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Helicobacter pylori Induces Macrophage Apoptosis by Activation of Arginase II

Alain P. Gobert,∗,† Yulan Cheng,∗,† Jian-Ying Wang,‡,†† Jean-Luc Boucher,∗ Jamie C. Newton,∗ and Keith T. Wilson2∗§

Helicobacter pylori infection induces innate immune responses in macrophages, contributing to mucosal inflammation and damage. Macrophage apoptosis is important in the pathogenesis of mucosal infections but has not been studied with H. pylori. NO derived from inducible NO synthase (iNOS) can activate macrophage apoptosis. Arginase competes with iNOS by converting L-arginine to L-ornithine. Since we reported that H. pylori induces iNOS in macrophages, we now determined whether this bacterium induces arginase and the effect of this activation on apoptosis. NF-kB-dependent induction of arginase II, but not arginase I, was observed by immunoblot analysis and qPCR in RAW 264.7 macrophages cocultured with H. pylori. The time course of apoptosis matched those of both arginase and iNOS activities. Surprisingly, apoptosis was blocked by the arginase inhibitors Nω-hydroxy-L-arginine or Nω-hydroxy-nor-l-arginine, but not by the iNOS inhibitor N-iminoethyl-L-lysine. These findings were confirmed in peritoneal macrophages from iNOS-deficient mice and were not dependent on bacterial-macrophage contact. Ornithine decarboxylase (ODC), which metabolizes L-ornithine to polyamines, was also induced in H. pylori-stimulated macrophages. Apoptosis was abolished by inhibition of ODC and was restored by the polyamines spermidine and spermine. We also demonstrate that arginase II expression is up-regulated in both murine and human H. pylori gastritis tissues, indicating the likely in vivo relevance of our findings. Therefore, we describe arginase- and ODC-dependent macrophage apoptosis, which implicates polyamines in the pathophysiology of H. pylori infection.


Helicobacter pylori is a Gram-negative, microaerophilic bacterium that selectively colonizes the human stomach. The current prevalence of H. pylori is~40% of the population of the U.S. (1) and is substantially higher in underdeveloped regions. An important hallmark of H. pylori infection is that it induces a vigorous gastric mucosal immune response that fails to eradicate the organism, and the infection persists for the life of the host. The host response can result in mucosal damage that may lead to clinical consequences of ulcer disease or cancer. In addition to a Th1-specific lymphocyte response (2), H. pylori infection induces activation of an innate response program in macrophages (3), which includes increased expression of inducible NO synthase (iNOS) (4, 5), and production of inflammatory mediators such as reactive oxygen intermediates, TNF-α, and IL-1 (6).

An important part of the induction of mucosal damage by H. pylori involves activation of apoptosis. It has been shown that H. pylori in contact with the gastric epithelium induces substantial apoptosis of these cells in vivo (7) and in vitro (8). Although H. pylori is considered a noninvasive pathogen, its products have been shown to reach the lamina propria (9), and when crypt abscesses and other forms of direct epithelial damage occur, bacteria can reach mucosal macrophages. However, the ability of H. pylori to induce macrophage apoptosis has not been examined. The death of macrophages could contribute to the mucosal inflammation by causing the release of preformed proinflammatory cytokines, such as IL-1 (10). In addition, the loss of activated macrophages is likely to decrease the effective immune response to the pathogen. This may limit the inflammation under some conditions but would contribute to the inability of the host to eliminate the infection.

Apoptosis of macrophages can be induced by numerous effector molecules, including NO (11). Because we have previously shown that iNOS expression and activity are induced by H. pylori in macrophages in vitro (4), and that mucosal macrophages are a major source of iNOS in H. pylori infection in vivo (5), we reasoned that modulation of NO production in response to H. pylori would regulate levels of macrophage apoptosis. Arginases are enzymes that can be induced in macrophages by bacterially derived products (12) and are natural competitive inhibitors of iNOS, because they metabolize the same substrate, L-arginine (13). Therefore, arginases can regulate the biological effects of NO (14, 15).
and have been reported to inhibit NO-dependent apoptosis in RAW 264.7 macrophage cells (16). Importantly, arginase converts l-arginine to l-ornithine, which is then acted upon by ornithine decarboxylase (ODC) to produce polyamines. The biogenic polyamines, putrescine, spermidine, and spermine, have been shown to have numerous biological functions, including inhibition of monocyte activation (17), stimulation of cellular migration (18) and proliferation (19), and regulation of apoptosis (20).

