l-Arginine Availability Regulates Inducible Nitric Oxide Synthase-Dependent Host Defense against *Helicobacter pylori*

Rupesh Chaturvedi, Mohammad Asim, Nuruddeen D. Lewis, Holly M. Scott Algood, Timothy L. Cover, Preston Y. Kim and Keith T. Wilson

***Infect. Immun.* 2007, 75(9):4305. DOI: 10.1128/IAI.00578-07.**
Published Ahead of Print 11 June 2007.

INFECTION AND IMMUNITY, Sept. 2007, p. 4305–4315 Vol. 75, No. 9
0019-9567/07/$08.00+0 doi:10.1128/IAI.00578-07

L-Arginine Availability Regulates Inducible Nitric Oxide Synthase-Dependent Host Defense against *Helicobacter pylori*▽

Rupesh Chaturvedi,[1,2] Mohammad Asim,[1,2] Nuruddeen D. Lewis,[2,3] Holly M. Scott Algood,[1,2] Timothy L. Cover,[1,2,4] Preston Y. Kim,[5] and Keith T. Wilson[1,2,3]*

Veterans Affairs Tennessee Valley Healthcare System, Nashville, Tennessee 37212[1]; Department of Medicine,[2] Department of Cancer Biology,[3] and Department of Microbiology and Immunology,[4] Vanderbilt University School of Medicine, Nashville, Tennessee 37232; and Department of Medicine, University of Maryland School of Medicine, Baltimore, Maryland 21201[5]

Received 20 April 2007/Accepted 5 June 2007

***Helicobacter pylori* infection of the stomach causes an active immune response that includes stimulation of inducible nitric oxide (NO) synthase (iNOS) expression. Although NO can kill *H. pylori*, the bacterium persists indefinitely, suggesting that NO production is inadequate. We determined if the NO derived from iNOS in macrophages was dependent on the availability of its substrate, L-arginine (L-Arg). Production of NO by *H. pylori*-stimulated RAW 264.7 cells was dependent on the L-Arg concentration in the culture medium, and the 50% effective dose for L-Arg was 220 μM, which is above reported plasma L-Arg levels. While iNOS mRNA induction was L-Arg independent, iNOS protein increased in an L-Arg-dependent manner that did not involve changes in iNOS protein degradation. L-Lysine, an inhibitor of L-Arg uptake, attenuated *H. pylori*-stimulated iNOS protein expression, translation, NO levels, and killing of *H. pylori*. While L-Arg starvation suppressed global protein translation, at concentrations of L-Arg at which iNOS protein was only minimally expressed in response to *H. pylori*, global translation was fully restored and eukaryotic translation initiation factor α was dephosphorylated. *H. pylori* lacking the gene *rocF*, which codes for a bacterial arginase, induced higher levels of NO production by increasing iNOS protein levels. When murine gastric macrophages were activated with *H. pylori*, supraphysiologic levels of L-Arg were required to permit iNOS protein expression and NO production. These findings indicate that L-Arg is rate limiting for iNOS translation and suggest that the levels of L-Arg that occur in vivo do not permit sufficient NO generation by the host to kill *H. pylori*.**

Helicobacter pylori is a gram-negative, microaerophilic bacterium that colonizes the human stomach, infects ~30 to 40% of the population in the United States (46), and has a higher prevalence in underdeveloped regions. *H. pylori* infection induces a chronic lymphocytic response and an innate immune response in neutrophils, monocytes, and macrophages (21–23, 33–35, 47). However, this response is ineffective, as the bacterium generally persists for the life of the host, leading to chronic gastritis, peptic ulcers, gastric adenocarcinoma, and lymphoma. Although *H. pylori* has often been considered a noninvasive pathogen, early studies indicated that its antigens are present in the lamina propria (33), recent studies have demonstrated the ability of *H. pylori* to invade gastric epithelial cells in vitro (2) and in vivo (42), and bacteria have been shown to be bound to erythrocytes in microvessels of the lamina propria (3). Furthermore, it has now been shown by transmission electron microscopy and immunogold detection that *H. pylori* is in direct contact with immune cells of the lamina propria in the majority of cases of gastritis and gastric cancer (38). Additionally, this bacterium can induce innate immune response genes and cause apoptosis in macrophages even when it is separated by filter supports or when water extracts or lysates are used (21, 23).

Inducible nitric oxide (NO) synthase (iNOS) expression can result in high-output production of NO, which is well known to be an important effector molecule in the innate immune response to pathogens due to its antimicrobial capability (16). We have demonstrated that *H. pylori* induces iNOS and NO production in macrophages (6, 21–23, 47). NO can kill the bacterium in vitro (6, 22, 30), but *H. pylori* survives in the human stomach despite detection of iNOS in the infected gastric mucosa (19). It is important to note that even when *H. pylori* is separated from the mucosal macrophages, NO itself is freely diffusible and can be detected systemically under conditions of infection or other forms of stress where iNOS is upregulated (for a review, see reference 17). As an example, in mice with colitis induced by *Citrobacter rodentium* infection in the colon there is a marked increase in serum NO levels (20). Moreover, when *H. pylori* is physically separated from macrophages by transwell filter supports in vitro, bacteria are still killed in an NO-dependent manner (6, 22). All of these findings have suggested to us that a factor contributing to the persistence of *H. pylori* in the stomach may be that the amount of NO made in the gastric mucosa may not be sufficient to kill the infecting *H. pylori*.

The iNOS gene is a highly inducible gene, and its transcription is readily upregulated by a variety of inflammatory stimuli (32, 49), including *H. pylori* (19, 21–23, 47). However, it has also been noted that the amount of NO produced by iNOS can depend greatly on the amount of the substrate, L-arginine (L-Arg). The term "arginine paradox" (18, 31) has been used to describe the finding that addition of L-Arg to cells can result in

* Corresponding author. Mailing address: Department of Medicine, Division of Gastroenterology, Vanderbilt University School of Medicine, 1030C MRB IV, 2215B Garland Ave., Nashville, TN 37232. Phone: (615) 343-5675. Fax: (615) 343-6229. E-mail: keith.wilson@vanderbilt.edu.
▽ Published ahead of print on 11 June 2007.

increased NO generation by iNOS at concentrations at which the enzyme should be saturated, even concentrations that are much greater than the K_m of iNOS, which has been reported to be in the range from 2.8 to 16 μM (25, 45). An additional component of this paradox stems from the concept that there appears to be a pool of intracellular L-Arg that is not accessible to iNOS and that uptake of extracellular L-Arg is required for substantive iNOS-derived NO generation (5, 11, 39).

We sought to determine the role of L-Arg availability in the macrophage response to *H. pylori* infection. Our data show that *H. pylori*-stimulated iNOS mRNA expression does not require L-Arg, but *H. pylori*-stimulated iNOS protein translation and NO production are entirely dependent on the L-Arg concentration such that levels substantially higher than the reported mammalian plasma levels (~100 μM) (4, 20, 43) are needed to maximize the host response. Furthermore, limitation of L-Arg availability inhibits iNOS translation and thus attenuates killing of *H. pylori*. These studies are the first studies to show that the host response of macrophages to a bacterial pathogen is dependent on L-Arg-driven iNOS translation.

MATERIALS AND METHODS

Reagents. All reagents used for cell culture, RNA extraction, and reverse transcription (RT)-PCR were obtained from Invitrogen. All other chemicals were purchased from Sigma.

Bacteria, cells, and culture conditions. *H. pylori* SS1 was grown and used as described previously (7, 47), and the multiplicity of infection (MOI) was defined as the ratio of bacteria to eukaryotic cells. Macrophages were activated with either live bacteria (6–8, 21–23) or *H. pylori* lysates prepared with a French press (7, 47), and MOIs were determined in lysates as described previously (47). For bactericidal studies, live *H. pylori* was separated from macrophages by filter supports (pore size, 0.4 μm; Transwell; Corning Inc., Corning, NY). When live bacteria were used, medium without antibiotics was used as described previously (6, 22). In some experiments an isogenic mutant of SS1 was used that is deficient in the *rocF* gene, which codes for *H. pylori* arginase, as described previously (22). The murine macrophage cell line RAW 264.7 was maintained in complete Dulbecco's modified Eagle's medium (DMEM) (7, 47). For experiments, cells were washed with phosphate-buffered saline (PBS) and incubated in L-Arg-free, phenol red-free DMEM (AthenaES, Baltimore, MD) without serum containing 0.3% bovine serum albumin for 2 h. The medium was removed, and fresh medium having the same composition with various concentrations of L-Arg was added. These experiments were conducted under serum-free conditions to avoid contamination with additional L-Arg. In additional experiments, cells were also cultured in DMEM without L-methionine, L-cystine (the stable oxidized form of L-cysteine), and L-leucine (Millipore, Billerica, MA) with different amounts of these amino acids added back.

Measurement of NO. The concentration of the oxidized metabolite of NO, nitrite (NO_2^-), was assessed by the Griess reaction (6, 21–23, 47). For the experiments with RAW 264.7 cells, 1×10^6 cells/ml of cell culture medium was plated, and data were expressed as micromolar concentrations of NO_2^-.

RT-PCR. iNOS mRNA expression was measured by RT-PCR as described previously (7). Two different sets of primers were used. The first set was used exactly as reported previously (6, 21, 23), generating a 499-bp product. The second set of primers consisted of sense primer 5′-CACCTTGGAGTTCACCC AGT-3′ and antisense primer 5′-ACCACTCGTACTTGGGATGC-3′, and the product size was 170 bp. The primer sequences for β-actin were those described previously (21). For the 170-bp iNOS product, 1 μg RNA was reverse transcribed using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Each PCR was conducted with 1 μl of cDNA and HotMasterMix (Eppendorf, Westbury, NY). Fifteen picomoles each of the sense and antisense primers and 3 pmol each of the β-actin primers (7, 21) were used. One PCR cycle consisted of the following: 95°C for 30 s, 60°C for 1 min, 50 s, and 72°C for 30 s. The total number of cycles was 32. A final elongation step of 7 min at 72°C was used for each reaction.

