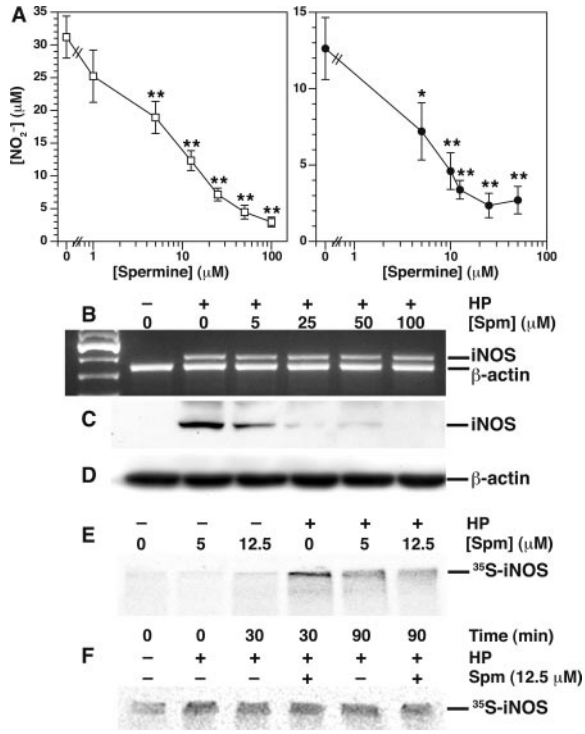

FIG. 1. **Effect of spermine on iNOS expression and NO_2^- production in *H. pylori*-stimulated macrophages.** *A*, inhibition of NO_2^- production by spermine in RAW 264.7 cells (*left*) and mouse peritoneal macrophages (*right*). Cells were cocultured with *H. pylori* at a multiplicity of infection of 10 in RAW 264.7 cells and 100 in peritoneal macrophages. Spermine was added at the same time as *H. pylori* and NO_2^- measured after 24 h. The level of NO_2^- in unstimulated control macrophages was 2.19 ± 0.25 μM in RAW 264.7 cells and 1.59 ± 0.30 μM in peritoneal macrophages. *, $p < 0.05$; **, $p < 0.01$ *versus* no spermine, $n = 5$–8 experiments in duplicate for RAW 264.7 cells and $n = 3$–6 mice at each concentration for peritoneal macrophages. Results shown are for stimulation with intact bacteria; similar results were obtained using lysates of *H. pylori*. *B*, iNOS mRNA expression in RAW 264.7 cells was assessed by RT-PCR at 6 h in the presence of the concentrations of spermine (*Spm*) as marked. iNOS (130 kDa) (*C*) and β-actin (42 kDa) (*D*) protein levels were assessed in RAW 264.7 cells by Western blotting with cell lysates harvested at 24 h. *E*, iNOS translation analysis. After treatment with the conditions noted, RAW 264.7 cells were labeled with [^{35}S]methionine, and iNOS protein was immunoprecipitated, resolved on SDS-PAGE and phosphorimaged. *F*, pulse-chase experiment. RAW 264.7 macrophages were cocultured with or without *H. pylori* for 20 h, [^{35}S]methionine was added for 4 h, and then the effect of spermine (12.5 μM) addition for 30 min or 90 min on *de novo* synthesized iNOS protein was determined. In *B*–*F*, representative experiments are shown, similar results were observed in at least three experiments.

inhibition of iNOS protein, there was still 90% viability in the presence of *H. pylori*. Additionally, incorporation of [^{35}S]methionine into total protein was unaffected by spermine, indicating that the effect on iNOS translation was specific.

Because we identified that spermine was inhibiting iNOS via an effect on iNOS protein, we directly tested the effect of spermine on *de novo* iNOS protein synthesis. *H. pylori* stimulated translation of iNOS that was significantly attenuated by spermine (Fig. 1*E*). We then conducted pulse-chase experiments (Fig. 1*F*) to determine whether spermine affected iNOS protein stability; addition of spermine to cells after 24-h stimulation with *H. pylori* did not inhibit levels of immunoprecipitated iNOS protein. Taken together, these results indicate that the inhibition of iNOS protein expression by spermine occurs at the level of protein translation and not by an effect on stability. To confirm this conclusion, we stimulated cells with *H. pylori* for 24 h, washed the cells to remove bacteria, and incubated them for an additional 24 h in the presence of spermine. Both NO_2^- and iNOS protein levels were identical in the presence