We hypothesized that arginase is induced by *H. pylori* in macrophages, and that it modulates apoptosis in these cells. To determine the respective roles of the iNOS and arginase-ODC pathways in *H. pylori*-induced apoptosis, we have used selective inhibitors of iNOS, arginase, and ODC, and peritoneal macrophages from wild-type (WT) and iNOS-/- C57BL/6 mice. Our results show, for the first time, that *H. pylori* induces apoptosis in macrophages by a process that occurs independently of iNOS expression but is mediated by activation of the arginase-ODC metabolic pathway.

**Materials and Methods**

**Reagents**

All reagents for cell culture, RNA extraction, RT-PCR, and Northern blotting were obtained from Life Technologies (Grand Island, NY). The NF-κB inhibitor, Z-Leu-Leu-Leu-CHO (MG-132), the protein kinase A (PKA) inhibitor, 3-1-(3-p-bromocinnamino)laminomethyl)-5-isouquinolinesulfonamide (HS99), and the protein kinase C (PKC) inhibitor, 2-[1-(3-dimethylaminopropyl)-5-methoxyindol-3-yl]-3-(1H-indol-3-yl) maleimide sulfonamide (H89), and the protein kinase C (PKC) inhibitor, 2-[1-(3-dimethylaminopropyl)-5-methoxyindol-3-yl]-3-(1H-indol-3-yl) maleimide (Go6983), were purchased from Calbiochem (San Diego, CA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

**Bacteria**

*H. pylori* SS1, a mouse-adapted human strain (22), was used. Bacteria were passaged on *Brucella* agar plates containing 10% sheep blood and were maintained in microaerobic conditions. For the experiments *H. pylori* maintained under microaerobic conditions. For the experiments identified macrophages (24), DMEM without serum, supplemented with 4 g/L N speciﬁc dimethylaminopropyl)-5-methoxyindol-3-yl]-3-(1H-indol-3-yl) maleimide sulfonamide (H89), and the protein kinase C (PKC) inhibitor, 2-[1-(3-dimethylaminopropyl)-5-methoxyindol-3-yl]-3-(1H-indol-3-yl) maleimide (Go6983), were purchased from Calbiochem (San Diego, CA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

**Mice, cells, and culture conditions**

The murine macrophage cell line RAW 264.7 was maintained in DMEM supplemented with 10% FBS, 1% penicillin-streptomycin, 1 mM sodium pyruvate, and 10 mM HEPES at 37°C in a humidified 5% CO2 atmosphere. For the coculture experiments RAW 264.7 cells were plated in the same medium without penicillin-streptomycin for 2 h, and *H. pylori* was then added to macrophages at a multiplicity of infection (MOI) of 10. To separate bacteria from macrophages, filter supports (0.2-μm pore size; Transwell; Nunc, Naperville, IL) were used, and under these conditions an MOI of 100 was used.

Experiments were also conducted with peritoneal macrophages isolated as previously described (23) from WT or iNOS-deficient C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME). *H. pylori* was added at an MOI of 10. Because serum has been shown to enhance arginase activity in resident macrophages (24), DMEM without serum, supplemented with 4 g/L BSA, 1 mM sodium pyruvate, and 10 mM HEPES, was used for these cultures.

For *H. pylori* mouse infection studies, C57BL/6 × Sv129 WT mice (The Jackson Laboratory) were inoculated with 106 CFU *H. pylori* SS1 every other day three times by gavage, and gastric tissues were harvested 4 mo later. Some mice were also gavaged with the same concentrations of nonpathogenic *Escherichia coli* strain DH5α as a control.