Real-time PCR. For iNOS the 170-bp product primers were used as described above. For cyclooxygenase 2 (COX-2), the primers were sense primer 5′-CCCC CACAGTCAAAGACACT-3′ and antisense primer 5′-GGCAATGCGGTTCT GATACT-3′. The primer sequences for ornithine decarboxylase (ODC) and β-actin were sequences described previously (21). cDNA was synthesized as described above, and 2 μl was used for real-time PCR for iNOS, COX-2, ODC, and β-actin with iQ SYBR green Supermix (Bio-Rad). The thermal cycling conditions and the method used to calculate relative expression have been described previously (7).

Immunoblot analysis. RAW 264.7 cells were treated with either live or lysed *H. pylori* for 24 h. In some experiments lactacystin (Calbiochem, San Diego, CA), a proteosomal inhibitor, was used. Cells were lysed and Western blotting for iNOS and β-actin was performed as described previously (6). Phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) was detected with a polyclonal antibody to phospho-eIF2α (Ser51; 1:1,000; Biosource, Camarillo, CA). COX-2 was detected with a mouse monoclonal antibody from BD Transduction Labs (San Jose, CA) (1:500), and ODC was detected with a polyclonal antibody from Santa Cruz Biotechnology (Santa Cruz, CA) (1:250).

Detection of iNOS protein by flow cytometry. After stimulation cells were washed with PBS, fixed in 0.1% paraformaldehyde, and permeabilized with 100% methanol on ice. Cells were then incubated with polyclonal rabbit anti-iNOS antibody (BD Transduction Laboratories) at a 1:200 dilution for 30 min at room temperature, which was followed by staining with goat anti-rabbit secondary antibody conjugated to fluorescein isothiocyanate. A total of 3×10^4 cells were acquired with a BD LSRII system (BD Biosciences) and analyzed using CELLQuest software (BD Biosciences).

Translation analysis for iNOS. After 18 h of coculture of RAW 264.7 macrophages with or without *H. pylori* in the presence and absence of L-lysine (L-Lys), proteins were labeled with [^{35}S]methionine and analyzed for iNOS translation exactly as described previously (6).

Global translation analysis. RAW 264.7 macrophages were cultured in L-Arg-free medium as described above, with different levels of L-Arg added. In additional experiments, cells were also cultured in medium with controlled concentrations of the amino acids L-methionine, L-cystine, and L-leucine. After 18 h of coculture with *H. pylori*, 20 μCi/ml of [^{35}S]methionine (specific activity, >1,000 Ci/mmol) was added for 4 h. Cells were washed with PBS and lysed in radioimmunoprecipitation assay buffer. Proteins were precipitated with 10% trichloroacetic acid (TCA), resuspended in radioimmunoprecipitation assay buffer, and blotted on nitrocellulose paper. The paper was then washed with 10% TCA and 70% ethanol. After drying, radioactivity was measured with a liquid scintillation counter (Tri-Carb 2100TR; PerkinElmer, Boston, MA). Lysates were also resolved by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and phosphorimaged (Cyclone; PerkinElmer).

Gastric macrophage isolation. Male 8-week-old mice were sacrificed by CO_2 inhalation. The stomach was rapidly removed from each mouse by excising between the distal end of the esophagus at the gastroesophageal junction and the proximal duodenum (near the pyloric sphincter). The paragastric lymph nodes were removed from the gastric wall. The forestomach (nonglandular portion) was removed from the glandular stomach and discarded. The glandular stomach was opened, rinsed gently in PBS, and cut longitudinally. To dissociate the gastric tissue, the glandular stomach was cut with scissors into 2-mm pieces and digested for 20 min with 1 mg/ml dispase, 0.25 mg/ml collagenase A, and 25 U/ml DNase (Roche Diagnostics, Indianapolis, IN) at 37°C. The suspension was passed through a 70-μm cell strainer (BD Biosciences, San Diego, CA). Cells were harvested by centrifugation and washed with PBS containing 4% fetal bovine serum. Cells were resuspended in cold IMag buffer (0.05% bovine serum albumin and 2 mM EDTA in PBS), and viable cells were counted with trypan blue.

Next, 1×10^7 cells were labeled with biotin-conjugated anti-mouse F4/80 antibody (CALTAG Laboratories, Burlingame, CA) using 100 μl of a 1:200 dilution on ice for 30 min. Cells were washed with 1 ml of IMag buffer and incubated with 50 μl of streptavidin conjugated with magnetic beads (BD Biosciences) on ice for 30 min. Cells were brought to a volume of 1 ml with cold IMag buffer, and cell suspensions were applied to an IMagnet (BD Biosciences) for 6 min at room temperature. The negative fraction was carefully removed, washed with 1 ml of cold IMag buffer, and then reapplied to the IMagnet for 5 min. The supernatant was removed, and the cells were resuspended in IMag buffer and reapplied to the IMagnet for 5 min. The positive fraction was resuspended in DMEM cell culture medium without L-Arg, phenol red, or serum. Viable cells were counted and plated in 96-well plates in the presence of different concentrations of L-Arg for experiments. In some experiments, the positive fraction was incubated with fluorescein isothiocyanate-conjugated monoclonal antibody to the leukocyte marker CD11b and allophycocyanin-conjugated monoclonal antibody to Gr-1, a marker for granulocytes. Cells were analyzed by flow cytometry to determine the percentage of positive cells using a BD LSRII system (BD Biosciences).

Isolation of total RNA from gastric macrophages and real-time PCR. A total of 2×10^4 cells/well were plated in 96-well plates and activated for 6 h with *H. pylori* lysate. Total RNA was isolated using a VERSAGENE 96-well RNA purification kit (Gentra Systems, Minneapolis, MN) according to the manufac-

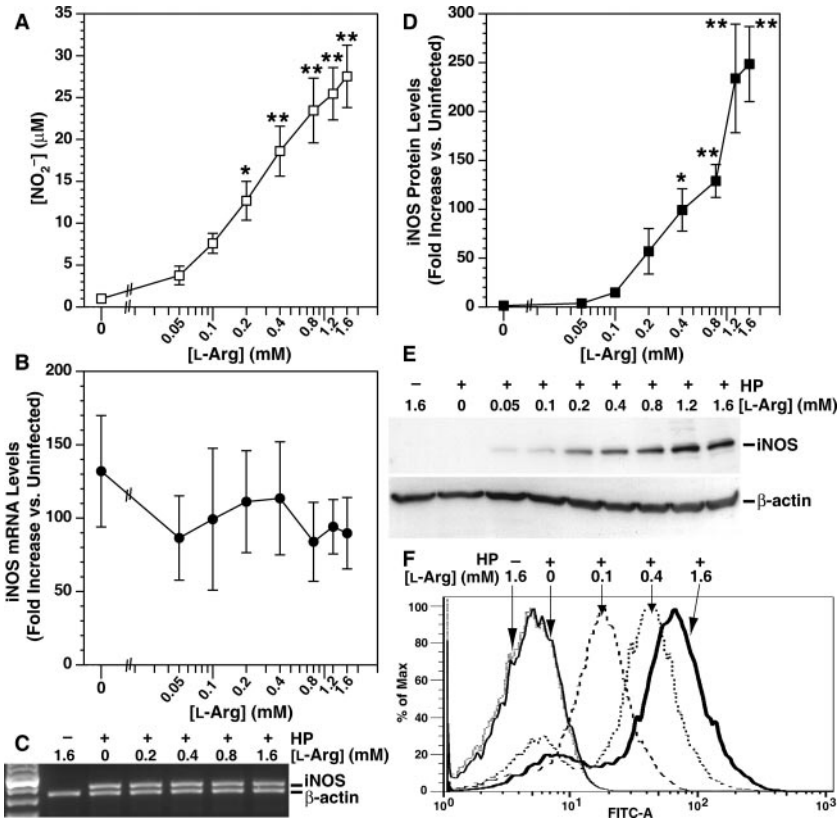

FIG. 1. Effect of L-Arg on iNOS expression in *H. pylori*-stimulated macrophages. RAW 264.7 macrophages were stimulated with intact *H. pylori* at an MOI of 10. Experiments were conducted in arginine- and serum-free DMEM to which known concentrations of L-Arg were added. (A) L-Arg-dependent increase in NO_2^- levels in cells stimulated with *H. pylori* for 24 h. One asterisk indicates a *P* value of <0.05 and two asterisks indicate a *P* value of <0.01 for a comparison with the preparation with no L-Arg (0 mM). Thirteen separate experiments were performed in duplicate. (B and C) iNOS mRNA expression assessed by real-time PCR (B) and RT-PCR (C) at 6 h after stimulation in the presence of the concentrations of L-Arg indicated. (D) Densitometry for Western blotting for iNOS. One asterisk indicates a *P* value of <0.05 and two asterisks indicate a *P* value of <0.01 for a comparison with the preparation with no L-Arg (0 mM). For panels B and D three separate experiments were performed. (E) Representative Western blot analysis performed for iNOS (130 kDa) (upper panel) and β-actin (42 kDa) (lower panel) with cells harvested at 24 h after stimulation. Thirty micrograms of total protein was loaded per lane. (F) iNOS protein levels assessed by flow cytometry as detected using a fluorescein isothiocyanate-conjugated secondary antibody. Representative fluorescence tracings from two separate experiments performed in duplicate are shown. HP, *H. pylori*; FITC-A, fluorescein isothiocyanate area.

turer's instructions. cDNA was synthesized from RNA with an iScript cDNA synthesis kit (Bio-Rad). Three microliters of cDNA was used for a real-time PCR for iNOS and β-actin, which was performed as described previously (7).