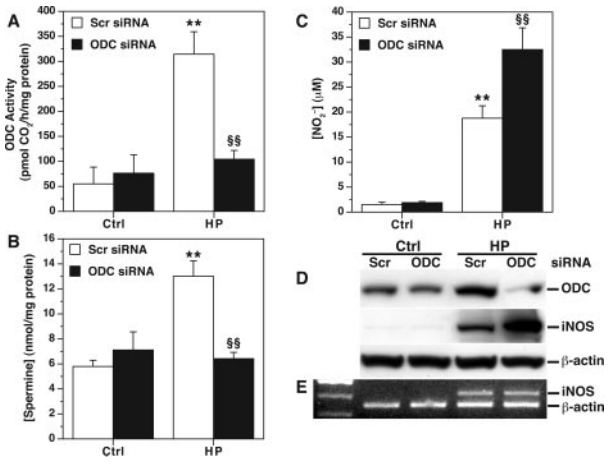

FIG. 2. **Transfection of ODC siRNA increases iNOS protein expression and NO_2^- production in *H. pylori*-stimulated macrophages.** RAW 264.7 macrophages were transiently transfected with duplex ODC siRNA or scrambled siRNA. Cells were stimulated with *H. pylori* at multiplicity of infection of 10, and the following were measured: ODC activity (*A*), spermine levels (*B*), NO_2^- production (*C*), protein expression of ODC and iNOS by Western blotting (*D*), and iNOS mRNA expression (*E*). *A*, *C*, and *D* were at 24 h after stimulation; *B* was at 12 h; and *E* was at 6 h. *A*—*C*: **, $p < 0.01$ *versus* unstimulated scrambled siRNA control; §§, $p < 0.01$ *versus H. pylori* + scrambled siRNA. In *A*–*D* and *E*, $n = 4$ and 2 separate experiments, respectively.

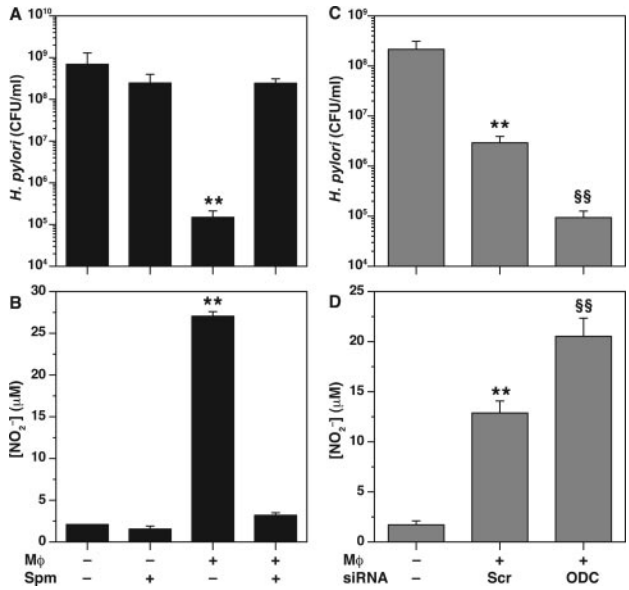

FIG. 3. **Spermine inhibits NO-mediated killing of *H. pylori* by macrophages.** *H. pylori* placed above transwell filter supports were incubated with or without 1×10^6 macrophages/ml in complete DMEM for 24 h at multiplicity of infection of 100. Colony-forming units were determined after 24-h coculture. *A*, effect of exogenous spermine addition on *H. pylori* survival. *B*, NO_2^- levels for the conditions in *A*. *A* and *B*: **, $p < 0.01$ *versus H. pylori* alone, *H. pylori* + spermine, or *H. pylori* + macrophages + spermine, $n = 4$. *C*, effect of transient transfection of ODC siRNA on *H. pylori* survival. *D*, NO_2^- levels for the conditions in *C*. *C* and *D*: **, $p < 0.01$ *versus H. pylori* alone; §§, $p < 0.01$ *versus H. pylori* + macrophages transfected with scrambled siRNA, $n = 4$.

and absence of spermine, indicating that spermine had no effect on preformed iNOS (data not shown).