**RT-PCR**

RAW 264.7 macrophages were seeded at 2 × 105/well in six-well plates, and murine peritoneal macrophages were seeded at 5 × 105/well in 24-well plates. After stimulation, total RNA was isolated using TRIzol reagent. RNA was also extracted from mouse and human tissues by homogenization in TRIzol reagent. The human tissue samples were from patients with *H. pylori* gastritis, as previously described (5). Subsequently, 2 μg RNA from each sample was reverse transcribed using 50 U Superscript II reverse transcriptase. PCR was conducted using 2 μl cDNA and 1 U Taq DNA polymerase. For iNOS, arginase I, and arginase II, 15 pmol each of 5′ and 3′ primers were used with 3 pmol each of β-actin primers in a multiplex reaction (5, 25). For ODC, PCR was performed with 6 and 18 pmol each of ODC primers and β-actin primers, respectively. One PCR cycle consisted of the following: 94°C for 1 min, 60°C for 1.5 min, and 72°C for 1.8 min. For RAW 264.7 cells, the total cycle numbers were 35 for iNOS and arginase I, 25 for arginase II, and 20 for ODC. For peritoneal macrophages, the total cycle numbers were 27 for arginase I and arginase II and 37 for β-actin. For mouse tissues, the total cycle numbers were 27 for arginase I and 28 for arginase II. For human tissues, the total cycle numbers were 40 for arginase I and 28 for arginase II. A final elongation step of 7 min at 72°C was used for each reaction. Sense and antisense primer sequences and PCR product sizes were as follows: murine iNOS, 5′-GCTTCGCCTCTG GAAAG-3′ and 5′-TCACTGCAGAAACCTT-3′, 499 bp; murine arginase I, 5′-AGAAAGGGCGCATACCT-3′ and 5′-CACCCTCTCT GCTGTCTTCC-3′, 201 bp; murine arginase II, 5′-ACAGGGTGCTGTC AGCCTCT-3′ and 5′-TGATCCAGCACGACCAATCA-3′, 298 bp; human arginase I, 5′-CCCTTGGTGACATCCTAATA-3′ and 5′-GACTCC AAATACGGGTGA-3′, 201 bp; human arginase II, 5′-GACAGC GGACACTGGTCTT-3′ and 5′-CGTCTCCATGACCTTCTG-3′, 499 bp; human ODC, 5′-CAGCAGGCTTCCTTGGGAAC-3′ and 5′-CATGGATT CGACGGATTA-3′, 602 bp; and murine/human β-actin, 5′-CCAGAG CAAGAGGATTCTCC-3′ and 5′-CTGTTGGTTGAAGGCTGTA-3′, 436 bp. PCR products were run on 2% agarose gels with 0.4 μg/ml ethidium bromide. Stained bands were visualized under UV light and photographed with a digital gel documentation system (EDAS 290 and 1D software; Kodak Digital Science, Rochester, NY).

**Northern blot analysis**

RAW 264.7 macrophages were seeded at 104/ml in 75-cm2 flasks. After incubation with *H. pylori*, total RNA was extracted with TRIzol, and 10 μg RNA/lane was separated on 1% agarose gels. Northern blot analysis was performed as previously described (4, 15). For iNOS mRNA detection, a 3P random primer-labeled full-length (3.9-kb) cDNA probe for murine iNOS (obtained from Q. W. Xie and C. Nathan, Cornell University Medical College, Ithaca, NY) was used. The arginase II cDNA probe (298 bp) was generated by RT-PCR amplification of arginase II derived from RAW 264.7 macrophage RNA. The concentration and loading of RNA were standardized by hybridization with a cDNA probe (1.1 kb) for GAPDH (Clontech Laboratories, Palo Alto, CA). Densitometric analysis of band intensity was determined using National Institutes of Health Image version 1.62 (http://rsb.info.nih.gov/nih-image/).