Analysis of NO production and iNOS protein expression in gastric macrophages. A total of 4×10^4 cells/well were plated in 96-well plates and activated with *H. pylori* lysate in the presence of various concentrations of L-Arg in serum-free DMEM. After 48 h, supernatants were removed and the NO_2^- concentration was assayed by using the Griess reaction as described above. NO_2^- concentrations are reported below as micromolar concentrations adjusted to a cell concentration of 1×10^6 cells/ml. iNOS protein was assessed by flow cytometry exactly as described above for the RAW 264.7 cells except that 5×10^3 cells were acquired for analysis.

Statistical analysis. Quantitative data are expressed below as means ± standard errors. For comparisons between multiple groups the Student-Newman-Keuls multiple-comparison test was used, and for single comparisons between two groups the Student *t* test was used.

RESULTS

***H. pylori*-induced NO production in macrophages is dependent on the L-Arg concentration and occurs through an effect on iNOS protein expression.** Because macrophage iNOS has been found to be regulated at multiple levels (6, 15, 31) and L-Arg availability has been shown to affect iNOS at the post-transcriptional level (15, 31), we cocultured *H. pylori* and RAW 264.7 macrophages in media containing different concentrations of L-Arg and compared iNOS mRNA, protein, and NO levels under these different conditions. We tested a broad range of L-Arg levels (from 0 to 1.6 mM) and found that NO production in response to *H. pylori* occurred in a concentration-dependent manner (Fig. 1A). With *H. pylori* stimulation, the levels of NO_2^-, the oxidized metabolite of NO, were the same as those in unstimulated cells when no L-Arg was present in the medium and continued to increase until the maximum level of L-Arg tested, 1.6 mM, was reached. From these data we estimated that the 50% effective dose of L-Arg is 220 μM, which is higher than previously reported plasma L-Arg levels (4, 20, 43). In contrast, when iNOS mRNA was assessed, there was substantial induction (132-fold) when *H. pylori* was added in the absence of L-Arg, and the level was not significantly affected by addition of L-Arg (Fig. 1B and 1C). This suggested an additional level of regulation. When iNOS protein levels were assessed by Western blotting (Fig. 1D and 1E), there was an absence of iNOS without L-Arg in the medium and there

was a steady increase in the iNOS level with L-Arg addition. Based on densitometry of Western blots (Fig. 1D), it was readily apparent that the iNOS protein levels were only minimally increased with 0.1 mM L-Arg, the concentration in plasma of both mice and humans (4, 20, 43), and that the levels continued to increase with addition of L-Arg in a manner similar to that observed for the increase in NO_2^- levels shown in Fig. 1A, completely distinct from the lack of increase in iNOS mRNA levels. To confirm the findings obtained with the Western blots, we also performed flow cytometry for iNOS protein (Fig. 1F) and found that with *H. pylori* stimulation there was no increase in iNOS protein in the absence of L-Arg and that there was a concentration-dependent increase at the levels of L-Arg assessed.

These findings are not attributable to an effect of the concentration of L-Arg in the medium on *H. pylori*, because we have found that higher levels of L-Arg do not enhance the viability or growth of *H. pylori* in DMEM, and the same effect of L-Arg on iNOS protein and NO production still occurred even when macrophages were activated with French-pressed *H. pylori* lysates rather than live bacteria (data not shown). It should also be noted that in unstimulated cells, even when they were cultured with the maximum concentration of L-Arg in the medium (1.6 mM) used in our studies, there was no induction of iNOS mRNA (Fig. 1B) or protein (Fig. 1C), and the NO_2^- levels were also not increased (data not shown).

Absence of iNOS protein at low L-Arg concentrations is not due to instability of the protein. Because we found that iNOS protein was not detectable in the absence of L-Arg despite the induction of iNOS mRNA expression, we considered the possibility that L-Arg deprivation results in decreased stability of the iNOS protein. We therefore compared iNOS protein levels by Western blotting in the absence and presence of a specific proteosomal inhibitor, lactacystin, that prevents proteosome-dependent degradation of iNOS (37). To avoid the inhibition of NF-κB activation that can occur with pretreatment with lactacystin (37), cells were treated with lactacystin and *H. pylori* at the same time. Additionally, because proteosomal inhibitors can alter protein stability in bacteria (40), we used lysates of *H. pylori* in these experiments. When cells were treated with *H. pylori* but deprived of L-Arg, addition of lactacystin failed to reveal that any iNOS protein was being made and then degraded (Fig. 2). Addition of lactacystin to unstimulated cells exposed to high levels of L-Arg also did not reveal any iNOS protein, indicating that there was not basal iNOS protein being made that was rapidly degraded. When cells were stimulated with *H. pylori* in the presence of either 0.4 or 1.6 mM L-Arg, lactacystin did not enhance the levels of the 130-kDa iNOS monomer, but it allowed detection of the dimeric form of iNOS that is normally degraded by proteosomes (Fig. 2). It should be noted that the iNOS dimer has been shown to be undisruptable in the presence of proteosomal inhibitors (29). As expected, the total amount of iNOS (dimer plus monomer) in *H. pylori*-stimulated cells cultured with L-Arg was greater in the cells treated with lactacystin than in cells not exposed to the proteosomal inhibitor. Taken together, these data indicate that L-Arg does not affect iNOS protein expression by an effect on protein stability.

L-Arg availability regulates NO production by an effect on iNOS protein translation. The data presented in Fig. 1 and 2 suggested that NO generation by *H. pylori*-stimulated macrophages requires induction of iNOS mRNA expression and is also dependent on a posttranscriptional effect of L-Arg that might be due to an effect on protein translation. To further verify the importance of L-Arg availability, we stimulated macrophages with *H. pylori* in the presence and absence of L-Lys, which is an inhibitor of L-Arg uptake into cells that also depletes intracellular L-Arg (9). L-Lys significantly attenuated NO production to the same low level regardless of the amount of L-Arg added (Fig. 3A). In contrast, iNOS mRNA expression was increased by *H. pylori*, regardless of the concentration of L-Arg or the presence of L-Lys (Fig. 3B). However, L-Lys treatment of *H. pylori*-stimulated cells completely blocked the iNOS protein expression that was otherwise detected in the presence of L-Arg (Fig. 3C). When iNOS translation was assessed by [^{35}S]methionine incorporation, the level was very low both in unstimulated cells and in cells activated with *H. pylori* but deprived of L-Arg, and there was a concentration-dependent increase in stimulated cells exposed to L-Arg (Fig. 3D) that paralleled the iNOS protein and NO_2^- levels. Additionally, iNOS translation was markedly attenuated with addition of L-Lys, which matched the loss of iNOS protein expression. These data indicate that L-Arg is required for iNOS translation and that L-Arg must be taken up from the extracellular environment for the iNOS translation to occur.

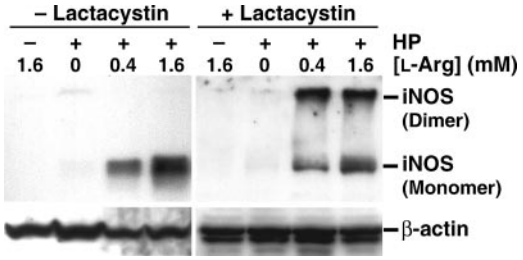

FIG. 2. Effect of inhibition of proteosomal degradation on iNOS protein levels. Lactacystin (10 μM) was added at the same time as *H. pylori* lysate (MOI, 100), and RAW 264.7 cells were harvested at 24 h. Western blotting was performed for iNOS and β-actin using the same polyclonal antibodies used for Fig. 1. Similar results were obtained in two experiments. HP, *H. pylori*.

Global protein translation requires a minimum level of L-Arg but is not concentration dependent at concentrations above 0.1 mM. Because amino acid starvation has been shown to suppress protein translation (41, 44), we determined whether the effects that we observed on iNOS could be nonspecific. We performed an analysis of total protein translation in RAW 264.7 cells by labeling proteins with [^{35}S]methionine and precipitating them with TCA (Fig. 4). When [^{35}S]methionine incorporation was assessed by scintillation counting (Fig. 4A) and by SDS-PAGE (Fig. 4B), both techniques demonstrated that there was suppression of translation in the absence of L-Arg, regardless of whether *H. pylori* was present in the medium. Addition of L-Arg enhanced total translation, and in the presence of *H. pylori* there was no further increase in protein translation at levels of L-Arg above 0.1 mM, the physiologic level of L-Arg in mice and humans (4, 20, 43). These data indicate that the concentration-dependent increase in the iNOS protein level that we observed (Fig. 1D to F) at concentrations of L-Arg above 0.1 mM cannot be attributed to changes in total protein translation.

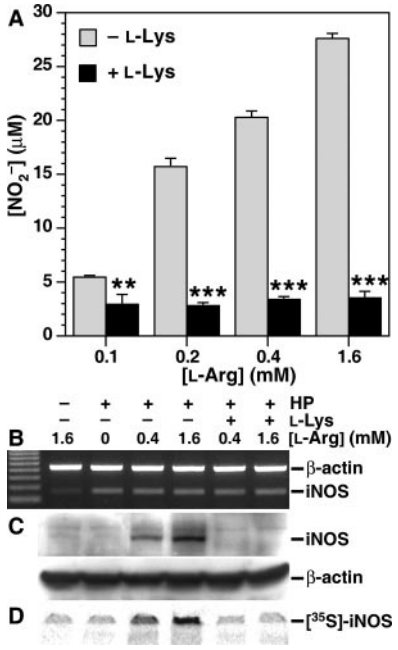

FIG. 3. iNOS protein translation is dependent on L-Arg uptake. L-Lys (20 mM), an inhibitor that blocks L-Arg uptake, was added to RAW 264.7 cells at the same time as intact *H. pylori* (MOI, 100). (A) L-Lys inhibits L-Arg-dependent induction of NO_2^- production. Two asterisks indicate a *P* value of <0.01 and three asterisks indicate a *P* value of <0.001 for a comparison with the preparation with no L-Lys ($n = 4$). (B) L-Lys had no effect on iNOS mRNA expression as assessed by RT-PCR at 6 h after stimulation. (C and D) L-Lys inhibited iNOS protein expression and iNOS translation, respectively. In panel C, Western blotting was performed at 24 h after stimulation. In panel D, cells were cultured with or without *H. pylori* at the concentrations of L-Arg indicated and in the presence or absence of L-Lys for 18 h, [^{35}S]methionine was added for 4 h, and iNOS protein was immunoprecipitated, resolved by SDS-PAGE, and phosphorimaged. In panels B to D the results of representative experiments are shown; similar results were obtained in at least two experiments in each case. HP, *H. pylori*.