We also assessed the effect of the two other biogenic polyamines on *H. pylori*-stimulated NO production and iNOS protein levels. Putrescine had no inhibitory effect, while spermidine had a modest effect that was less marked than spermine. At 12.5 μM, spermidine inhibited NO_2^- levels by 36.0 ± 2.6% *versus* 74.1 ± 7.6% for spermine ($p < 0.01$). A similar difference was detected by Western blotting (data not shown).

Specific Knockdown of ODC by siRNA Increases H. pylori-stimulated iNOS Protein Expression and NO Production—We have reported that *H. pylori* induces ODC expression and activity (13). We therefore transfected cells with an siRNA duplex specific for ODC or scrambled control siRNA. Transfection of ODC siRNA caused a significant reduction in *H. pylori*-stimulated ODC activity (Fig. 2*A*) and spermine levels (Fig. 2*B*) that resulted in a significant potentiation of NO production (Fig. 2*C*). Western blot analysis (Fig. 2*D*) demonstrated that in *H. pylori*-stimulated macrophages, transfection of ODC siRNA resulted in a significant knockdown of ODC protein expression and a concomitant marked increase in iNOS protein levels. Consistent with the data in Fig. 1, ODC siRNA had no effect on *H. pylori*-stimulated iNOS mRNA expression (Fig. 2*E*).

Spermine Prevents Killing of H. pylori by Macrophages—Since we have reported that *H. pylori* are killed by macrophages by an NO-dependent mechanism (7), we determined whether inhibition of *H. pylori*-stimulated macrophage iNOS expression and NO production by spermine impairs host defense against *H. pylori*. When *H. pylori* were cocultured in complete DMEM with macrophages for 24 h, there was a 3-log order killing of *H. pylori* compared with bacteria cultured in medium alone (Fig. 3*A*). Addition of spermine to cocultures prevented this killing. Spermine added to *H. pylori* alone in the absence of macrophages had no effect on *H. pylori* levels. There was an inverse correlation between bacterial concentration and NO production, such that killing was associated with increased NO_2^- levels and spermine rescue with loss of NO generation (Fig. 3*B*). When macrophages were transfected with ODC siRNA and cocultured with *H. pylori*, there was a significant enhancement of bacterial killing compared with scrambled siRNA control (Fig. 3*C*). There was again a clear correlation between killing and NO levels generated in these cells (Fig. 3*D*).

DISCUSSION

We demonstrated that spermine inhibits *H. pylori*-stimulated NO production by inhibition of iNOS protein translation. Production of polyamines by ODC in *H. pylori*-stimulated macrophages acts to down-regulate iNOS, since knockdown of ODC by RNA interference resulted in increased iNOS protein expression and NO production. We have shown in macrophages that putrescine synthesis is not induced by *H. pylori*, while spermidine and spermine are increased (13). Since addition of spermine had a greater inhibitory effect on iNOS than spermidine, it is most likely that the main effect of the ODC siRNA on iNOS is due to inhibition of spermine generation. Consistent with this, addition of spermine (25 μM) to macrophages transfected with ODC siRNA (data not shown), or prevention of spermine catabolism, discussed below, potently inhibited NO generation.

Spermine has been reported to inhibit NO production in LPS-stimulated J774 macrophages (12, 16). However, in these studies, the effect on iNOS itself was not studied. Also in this model NO production and inhibition with spermine depended on the presence of serum. In our model, *H. pylori* stimulates iNOS by an LPS-independent mechanism (4). When we replaced serum with bovine serum albumin, there was no loss of *H. pylori* stimulated NO production, and the inhibitory effect of spermine was maintained (data not shown).