**Immunoblotting for arginase II**

After coculture with *H. pylori*, RAW 264.7 macrophages were lysed in the presence of protease inhibitors (26). Mouse tissues were homogenized in the same lysis buffer. Protein concentrations of 12,000 x 1 g supernatants were measured using the DC Protein Assay kit (Bio-Rad, Hercules, CA), and 100 μg/lane was separated by SDS-PAGE using a 12% gel and transferred onto Immobilon-P membranes (Millipore, Bedford, MA) by electroblotting. Membranes were blocked overnight at room temperature using PBS containing 0.1% Tween and 5% nonfat dry milk. Arginase II protein was detected by incubation of blots with a polyclonal Ab to rat mammary gland arginase II (27), which does not cross-react with arginase I by immunoblotting. The primary Ab was used at a dilution of 1:500 for 2 h at room temperature, followed by a sheep anti-rabbit Ab conjugated to HRP (1:2000). Chemiluminescent detection was performed using the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and exposure to Hyperfilm ECL (Amersham, Little Chalfont, U.K.).

**Determination of arginase activity**

Colorimetric detection of macrophage arginase activity was determined as previously described (14). RAW 264.7 macrophages were lysed in 50 μl of a solution containing 0.1% Triton X-100, 0.01% aprotinin, 0.01% pepstatin, and 0.01% aprotinin. MinCl (10 mM for 10 min at 55°C) and l-arginine (0.5 M for 1 h at 37°C) were successively added, and the reaction was stopped by adding an acid solution. The concentration of urea synthesized by arginase metabolism was determined at 540 nm after addition of anti-isonitrosopropiophenone for 45 min at 100°C.
By Northern blot analysis. As shown in Fig. 1B expression of both iNOS and arginase II was increased in RAW basal expression at 6 h and later time points. To confirm arginase II protein in RAW 264.7 cells was significantly enhanced with unstimulated cells, by 7-fold for arginase II and 15-fold were up-regulated in H. pylori A 12 h (Fig. 1A) after 2 h of coculture (Fig. 1A). iNOS gene product was not expressed in control macrophages or decreased minimally from this level at 6 and 12 h (Fig. 1A). The mRNA expression was examined after 6 h by RT-PCR. Arginase II was up-regulated by 8-fold after 2 h and was significantly enhanced after 12 h of stimulation (Fig. 2A). In contrast, H89 and Go 6983 (10 μM) were added to macrophages 30 min before infection with H. pylori. Arginase II mRNA expression was examined after 6 h by RT-PCR.

The signaling mechanism responsible for the expression of arginase II mRNA in RAW 264.7 cells in response to H. pylori was investigated. Because the induction of arginase enzymes has been linked to increased activity of NF-κB and protein kinases, we assessed each of these pathways. Up-regulation of arginase II mRNA expression in response to H. pylori was completely inhibited by the NF-κB inhibitor MG-132 (Fig. 1D). In contrast, H89 and Go 6983, specific inhibitors of PKA and PKC, respectively, did not affect arginase II expression.

Assay for ODC activity

ODC activity was determined by a radiometric analysis in which the amount of [14C]O2 liberated from l-[14C]ornithine was estimated as previously described (28). Cells were lysed in 500 μl of a solution of 1 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.05 mM pyridoxal-5’-phosphate, and 5 mM DTT. Cells were frozen and thawed three times, then sonicated. After centrifugation at 12,000 × g for 15 min, 300 μl of the supernatant was incubated at 37°C with 10 nmol l-[14C]ornithine (sp. act. 47.7 mC/mmol; NEN, Boston, MA) for 15 min. The [14C]O2 liberated by the activity of the ODC was trapped on filter paper impregnated with 20 μl 2 N NaOH. The reaction was stopped with 300 μl 10% TCA, and [14C]O2 present on the filter paper was measured by liquid scintillation spectroscopy. Aliquots of the 12,000 × g supernatants were assayed for protein concentration using the DC Protein Assay kit.

Measurement of NO concentration

The concentration of the oxidized product of NO, nitrite (NO2−), was assessed by the Griess reaction, as we have previously described (4, 15).