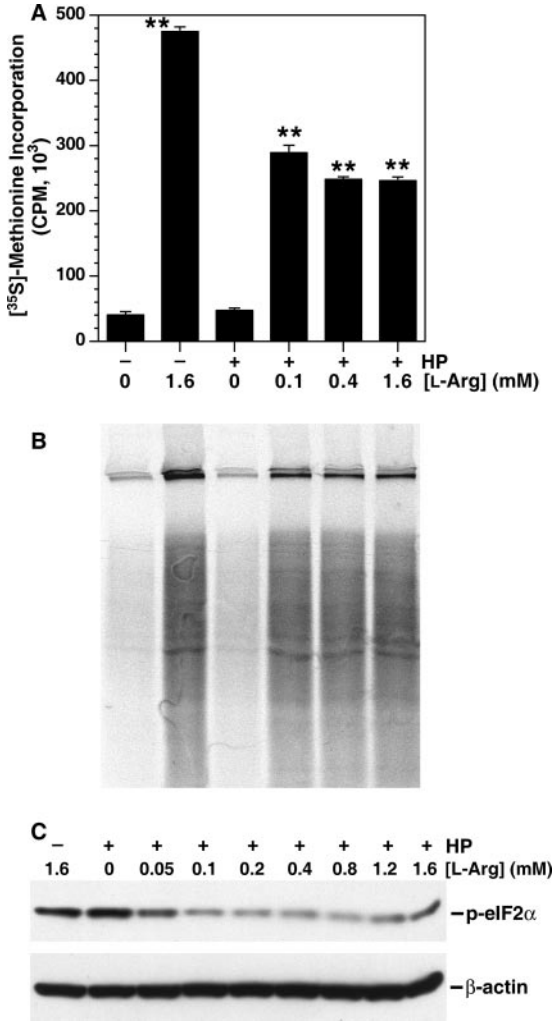

FIG. 4. Effect of L-Arg on global protein translation and dephosphorylation of eIF2α. (A and B) Total translation was measured by scintillation counting of [^{35}S]methionine in TCA-precipitable protein (A) and by SDS-PAGE and phosphorimaging (B). Note that the conditions indicated in panel A also apply to panel B. In panel A, one asterisk indicates a *P* value of <0.05 and two asterisks indicate a *P* value of <0.01 for a comparison with unstimulated cells cultured in the absence of L-Arg ($n = 3$). (C) Western blot obtained using a polyclonal antibody to phospho-eIF2α Ser51 (40 kDa) (upper panel) and β-actin (42 kDa) (lower panel). HP, *H. pylori*.

Levels of amino acids, including L-Arg, are known to alter the phosphorylation status of eIF2α (41, 44). The dephosphorylated form enhances translation, whereas the phosphorylated form is considered to be a global inhibitor of protein translation (41, 44). Dephosphorylation of eIF2α has been associated with facilitation of iNOS translation in brain astrocytes (31), but this phenomenon has not been previously studied in immune cells or in cells exposed to an infectious stimulus. We therefore assessed levels of the eIF2α phosphoprotein (Fig. 4C) and found that the levels were high in unstimulated cells even in the presence of high levels of L-Arg, indicating that L-Arg alone is unlikely to be sufficient to activate protein translation in macrophages. In cells activated with *H. pylori*, phospho-eIF2α levels rapidly decreased as L-Arg levels were increased from 0 to 0.1 mM, but there was not a significant change at higher concentrations of L-Arg. These data are consistent with the increase in global protein translation at 0.1 mM L-Arg that did not increase further at higher concentrations of L-Arg that we observed (Fig. 4A and 4B). However, these results are not consistent with the continued increase in iNOS protein expression (Fig. 1D to F and 3C) and iNOS protein translation (Fig. 3D) with concentrations of L-Arg above 0.1 mM, indicating that L-Arg may have specific effects on iNOS protein translation.

Other *H. pylori*-inducible proteins do not exhibit L-Arg dependence at concentrations above 0.1 mM. To further establish the specificity of the effect of L-Arg availability on iNOS, we studied two other genes that we have previously demonstrated to be induced by *H. pylori*, namely the genes encoding COX-2 (19, 35) and ODC (6–8, 21). As shown in Fig. 5A and 5C, *H. pylori* induced mRNA expression of COX-2 and ODC, respectively, in the absence of L-Arg, and there was no significant effect of addition of L-Arg on mRNA levels. When protein levels were assessed, for both COX-2 (Fig. 5B) and ODC (Fig. 5D) there was a lack of induction in the absence of L-Arg, but the protein levels were markedly increased at 0.1 mM L-Arg and did not increase further at higher concentrations of L-Arg, similar to the global protein translation data shown in Fig. 4A

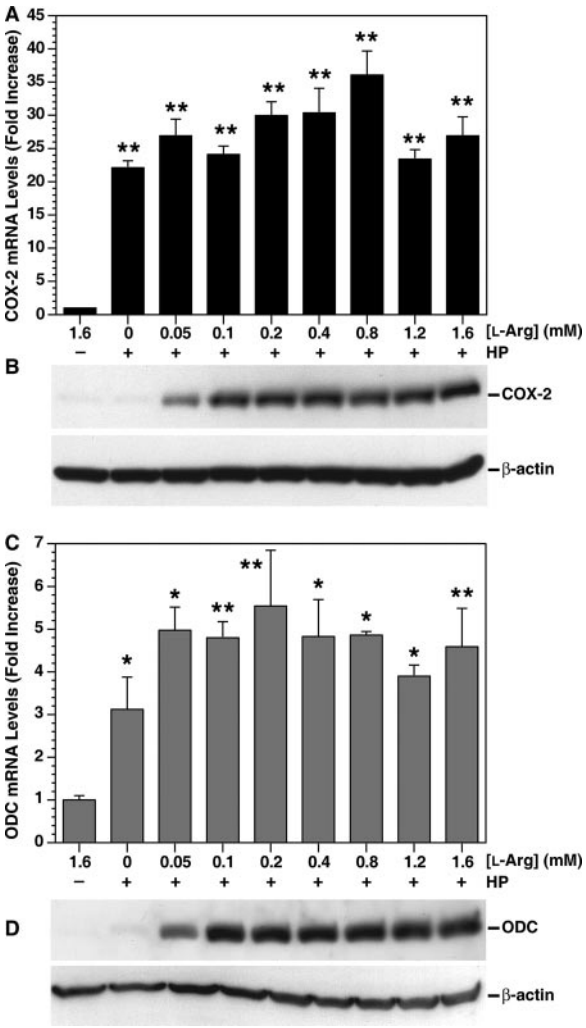

FIG. 5. *H. pylori*-stimulated expression of COX-2 and ODC in the presence of different concentrations of L-Arg. (A) COX-2 mRNA expression assessed by real-time PCR. The data are expressed as fold increases compared with unstimulated cells. (B) Western blot for COX-2 (upper panel) (72 kDa) and β-actin (lower panel). (C) Real-time PCR for ODC. (D) Western blot for ODC (upper panel) (53 kDa) and β-actin (lower panel). In panels A and C, two asterisks indicate a P value of <0.01 and three asterisks indicate a P value of <0.001 for a comparison with unstimulated cells ($n = 3$). In panel B, the blot was the same as that used in Fig. 4C after stripping and reprobing; hence, the β-actin is the same as that in Fig. 4C. In panel D, the blot was the same as that used in Fig. 1E; hence, the β-actin is the same as that in Fig. 1E. For panels B and D, similar results were obtained in two experiments. HP, *H. pylori*.

and 4B. Thus, these data are consistent with the requirement for a threshold of 0.1 mM L-Arg, which is known to be the physiologic level of L-Arg in vivo (4, 20, 43), for maximal protein expression of COX-2 and ODC, which is clearly different than the case for iNOS, for which there was minimal protein expression at 0.1 mM L-Arg and a marked increase in the iNOS level at higher levels of L-Arg.

Global protein translation and iNOS protein expression requires a minimal level of other amino acids, but the effect is not concentration dependent. To determine whether the effect of L-Arg was specific for this amino acid, we performed experiments in which medium deficient in either the essential amino acid methionine or leucine or the stable form of the nonessential amino acid cysteine (cystine) was used. When global translation was assessed (Fig. 6A and 6B), in the absence of each amino acid there was suppression of translation in *H. pylori*-stimulated cells compared to the translation in control medium in which these amino acids were replete. However, addition of individual amino acids to concentrations found in standard DMEM (0.2, 0.2, and 0.8 mM for methionine, cystine, and leucine, respectively) restored translation, and in fact addition of levels that were only 25% of the standard concentrations in DMEM also allowed normal levels of global translation (Fig. 6A and 6B). *H. pylori*-stimulated iNOS mRNA expression levels did not depend on the level of these amino acids (Fig. 6C), exactly as we observed with L-Arg. NO production was attenuated by the absence of methionine, cystine, or leucine (Fig. 6D). However, at the levels of the amino acids that were 25% of the standard DMEM levels, there was maximization of NO_2^- levels that was paralleled by similar expression of iNOS protein that did not increase further with higher concentrations of these amino acids (Fig. 6E).