Polyamine oxidation can occur by acetylation of spermine or spermidine by spermidine/spermine N^1-acetyltransferase prior to back-conversion by acetyl polyamine oxidase (17, 18) or by direct conversion of spermine to spermidine by polyamine oxidase 1 (PAO1), also called spermine oxidase (19, 20). We have recently demonstrated that *H. pylori* up-regulates expression and activity of PAO1 in macrophages, which acts to regulate intracellular spermine levels in activated cells (13). Inhibition

of polyamine oxidation with MDL 72527 resulted in a significant inhibition of *H. pylori* stimulated NO production and enhanced the inhibitory effect of spermine (data not shown). Similarly, we have found that in macrophages stimulated with *H. pylori*, cells transfected with PAO1 siRNA that prevents spermine metabolism have a marked inhibition of NO production, and conversely, transfection with PAO1 cDNA that causes spermine oxidation to spermidine results in potentiation of NO levels.[2] These findings indicate that it is spermine itself and not an oxidized product or spermidine that inhibits iNOS.

Knockdown of ODC could lead to increased iNOS and NO levels by shunting of L-arginine substrate away from the arginase-ODC pathway and back toward iNOS, since increased L-arginine availability has been reported to increase iNOS translation (21, 22). However, since spermine has a negative feedback effect on ODC (23), if this were true, addition of spermine or prevention of spermine degradation would be expected to increase iNOS, whereas we have observed the opposite effect. We used RNA interference to inhibit ODC, because α-difluoromethylornithine (DFMO), the pharmacologic inhibitor of ODC can increase spermine levels, which has been attributed to increasing *S*-adenosylmethionine decarboxylase (24). We have observed that in *H. pylori*-stimulated RAW 264.7 cells, DFMO inhibited putrescine and spermidine generation and increased spermine levels; in parallel there was inhibition of iNOS protein expression and NO production (data not shown). While there is a report of DFMO increasing iNOS protein and NO levels in LPS-stimulated J774.2 macrophages (25), but the effect only occurred when cells were pretreated for at least 24 h with DFMO, and the same group also reported inhibition of iNOS when DFMO was used concurrently with LPS stimulation (26).

The increase in iNOS and NO levels with ODC siRNA could be due to rescue of macrophages from apoptosis. We reported that inhibition of ODC (5) or of spermine oxidation (13) blocks *H. pylori*-induced apoptosis and restores cell viability; ODC siRNA has a similar effect (data not shown). However, we have strong evidence that changes in iNOS and NO levels occur independently of effects on apoptosis in *H. pylori*-stimulated cells: 1) inhibition of PAO1 by siRNA or MDL 72527 reduces apoptosis (13) but actually decreases iNOS/NO levels[2]; 2) overexpression of PAO1 increases *H. pylori*-stimulated iNOS/NO[2], despite causing macrophage apoptosis (13); and 3) prevention of apoptosis by inhibition of arginase does not further increase *H. pylori*-stimulated NO levels (5). Moreover, our Western blot analysis in Fig. 2 utilized equal amounts of protein loaded per lane that was verified by blotting for β-actin, indicating that the increase in iNOS protein levels cannot be explained simply by more cells contributing to the amount of iNOS.

Similar to our results, spermine has been shown to inhibit TNF-α and MIP-1α generation in monocytes by a post-transcriptional effect that was independent of the presence of serum or an oxidized product (11). While spermine is known to inhibit the translation of its own biosynthetic enzymes (23), we are the first to report that spermine regulates protein translation of an important immune response gene, namely iNOS. Although iNOS is a transcriptionally regulated gene (27), evidence of translational control has emerged, such that reduction of L-arginine availability by arginase (21, 22) reduces iNOS protein translation. We have found that spermine inhibits *H. pylori*-induced L-arginine uptake,[2] but spermine may have direct effects on iNOS translation because arginase inhibitors did not result in increased NO production in *H. pylori* stimulated macrophages in DMEM with serum (5).

We show that *H. pylori* are killed by macrophages separated from the bacteria by a filter, mimicking the situation *in vivo*, and that NO levels (measured as NO_2^-) are inversely correlated with bacterial levels, consistent with our previous report that iNOS$^{-/-}$ macrophages failed to kill *H. pylori* (7), and our findings that iNOS$^{-/-}$ mice infected with *H. pylori* have increased bacterial colonization and gastritis severity.[3] Our killing studies indicate that the induction of ODC by *H. pylori* contributes to the persistence of the bacterium. When combined with our studies implicating spermine and its oxidation product, H_2O_2, in apoptosis (5, 13) and DNA damage (28), we contend that the induction of ODC by *H. pylori* is a key cause of the dysregulation of the innate immune response to this pervasive pathogen.

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