Apoptosis analysis

Macrophage apoptosis was determined by two methods. ELISA. RAW 264.7 cells (2 × 105 cells/well) and peritoneal macrophages (104 cells/well) were cultured in 24- and 96-well plates, respectively, in the presence or the absence of H. pylori and various inhibitors. Floating and adherent cells were harvested after experiments, counted with a hemocytometer, and 104 cells were analyzed using the Cell Death Detection ELISA Plus kit from Roche Molecular Biochemicals (Indianapolis, IN), based on the determination of cytoplasmic histone-associated DNA fragments. According to the manufacturer’s instructions, in each experiment the OD of unstimulated control cells was assigned a value of 1.0, and the relative amount of apoptosis in experimental groups was determined as a ratio to control level.

DNA nick end labeling of cells. Macrophages (104/well) were cultured and stimulated in four-well plastic chamber slides (Nunc). At the end of the experiments, cells were washed with PBS and fixed with 4% formaldehyde. DNA fragmentation was analyzed by TUNEL assay using an In Situ Apoptosis Detection Kit ( Trevigen, Gaithersburg, MD). The percentage of apoptotic cells was determined after counting 10 microscope fields at a magnification of ×400.

Statistical analysis

For comparisons between multiple groups, the Student-Newman-Keuls test was used; and for single comparisons between two groups, Student’s t test was used.

Results

H. pylori SS1 stimulates both arginase II and iNOS in RAW 264.7 macrophages

After 0–12 h of coculture with H. pylori, mRNA levels of arginase I, arginase II, and iNOS were determined in macrophages by RT-PCR. Arginase II was up-regulated by 8-fold after 2 h and was decreased minimally from this level at 6 and 12 h (Fig. 1A). The iNOS gene product was not expressed in control macrophages or after 2 h of coculture (Fig. 1A) but was markedly induced at 6 h. We found no increase in the expression of type I arginase in macrophages stimulated for 2–12 h (Fig. 1A) or after 24 h with H. pylori (data not shown), and there was actually a decrease from basal expression at 6 h and later time points. To confirm that the expression of both iNOS and arginase II was increased in RAW 264.7 cells in response to H. pylori, we also assessed mRNA levels by Northern blot analysis. As shown in Fig. 1B, both transcripts were up-regulated in H. pylori-stimulated macrophages compared with unstimulated cells, by 7-fold for arginase II and 15-fold for iNOS.

Arginase II protein expression was further assessed by Western blot analysis. Compared with control macrophages, the level of arginase II protein in RAW 264.7 cells was significantly enhanced in response to H. pylori (Fig. 1C).
arginase was increased 7.7 ± 1.5-fold compared with that in control cells. The production of NO$\text{}_2^-$ by *H. pylori*-activated macrophages was not increased before 6 h and became significantly elevated after 12 h (Fig. 2B). After 18 and 24 h of coculture, NO$\text{}_2^-$ levels were increased by 11.9 ± 1.7- and 27.1 ± 2.8-fold vs the control value, respectively. Despite the significant increases in NO production, there was no loss of viability for the RAW 264.7 macrophages (data not shown).

To compare arginase and iNOS activities to *H. pylori*-stimulated macrophage programmed cell death, the time course of apoptosis was assessed. As shown in Fig. 2C, apoptosis was not detected between 2 and 12 h of coculture, but was significantly increased by 2.8 ± 0.87- and 3.4 ± 0.13-fold, at 18 and 24 h, respectively. The time point of 24 h was then used for all other experiments.

H. pylori-stimulated macrophage apoptosis is arginase dependent

Because both arginase and iNOS activities directly paralleled the induction of apoptosis, we assessed the effects of the arginase inhibitors, NOHA and norNOHA, and the selective inhibitor of iNOS, l-NIL, on *H. pylori*-stimulated macrophage apoptosis. Relative to the control level, the induction of apoptosis by *H. pylori* was inhibited by 78 and 95% with NOHA and norNOHA, respectively (Fig. 3). Surprisingly, macrophage apoptosis was not affected by l-NIL (Fig. 3) despite complete inhibition of NO production. NO$\text{}_2^-$ generation by RAW 264.7 macrophages was not enhanced by the arginase inhibitors, most likely due to excess l-arginine in the medium, as previously described (14, 15). NO$\text{}_2^-$ levels were as follows: control, 3.6 ± 1.2 μM; *H. pylori*, 21.7 ± 5.5 μM; *H. pylori* and l-NIL, 2.5 ± 1.0 μM; and *H. pylori* and norNOHA, 22.9 ± 8.2 μM. In addition, no alteration of the basal level of apoptosis was observed in control macrophages treated with iNOS or arginase inhibitors (data not shown).