Killing of *H. pylori* by macrophages is dependent on both the extracellular L-Arg concentration and macrophage uptake. Macrophage-derived NO is likely to be an important component of the host defense against *H. pylori*, since we have shown that lamina propria macrophages from an infected human stomach express iNOS (19) and NO can kill *H. pylori* in vitro (6, 22, 30). When *H. pylori* is cultured above a transwell filter support with macrophages below the support, there is induction of iNOS mRNA, iNOS protein, and NO release (23) and killing of the bacterium is NO dependent (22). Therefore, the effect of L-Arg concentration and availability on the ability of macrophages to kill *H. pylori* was assessed in this transwell model system. When *H. pylori* was cocultured with macrophages in L-Arg-free medium, there was no decrease in the number of viable *H. pylori* cells compared to the results when bacteria were added alone without macrophages (Fig. 7A). Addition of L-Arg to the medium bathing the macrophages allowed NO production to occur (Fig. 7B), and this restored bactericidal activity (Fig. 7A). Addition of L-Lys inhibited macrophage NO generation in the transwell system (Fig. 7B), and this prevented bacterial killing (Fig. 7A). Therefore, in this model that mimics the separation of *H. pylori* from macrophages in the stomach, the ability of the macrophages to kill *H. pylori* is dependent on entry of extracellular L-Arg into the macrophages. Further, these data suggest that factors that limit the amount of L-Arg that is able to enter the cell, whether through reduction of extracellular L-Arg or inhibition of uptake, compromise the ability of cells expressing iNOS mRNA to be able to exert an antibacterial effect on *H. pylori*.

***H. pylori* arginase inhibits iNOS protein expression.** *H. pylori* itself possesses a bacterial arginase, encoded by the gene *rocF*, which can reduce the availability of extracellular L-Arg to the host cells to the point where macrophage NO production and killing of *H. pylori* are markedly attenuated (6, 22, 30). In light of our present finding that uptake of extracellular L-Arg is required for iNOS translation, we sought to determine whether *H. pylori* arginase simply reduces macrophage NO production by reducing the amount of substrate for iNOS by competitive inhibition alone or whether the effect of the bacterial arginase is to reduce macro-

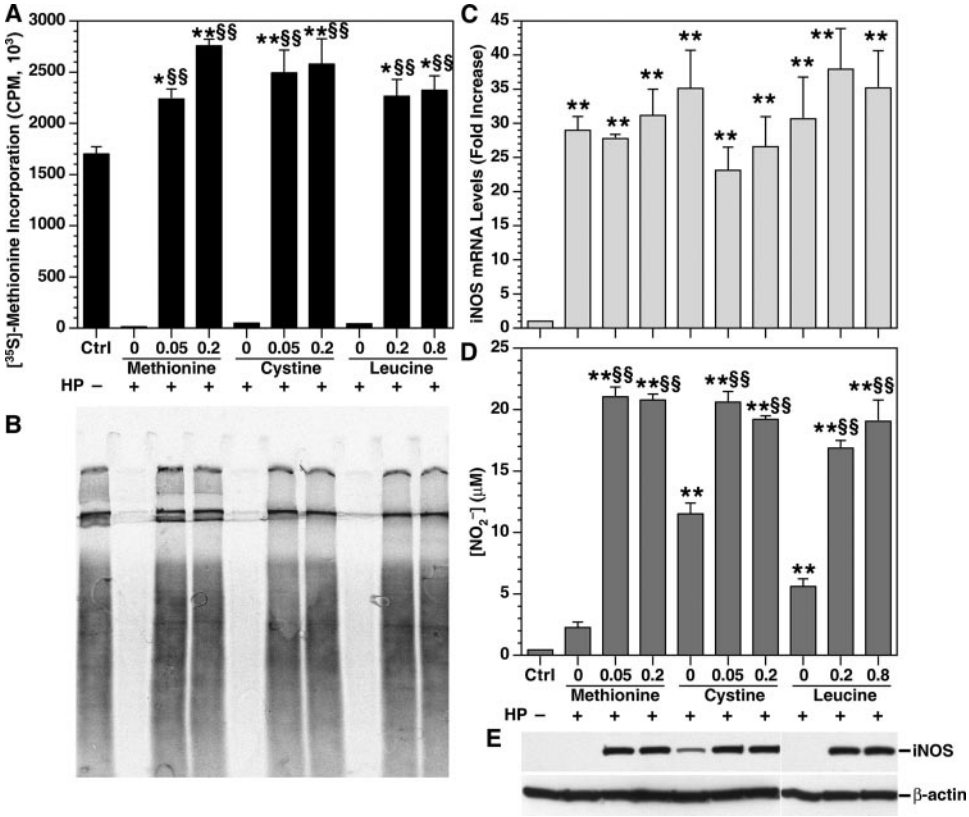

FIG. 6. Effect of other amino acids on global translation and iNOS protein expression. RAW 264.7 cells were cultured in medium deficient in the amino acids L-methionine, L-cystine, and L-leucine, to which each was individually added back at the concentrations indicated (in mM). Ctrl, unstimulated cells cultured in the medium to which L-methionine, L-cystine, and L-leucine were all added back at the concentrations found in standard DMEM (0.2, 0.2, and 0.8 mM, respectively). Total protein translation was measured by scintillation counting of [^{35}S]methionine in TCA-precipitable protein (A) and by SDS-PAGE and phosphorimaging (B). Note that the conditions indicated in panel A also apply to panel B. (C) iNOS mRNA levels assessed by real-time PCR. The data are expressed as the fold increases compared with unstimulated cells cultured in medium with replete amino acids. (D and E) NO_2^- levels (D) and iNOS Western blotting (E) for cells cultured with different concentrations of amino acids, as described above for panel A. Note that the conditions indicated in panel D apply to panels C and E. In panels A, C, and D, one asterisk indicates a P value of <0.05 and two asterisks indicate a P value of <0.01 for a comparison with the unstimulated control, and one section sign indicates a P value of <0.05 and two section signs indicate a P value of <0.01 for a comparison with the culture with none of the amino acid added back (0 mM). (A and C) $n = 3$; (D) $n = 3$ in duplicate. HP, *H. pylori*.

phage iNOS expression. We used 0.4 mM L-Arg in the medium for these experiments, since this is the concentration in most cell culture media that are normally used, such as DMEM, and we had found (Fig. 1 and 3) that this level of L-Arg results in a substantial level of iNOS protein expression and NO generation. Macrophages were cocultured with wild-type and isogenic *rocF* mutant *H. pylori* SS1 at various MOIs. As shown in Fig. 8A, there was a significant increase in the NO_2^- levels generated by the macrophages when they were activated with the *rocF* strain compared with the results obtained with the wild-type strain that was not associated with differences in iNOS mRNA levels (Fig. 8B), but there was an increase in iNOS protein levels (Fig. 8C) with deletion of bacterial arginase. These data indicate that *H. pylori* arginase can effectively limit iNOS protein expression by inhibiting L-Arg availability to host cells.

Gastric macrophages require exposure to excess L-Arg to express iNOS protein and generate NO. To further assess the relevance of our findings, we sought to verify that gastric tissue-derived macrophages express iNOS in response to *H. pylori* and to determine whether they also exhibit dependence on L-Arg availability for iNOS protein expression and NO production. Mouse stomachs were enzymatically digested, and in the initial experiments, we determined that there were $1.81 \pm 0.12 \times 10^5$ leukocytes isolated per stomach by positive selection with the α-integrin CD11b, 17.4% ± 1.6% of which stained positive for the macrophage marker F4/80, yielding a total of $3.12 \times 10^4 \pm 0.29 \times 10^4$ macrophages per mouse ($n = 6$).

We then proceeded with direct isolation of macrophages by using streptavidin-conjugated magnetic beads binding to biotinylated antibody to F4/80, as described in Materials and Methods. After this positive selection, cells were labeled with antibody to CD11b and assessed by flow cytometry to verify that the cells isolated were leukocytes (Fig. 9A) and that they did not contain neutrophils since they were negative for the marker Gr-1 (Fig. 9B). When cells were activated ex vivo with *H. pylori*, the results mirrored those obtained with the RAW 264.7 cell line, in that the NO production, measured as NO_2^-, was not increased above basal levels in the absence of L-Arg and increased in a concentration-dependent manner as L-Arg was added (Fig. 9C). iNOS mRNA expression was significantly

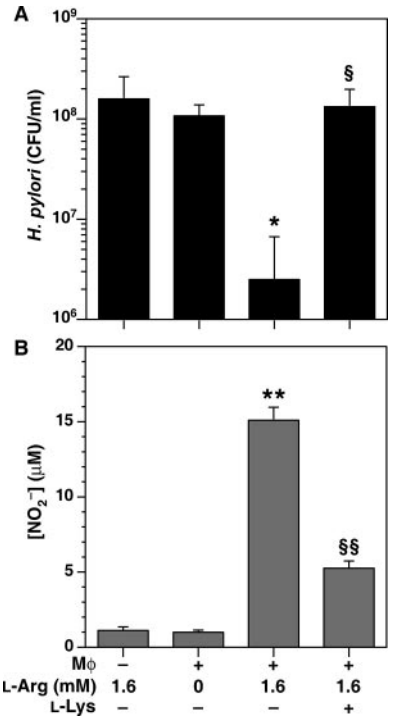

FIG. 7. Killing of *H. pylori* by macrophages is dependent on L-Arg availability. Live *H. pylori* placed above transwell filter supports was incubated with or without 1×10^6 macrophages (Mϕ)/ml in arginine- and serum-free DMEM with either 0 or 1.6 mM L-Arg for 24 h at an MOI of 100. The starting amount of bacteria in these experiments was 0.5×10^8 cells (1×10^8 cells/ml) added to 0.5×10^6 cells. CFU were determined after 24 h of coculture. (A) Effect of L-Arg and L-Lys on *H. pylori* survival. (B) NO_2^- levels for the conditions used for panel A. One asterisk indicates a P value of <0.05 and two asterisks indicate a P value of <0.01 for a comparison with *H. pylori* alone or *H. pylori* plus macrophages with 0 mM L-Arg. One section sign indicates a P value of <0.05 and two section signs indicate a P value of <0.01 for a comparison with *H. pylori* plus macrophages with 1.6 mM L-Arg without L-Lys ($n = 4$).

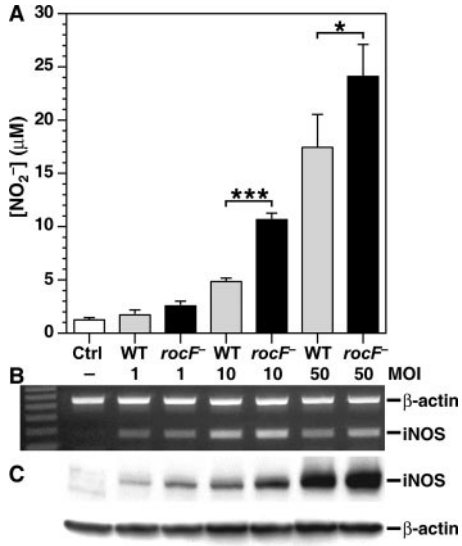

FIG. 8. *H. pylori rocF* deletion increases macrophage iNOS protein expression. Live wild-type (WT) and isogenic mutant *rocF* strains of *H. pylori* were cocultured with macrophages at the MOIs indicated. (A) NO_2^- levels. One asterisk indicates a P value of <0.05 and three asterisks indicate a P value of <0.001 for a comparison of the *rocF* mutant and the wild type ($n = 4$ to 7). (B) RT-PCR analysis of iNOS mRNA expression. (C) Western blot analysis of iNOS and β-actin protein levels. In panels B and C, the results of representative experiments are shown; similar results were obtained in at least two experiments for panel B and five experiments for panel C.

increased with *H. pylori* stimulation even in the absence of L-Arg and was not further increased with addition of L-Arg (Fig. 9D). In contrast, when iNOS protein levels were assessed by flow cytometry (Fig. 9E and 9F), we found that with *H. pylori* stimulation there was no increase in iNOS protein in the absence of L-Arg. With addition of 0.4 mM L-Arg there was only a slight increase in iNOS protein, and only at 1.6 mM L-Arg did the increase become significant. Taken together, these data suggest that when gastric macrophages are exposed to *H. pylori* products, high levels of L-Arg are required to generate iNOS protein.

DISCUSSION

In the current study we demonstrated that induction of iNOS by *H. pylori* is tightly regulated by L-Arg availability. Specifically, our data show that iNOS mRNA expression does not require L-Arg but that iNOS protein expression is very sensitive to L-Arg levels, since protein levels increased in a concentration-dependent manner and were effectively attenuated by inhibition of L-Arg uptake. Additionally, the protein expression closely paralleled levels of iNOS translation and was not attributable to an effect on protein stability. For effective host defense against *H. pylori*, uptake of extracellular L-Arg was required to generate sufficient NO to kill *H. pylori* in our coculture model. Importantly, we found that *H. pylori*-derived arginase can compete with host cells by inhibiting iNOS protein expression, and we showed that gastric macrophages also exhibit attenuation of iNOS protein expression at physiologic levels of L-Arg.

In our study, changes in iNOS protein expression and translation were not attributable simply to changes in global protein translation. Amino acid starvation is known to result in activation of the protein kinase GCN2, which then phosphorylates eIF2α at Ser51 and results in global suppression of protein translation (44). We found that under resting conditions, eIF2α was still heavily phosphorylated even with high levels of L-Arg, which may reflect a need for activation of the cells to dephosphorylate this protein. Our finding that eIF2α was dephosphorylated in the presence of *H. pylori* with 0.1 mM L-Arg was similar to the findings for astrocytes activated with cAMP plus gamma interferon (IFN-γ), where similar dephosphorylation was demonstrated at this concentration of L-Arg (31). However, our data differ in that the iNOS protein expression continued to increase at concentrations of L-Arg above 0.1 mM even though eIF2α dephosphorylation had already occurred. This indicates that other translational control mechanisms are likely to be involved. Dependence of iNOS protein expression on L-Arg availability has been reported in macrophages stimulated with lipopolysaccharide plus IFN-γ (15), but in those studies treatment with interleukin-13 was used to induce argi-

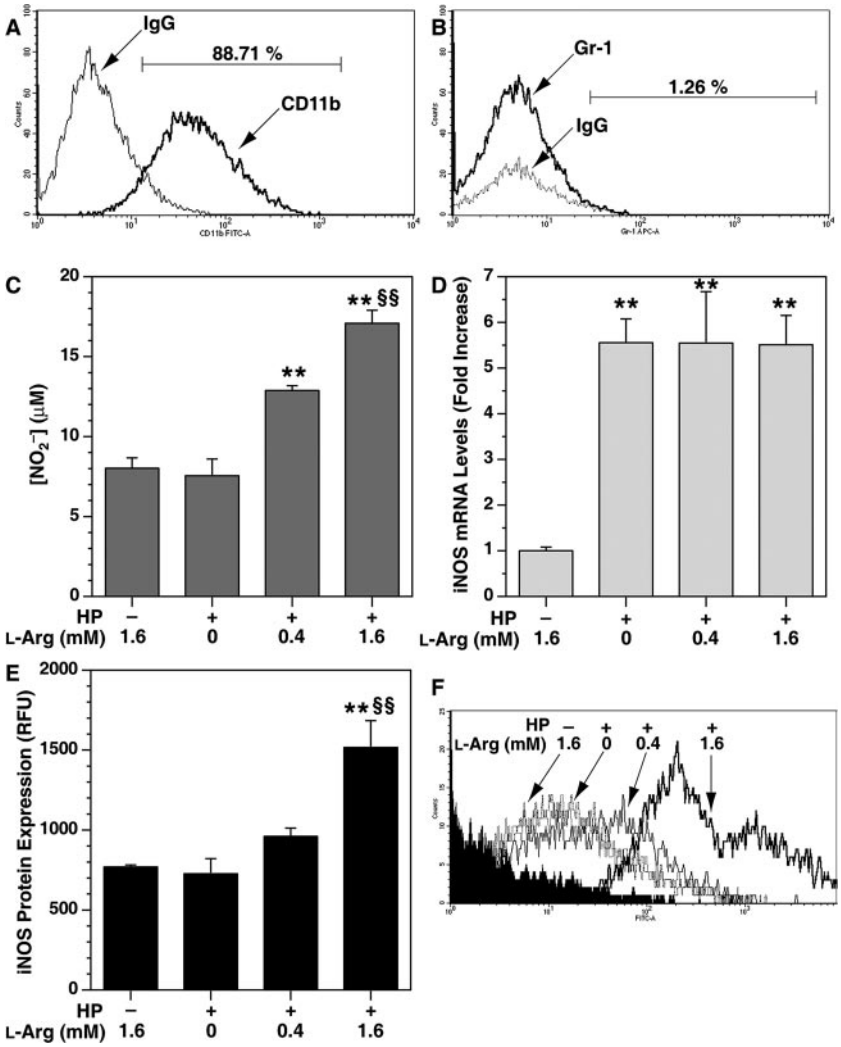

FIG. 9. Levels of iNOS expression and NO production in *H. pylori*-stimulated gastric macrophages. After positive selection of macrophages from the stomachs of C57BL/6 mice, cells were labeled with anti-CD11b antibody conjugated to fluorescein isothiocyanate (A) and antibody to Gr-1 conjugated to allophycocyanin (B) or isotype control immunoglobulin G (IgG), and cells were analyzed for fluorescence by flow cytometry. The percentages of CD11b-positive and Gr-1-positive cells are shown in panels A and B, respectively. In panels A and B, data representative of three mice are shown. For panels C to F cells were stimulated ex vivo with *H. pylori* lysate at an MOI of 100 at the concentrations of L-Arg indicated. For panel C the levels of NO_2^- were measured by the Griess reaction. For panel D the mRNA levels for iNOS were quantified by real-time PCR. iNOS protein levels were assessed by flow cytometry using a polyclonal antibody to iNOS. (E) Summary of data. (F) Representative data for one mouse. In panels C to E, two asterisks indicate a P value of <0.01 for a comparison with uninfected mice, and two section signs indicate a P value of <0.01 for a comparison with the preparation containing 0.4 mM L-Arg ($n = 3$ mice for each different experiment [namely, NO_2^-, mRNA, and protein]). HP, *H. pylori*; RFU, relative fluorescence units; FITC-A, fluorescein isothiocyanate area; APC-A, allophycocyanin area.

nase I and deplete L-Arg, and in addition to inhibition of iNOS translation, a reduction in iNOS protein stability was also reported in response to L-Arg reduction, an effect that we excluded in our studies. In our experimental systems, the expression of iNOS protein in response to *H. pylori* was regulated at the level of iNOS protein translation, and supplemental activation of arginase was not required to demonstrate the limitation of L-Arg availability. Regulation of the concentration of extracellular L-Arg, its uptake into cells, and endogenous competition due to induction of both arginase and ODC activity are all potentially important in determining the iNOS protein level in *H. pylori*-stimulated macrophages.

An important point raised by our data is that the production of NO in response to *H. pylori* in RAW 264.7 cells was increased at L-Arg concentrations up to 1.6 mM, with the iNOS protein level steadily increasing at L-Arg concentrations from 0.1 to 1.6 mM. Plasma L-Arg levels have been reported to be in the range from 65 to 130 μM in mice (20, 43) and in the range from 86 to 130 μM in humans (4), concentrations which are below our experimentally derived 50% effective dose for L-Arg (220 μM) in *H. pylori*-stimulated macrophages and represent a concentration range at which we found that the iNOS protein is just becoming detectable despite intact global protein translation levels. Furthermore, our data for the isolated gastric macrophages also strongly highlight the point that high levels of extracellular L-Arg are needed to allow iNOS protein to be

expressed. Our data also indicate that complete L-Arg starvation causes suppression of global protein translation and that the effects of L-Arg are specific to iNOS, because global translation, as well as the protein expression of other *H. pylori*-inducible genes, is maximized at 0.1 mM L-Arg, whereas the expression of the iNOS protein is just detectable at this L-Arg level and continues to increase at higher concentrations of L-Arg. Furthermore, depletion of other amino acids also suppresses global protein translation and iNOS protein expression, but iNOS levels and NO production are restored at levels well below those present in standard culture medium. Thus, the effect of L-Arg on iNOS appears to be specific. However, to address this matter further, one approach would be to conduct a proteomic analysis with *H. pylori*-stimulated macrophages. Such experiments are currently under way in our laboratory.