To confirm that *H. pylori*-stimulated apoptosis was NO independent, we assessed *H. pylori*-induced macrophage apoptosis in peritoneal macrophages from WT and iNOS$^{−/−}$ mice. For cells from both mouse genotypes, apoptosis was increased in a significant manner when the resident macrophages were cocultured with *H. pylori* (Fig. 4A), while NO$\text{}_2^-$ production was observed only with cells from WT mice. NO$\text{}_2^-$ levels were as follows: control WT cells, 3.7 ± 1.0 μM; *H. pylori* WT cells, 15.6 ± 0.8 μM; control iNOS$^{−/−}$ cells, 2.8 ± 0.2 μM; *H. pylori* iNOS$^{−/−}$ cells, 2.6 ± 1.5 μM. We also found that l-NIL treatment had no effect on *H. pylori*-stimulated apoptosis in WT peritoneal macrophages; compared with unstimulated control cells there were 1.96 ± 0.26- and 2.03 ± 0.33-fold increases in apoptosis for *H. pylori* alone and *H. pylori* plus l-NIL, respectively, by ELISA (p < 0.01 vs control for both). As in the RAW 264.7 cells, l-NIL completely abolished NO production, while norNOHA had no such effect. NO$\text{}_2^-$ levels were as follows: control, 3.7 ± 1.0 μM; *H. pylori*, 15.6 ± 0.8 μM; *H. pylori* plus l-NIL, 1.9 ± 0.4 μM; and *H. pylori* plus norNOHA, 13.6 ± 2.4 μM. As shown in Fig. 4B, up-regulation of arginase II, but not arginase I, was observed in response to *H. pylori* in resident macrophages from both WT and iNOS$^{−/−}$ mice.

**FIGURE 2.** *H. pylori* induces parallel increases in arginase activity, NO production, and apoptosis of RAW 264.7 cells. Macrophages were incubated with *H. pylori*, and at the times indicated arginase activity was determined by colorimetric assay of cell lysates (A), NO$\text{}_2^-$ concentrations were measured in the coculture supernatants (B), and apoptosis of macrophages was evaluated by ELISA (C). Data are the mean ± SEM of three experiments performed in duplicate. *, p < 0.05; **, p < 0.01 (vs control (0 h)); §§, p < 0.01 (vs 18 h).

H. pylori induces ODC in macrophages

To further explore the involvement of the arginase-ODC metabolic pathway in apoptosis, we assessed the expression and activity of ODC in response to *H. pylori*. Kinetic analysis of ODC mRNA levels showed that the basal expression of the gene in control macrophages was moderately up-regulated by 1.3-, 1.7-, and 2.1-fold levels showed that the basal expression of the gene in control macrophages was moderately up-regulated by 1.3-, 1.7-, and 2.1-fold. As shown in Fig. 5A, ODC mRNA levels were increased in a significant manner when the resident macrophages were cocultured with *H. pylori* (Fig. 5A), while NO$\text{}_2^-$ production was observed only with cells from WT mice. NO$\text{}_2^-$ levels were as follows: control WT cells, 3.7 ± 1.0 μM; *H. pylori* WT cells, 15.6 ± 0.8 μM; control iNOS$^{−/−}$ cells, 2.8 ± 0.2 μM; *H. pylori* iNOS$^{−/−}$ cells, 2.6 ± 1.5 μM. We also found that l-NIL treatment had no effect on *H. pylori*-stimulated apoptosis in WT peritoneal macrophages; compared with unstimulated control cells there were 1.96 ± 0.26- and 2.03 ± 0.33-fold increases in apoptosis for *H. pylori* alone and *H. pylori* plus l-NIL, respectively, by ELISA (p < 0.01 vs control for both). As in the RAW 264.7 cells, l-NIL completely abolished NO production, while norNOHA had no such effect. NO$\text{}_2^-$ levels were as follows: control, 3.7 ± 1.0 μM; *H. pylori*, 15.6 ± 0.8 μM; *H. pylori* plus l-NIL, 1.9 ± 0.4 μM; and *H. pylori* plus norNOHA, 13.6 ± 2.4 μM. As shown in Fig. 4B, up-regulation of arginase II, but not arginase I, was observed in response to *H. pylori* in resident macrophages from both WT and iNOS$^{−/−}$ mice.