Our findings lead to the question of why more iNOS is not expressed at levels of extracellular L-Arg that exceed the K_m of the enzyme. We have shown that the expression of iNOS protein is completely dependent on the ability of the cells to transport extracellular L-Arg into the cytoplasm based on our finding that L-Lys, an inhibitor of L-Arg uptake, abolished iNOS protein translation and iNOS protein expression. L-Arg uptake in macrophages has been attributed to the y^+ transport system (28). The cationic amino acid transporters (CAT) consist of CAT1, which is constitutively expressed and is involved in uptake of arginine for basic metabolism (39), and CAT2, which has the alternatively spliced isoforms CAT2A and CAT2B (10). CAT2 is the major transporter in macrophages (28), and the activity of this protein is required for NO production with activation by lipopolysaccharide plus IFN-γ (39). Previously, we have reported that the polyamine spermine, generated by the induction of ODC by *H. pylori*, inhibits macrophage NO production by blocking translation of iNOS (6). Further, we have found that *H. pylori* stimulates L-Arg transport into macrophages by inducing CAT2, but this uptake of L-Arg is effectively inhibited by spermine (R. Chaturvedi, P. Y. Kim, and K. T. Wilson, Gastroenterology **126:**A404, 2004).

Another potential mechanism limiting L-Arg availability for iNOS is the effect of the competing enzyme, arginase. First, *H. pylori*-derived arginase can efficiently consume L-Arg in the extracellular environment (22), and we show here that this actually leads to an effective reduction in iNOS protein expression. In cytokine-stimulated intestinal epithelial cells, *Giardia lamblia* has been shown to decrease NO generation by depletion of L-Arg, but in contrast to our data, there was no inhibition of iNOS protein expression (14). In addition, we have reported that *H. pylori* induces the mitochondrial form of arginase, namely arginase II, and that this results in an effective increase in arginase activity in macrophages (21). Further, our finding that polyamine levels are increased in response to *H. pylori* (7) indicates that the L-ornithine generated by arginase is converted into putrescine, followed by conversion to spermidine and spermine by constitutively expressed synthases, resulting in negative feedback regulation of iNOS translation (6).

Substantial amounts of L-Arg are also utilized in vivo by arginase I, the hepatic form of arginase that is essential for survival in its function of detoxifying ammonia in the urea cycle (26, 48). If arginase I is not present due to a congenital deficiency in humans (26) or in mice with an arginase I knockout (27), serum L-Arg levels can exceed 1 mM (13, 24), but this state is associated with profound neurotoxicity (13, 24, 26, 27). Additionally, L-Arg can also be consumed by other metabolic pathways to generate agmatine, creatine, and proline (36, 48). Thus, in mammals, systemic L-Arg levels cannot reach the levels that would optimize extracellular availability for iNOS. Nonetheless, the question remains, what might happen if mice were supplemented with L-Arg, as we have done previously in an infectious colitis model (20)? Consistent with systemic utilization of L-Arg by arginase I and the high levels of arginase II (21) and ODC (1) in *H. pylori* gastritis tissues, there was no enhancement of iNOS protein levels and there was no amelioration of colonization at 2, 4, and 6 months after inoculation when we treated C57BL/6 mice with 1% L-Arg in the drinking water during the entire course of chronic *H. pylori* infection (R. Chaturvedi, M. Asim, and K. T. Wilson, unpublished data).

It should also be recognized that limiting the availability of L-Arg for macrophages or perhaps other cells in the infected gastric mucosa may actually be beneficial for the host, because otherwise, high levels of NO generation could lead to uncontrolled tissue injury, as we have reported for the *C. rodentium* model of colitis (20) and as has been shown in numerous other models of inflammatory bowel disease (12). Additionally, the utilization of L-Arg by the arginase pathway results in apoptosis of both macrophages and gastric epithelial cells exposed to *H. pylori*, and the final common pathway is generation of oxidative stress by the metabolism of spermine, resulting in mitochondrial membrane depolarization and apoptosis (7, 50). Thus, the innate response to pathogens may include a brake on the influx of L-Arg to prevent this activation of the intrinsic pathway of apoptosis or uncontrolled iNOS translation. In summary, we suggest that L-Arg availability represents an important factor in the innate immune response to *H. pylori* via its role in regulating iNOS.

ACKNOWLEDGMENTS

This work was supported by grants from the Office of Medical Research, Department of Veterans Affairs (to K.T.W. and to T.L.C.), by Public Health Service grants DK53620 and DK63626 (to K.T.W.) and DK53623 (to T.L.C.) from the National Institute of Diabetes and Digestive and Kidney Diseases, and by a grant from the Crohn's & Colitis Foundation of America (to K.T.W.). N.D.L. was supported by Public Health Service grant CA009592 from the National Cancer Institute, and H.M.S.A. was supported by Public Health Service grant A107474 from the National Institute of Allergy and Infectious Diseases.

REFERENCES

1. **Alam, K., F. L. Arlow, C. K. Ma, and T. T. Schubert.** 1994. Decrease in ornithine decarboxylase activity after eradication of *Helicobacter pylori*. Am. J. Gastroenterol. **89:**888–893.
2. **Amieva, M. R., N. R. Salama, L. S. Tompkins, and S. Falkow.** 2002. *Helicobacter pylori* enter and survive within multivesicular vacuoles of epithelial cells. Cell. Microbiol. **4:**677–690.
3. **Aspholm, M., F. O. Olfat, J. Norden, B. Sonden, C. Lundberg, R. Sjostrom, S. Altraja, S. Odenbreit, R. Haas, T. Wadstrom, L. Engstrand, C. Semino-Mora, H. Liu, A. Dubois, S. Teneberg, A. Arnqvist, and T. Boren.** 2006. SabA is the *H. pylori* hemagglutinin and is polymorphic in binding to sialylated glycans. PLoS Pathog **2:**989–1001.
4. **Beaumier, L., L. Castillo, A. M. Ajami, and V. R. Young.** 1995. Urea cycle intermediate kinetics and nitrate excretion at normal and "therapeutic" intakes of arginine in humans. Am. J. Physiol. **269:**E884–E896.
5. **Bogle, R. G., A. R. Baydoun, J. D. Pearson, S. Moncada, and G. E. Mann.** 1992. L-Arginine transport is increased in macrophages generating nitric oxide. Biochem. J. **284:**15–18.
6. **Bussiere, F. I., R. Chaturvedi, Y. Cheng, A. P. Gobert, M. Asim, D. R. Blumberg, H. Xu, P. Y. Kim, A. Hacker, R. A. Casero, Jr., and K. T. Wilson.** 2005. Spermine causes loss of innate immune response to *Helicobacter pylori* by inhibition of inducible nitric-oxide synthase translation. J. Biol. Chem. **280:**2409–2412.

7. **Chaturvedi, R., Y. Cheng, M. Asim, F. I. Bussiere, H. Xu, A. P. Gobert, A. Hacker, R. A. Casero, Jr., and K. T. Wilson.** 2004. Induction of polyamine oxidase 1 by *Helicobacter pylori* causes macrophage apoptosis by hydrogen peroxide release and mitochondrial membrane depolarization. J. Biol. Chem. **279:**40161–40173.
8. **Cheng, Y., R. Chaturvedi, M. Asim, F. I. Bussiere, H. Xu, R. A. Casero, Jr., and K. T. Wilson.** 2005. *Helicobacter pylori*-induced macrophage apoptosis requires activation of ornithine decarboxylase by c-Myc. J. Biol. Chem. **280:**22492–22496.
9. **Closs, E. I., F. Z. Basha, A. Habermeier, and U. Forstermann.** 1997. Interference of L-arginine analogues with L-arginine transport mediated by the y+ carrier hCAT-2B. Nitric Oxide **1:**65–73.
10. **Closs, E. I., C. R. Lyons, C. Kelly, and J. M. Cunningham.** 1993. Characterization of the third member of the MCAT family of cationic amino acid transporters. Identification of a domain that determines the transport properties of the MCAT proteins. J. Biol. Chem. **268:**20796–20800.
11. **Closs, E. I., J. S. Scheld, M. Sharafi, and U. Forstermann.** 2000. Substrate supply for nitric-oxide synthase in macrophages and endothelial cells: role of cationic amino acid transporters. Mol. Pharmacol. **57:**68–74.
12. **Cross, R. K., and K. T. Wilson.** 2003. Nitric oxide in inflammatory bowel disease. Inflamm. Bowel Dis. **9:**179–189.
13. **Deignan, J. L., J. C. Livesay, P. K. Yoo, S. I. Goodman, W. E. O'Brien, R. K. Iyer, S. D. Cederbaum, and W. W. Grody.** 2006. Ornithine deficiency in the arginase double knockout mouse. Mol. Genet. Metab. **89:**87–96.
14. **Eckmann, L., F. Laurent, T. D. Langford, M. L. Hetsko, J. R. Smith, M. F. Kagnoff, and F. D. Gillin.** 2000. Nitric oxide production by human intestinal epithelial cells and competition for arginine as potential determinants of host defense against the lumen-dwelling pathogen Giardia lamblia. J. Immunol. **164:**1478–1487.
15. **El-Gayar, S., H. Thuring-Nahler, J. Pfeilschifter, M. Rollinghoff, and C. Bogdan.** 2003. Translational control of inducible nitric oxide synthase by IL-13 and arginine availability in inflammatory macrophages. J. Immunol. **171:**4561–4568.
16. **Fang, F. C.** 2004. Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. Nat. Rev. Microbiol. **2:**820–832.
17. **Fang, F. C.** 1997. Perspectives series: host/pathogen interactions. Mechanisms of nitric oxide-related antimicrobial activity. J. Clin. Investig. **99:**2818–2825.
18. **Forstermann, U., E. I. Closs, J. S. Pollock, M. Nakane, P. Schwarz, I. Gath, and H. Kleinert.** 1994. Nitric oxide synthase isozymes. Characterization, purification, molecular cloning, and functions. Hypertension **23:**1121–1131.
19. **Fu, S., K. S. Ramanujam, A. Wong, G. T. Fantry, C. B. Drachenberg, S. P. James, S. J. Meltzer, and K. T. Wilson.** 1999. Increased expression and cellular localization of inducible nitric oxide synthase and cyclooxygenase 2 in *Helicobacter pylori* gastritis. Gastroenterology **116:**1319–1329.
20. **Gobert, A. P., Y. Cheng, M. Akhtar, B. D. Mersey, D. R. Blumberg, R. K. Cross, R. Chaturvedi, C. B. Drachenberg, J. L. Boucher, A. Hacker, R. A. Casero, Jr., and K. T. Wilson.** 2004. Protective role of arginase in a mouse model of colitis. J. Immunol. **173:**2109–2117.
21. **Gobert, A. P., Y. Cheng, J. Y. Wang, J. L. Boucher, R. K. Iyer, S. D. Cederbaum, R. A. Casero, Jr., J. C. Newton, and K. T. Wilson.** 2002. *Helicobacter pylori* induces macrophage apoptosis by activation of arginase II. J. Immunol. **168:**4692–4700.
22. **Gobert, A. P., D. J. McGee, M. Akhtar, G. L. Mendz, J. C. Newton, Y. Cheng, H. L. Mobley, and K. T. Wilson.** 2001. *Helicobacter pylori* arginase inhibits nitric oxide production by eukaryotic cells: a strategy for bacterial survival. Proc. Natl. Acad. Sci. USA **98:**13844–13849.
23. **Gobert, A. P., B. D. Mersey, Y. Cheng, D. R. Blumberg, J. C. Newton, and K. T. Wilson.** 2002. Cutting edge: urease release by *Helicobacter pylori* stimulates macrophage inducible nitric oxide synthase. J. Immunol. **168:**6002–6006.
24. **Grody, W. W., R. M. Kern, D. Klein, A. E. Dodson, P. B. Wissman, S. H. Barsky, and S. D. Cederbaum.** 1993. Arginase deficiency manifesting delayed clinical sequelae and induction of a kidney arginase isozyme. Hum. Genet. **91:**1–5.
25. **Hevel, J. M., K. A. White, and M. A. Marletta.** 1991. Purification of the inducible murine macrophage nitric oxide synthase. Identification as a flavoprotein. J. Biol. Chem. **266:**22789–22791.
26. **Iyer, R., C. P. Jenkinson, J. G. Vockley, R. M. Kern, W. W. Grody, and S. Cederbaum.** 1998. The human arginases and arginase deficiency. J. Inherit. Metab. Dis. **21**(Suppl. 1)**:**86–100.
27. **Iyer, R. K., P. K. Yoo, R. M. Kern, N. Rozengurt, R. Tsoa, W. E. O'Brien, H. Yu, W. W. Grody, and S. D. Cederbaum.** 2002. Mouse model for human arginase deficiency. Mol. Cell. Biol. **22:**4491–4498.
28. **Kakuda, D. K., M. J. Sweet, C. L. Mac Leod, D. A. Hume, and D. Markovich.** 1999. CAT2-mediated L-arginine transport and nitric oxide production in activated macrophages. Biochem. J. **340:**549–553.
29. **Kolodziejski, P. J., M. B. Rashid, and N. T. Eissa.** 2003. Intracellular formation of "undisruptable" dimers of inducible nitric oxide synthase. Proc. Natl. Acad. Sci. USA **100:**14263–14268.
30. **Kuwahara, H., Y. Miyamoto, T. Akaike, T. Kubota, T. Sawa, S. Okamoto, and H. Maeda.** 2000. *Helicobacter pylori* urease suppresses bactericidal activity of peroxynitrite via carbon dioxide production. Infect. Immun. **68:**4378–4383.
31. **Lee, J., H. Ryu, R. J. Ferrante, S. M. Morris, Jr., and R. R. Ratan.** 2003. Translational control of inducible nitric oxide synthase expression by arginine can explain the arginine paradox. Proc. Natl. Acad. Sci. USA **100:**4843–4848.
32. **Lowenstein, C. J., E. W. Alley, P. Raval, A. M. Snowman, S. H. Snyder, S. W. Russell, and W. J. Murphy.** 1993. Macrophage nitric oxide synthase gene: two upstream regions mediate induction by interferon gamma and lipopolysaccharide. Proc. Natl. Acad. Sci. USA **90:**9730–9734.
33. **Mai, U. E., G. I. Perez-Perez, J. B. Allen, S. M. Wahl, M. J. Blaser, and P. D. Smith.** 1992. Surface proteins from *Helicobacter pylori* exhibit chemotactic activity for human leukocytes and are present in gastric mucosa. J. Exp. Med. **175:**517–525.
34. **Mai, U. E., G. I. Perez-Perez, L. M. Wahl, S. M. Wahl, M. J. Blaser, and P. D. Smith.** 1991. Soluble surface proteins from *Helicobacter pylori* activate monocytes/macrophages by lipopolysaccharide-independent mechanism. J. Clin. Investig. **87:**894–900.
35. **Meyer, F., K. S. Ramanujam, A. P. Gobert, S. P. James, and K. T. Wilson.** 2003. Cutting edge: cyclooxygenase-2 activation suppresses Th1 polarization in response to *Helicobacter pylori*. J. Immunol. **171:**3913–3917.
36. **Morris, S. M., Jr.** 2006. Arginine: beyond protein. Am. J. Clin. Nutr. **83:**508S–512S.
37. **Musial, A., and N. T. Eissa.** 2001. Inducible nitric-oxide synthase is regulated by the proteasome degradation pathway. J. Biol. Chem. **276:**24268–24273.
38. **Necchi, V., M. E. Candusso, F. Tava, O. Luinetti, U. Ventura, R. Fiocca, V. Ricci, and E. Solcia.** 2007. Intracellular, intercellular, and stromal invasion of gastric mucosa, preneoplastic lesions, and cancer by *Helicobacter pylori*. Gastroenterology **132:**1009–1023.
39. **Nicholson, B., C. K. Manner, J. Kleeman, and C. L. MacLeod.** 2001. Sustained nitric oxide production in macrophages requires the arginine transporter CAT2. J. Biol. Chem. **276:**15881–15885.
40. **Ruckdeschel, K., G. Pfaffinger, K. Trulzsch, G. Zenner, K. Richter, J. Heesemann, and M. Aepfelbacher.** 2006. The proteasome pathway destabilizes Yersinia outer protein E and represses its antihost cell activities. J. Immunol. **176:**6093–6102.
41. **Sattlegger, E., and A. G. Hinnebusch.** 2000. Separate domains in GCN1 for binding protein kinase GCN2 and ribosomes are required for GCN2 activation in amino acid-starved cells. EMBO J. **19:**6622–6633.
42. **Semino-Mora, C., S. Q. Doi, A. Marty, V. Simko, I. Carlstedt, and A. Dubois.** 2003. Intracellular and interstitial expression of *Helicobacter pylori* virulence genes in gastric precancerous intestinal metaplasia and adenocarcinoma. J. Infect. Dis. **187:**1165–1177.
43. **Shi, O., S. M. Morris, Jr., H. Zoghbi, C. W. Porter, and W. E. O'Brien.** 2001. Generation of a mouse model for arginase II deficiency by targeted disruption of the arginase II gene. Mol. Cell. Biol. **21:**811–813.
44. **Sood, R., A. C. Porter, D. A. Olsen, D. R. Cavener, and R. C. Wek.** 2000. A mammalian homologue of GCN2 protein kinase important for translational control by phosphorylation of eukaryotic initiation factor-2alpha. Genetics **154:**787–801.
45. **Stuehr, D. J., H. J. Cho, N. S. Kwon, M. F. Weise, and C. F. Nathan.** 1991. Purification and characterization of the cytokine-induced macrophage nitric oxide synthase: an FAD- and FMN-containing flavoprotein. Proc. Natl. Acad. Sci. USA **88:**7773–7777.
46. **Varanasi, R. V., G. T. Fantry, and K. T. Wilson.** 1998. Decreased prevalence of *Helicobacter pylori* infection in gastroesophageal reflux disease. Helicobacter **3:**188–194.
47. **Wilson, K. T., K. S. Ramanujam, H. L. T. Mobley, R. F. Musselman, S. P. James, and S. J. Meltzer.** 1996. *Helicobacter pylori* stimulates inducible nitric oxide synthase expression and activity in a murine macrophage cell line. Gastroenterology **111:**1524–1533.
48. **Wu, G., and S. M. Morris, Jr.** 1998. Arginine metabolism: nitric oxide and beyond. Biochem. J. **336:**1–17.
49. **Xie, Q. W., H. J. Cho, J. Calaycay, R. A. Mumford, K. M. Swiderek, T. D. Lee, A. Ding, T. Troso, and C. Nathan.** 1992. Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. Science **256:**225–228.
50. **Xu, H., R. Chaturvedi, Y. Cheng, F. I. Bussiere, M. Asim, M. D. Yao, D. Potosky, S. J. Meltzer, J. G. Rhee, S. S. Kim, S. F. Moss, A. Hacker, Y. Wang, R. A. Casero, Jr., and K. T. Wilson.** 2004. Spermine oxidation induced by *Helicobacter pylori* results in apoptosis and DNA damage: implications for gastric carcinogenesis. Cancer Res. **64:**8521–8525.

Editor: A. D. O'Brien