**FIGURE 3.** Regulation of *H. pylori*-mediated apoptosis by arginase. RAW 264.7 macrophages were cocultured for 24 h with *H. pylori* SS1 in the presence or the absence of l-NIL (50 μM), NOHA (1 mM), or norNOHA (100 μM). Macrophage apoptosis levels were assessed by ELISA. Values are expressed as the mean ± SEM of four experiments performed in duplicate. **, p < 0.01 (vs control macrophages); §§, p < 0.01 (vs *H. pylori*-stimulated macrophages).


we assessed enzymatic activity and found that it was increased by 10.6 ± 2.5-fold in macrophages infected with \( H. pylori \) for 18 h (Fig. 5B).

**ODC-dependent macrophage apoptosis**

To determine whether \( H. pylori \)-stimulated macrophage apoptosis is mediated by ODC, we examined the effect of the ODC inhibitor, DFMO, on apoptosis. As shown in Fig. 6A, compared with \( H. pylori \) stimulation alone, the apoptosis observed in macrophages cultured with the bacteria was abolished by DFMO. When DFMO was added to unstimulated macrophages, basal apoptosis was inhibited by 22 ± 4% (< 0.01 vs control; data not shown). Inhibition of \( H. pylori \)-stimulated apoptosis by DFMO was significantly reversed using the arginase inhibitor DFMO when \( H. pylori \) alone (Fig. 6B)

![FIGURE 4.](http://www.jimmunol.org/)

**FIGURE 4.** \( H. pylori \)-stimulated apoptosis of peritoneal macrophages is dependent on arginase II. A. Effect of \( H. pylori \) and norNOHA on macrophage apoptosis. Macrophages from WT ( [] ) or iNOS \(-/-\) ( [] ) mice were cocultured with \( H. pylori \) in DMEM-BSA in the presence or the absence of norNOHA, a specific arginase inhibitor. At 24 h apoptosis levels were measured by ELISA. Each bar represents the mean ± SEM of data collected from duplicate experiments with four mice. **, p < 0.01 (vs control macrophages); $, p < 0.05; §§, p < 0.01 (vs \( H. pylori \) alone). B. Arginase mRNA expression in macrophages from WT and iNOS \(-/-\) mice. Peritoneal macrophages were incubated with \( H. pylori \) for 6 h. Arginase I and arginase II mRNA levels were assessed by RT-PCR.

**Confirmation by TUNEL assay that arginase and ODC regulate \( H. pylori \)-stimulated macrophage apoptosis**

The effects of arginase and ODC inhibition on TUNEL staining are shown in Fig. 7. While cells exposed to \( H. pylori \) alone (Fig. 7B) had frequent cellular changes consistent with apoptosis, in the presence of norNOHA (Fig. 7C) or DFMO (Fig. 7D) there was a marked reduction of morphologic features of apoptosis. When quantified (Fig. 7E), the percentage of apoptotic cells was increased 2-fold above control levels with \( H. pylori \) alone; this increase in apoptosis was inhibited by 146 and 85% with norNOHA and DFMO, respectively.

**H. pylori-released products induce arginase- and ODC-dependent macrophage apoptosis**

Up-regulation of arginase II in RAW 264.7 cells was also observed when \( H. pylori \) were separated from macrophages by a filter support (Fig. 8A). These data suggest that \( H. pylori \)-derived effector molecules are potent activators of arginase II. In addition, factors released from \( H. pylori \) are capable of reproducing the same levels of apoptosis (Fig. 8B) as the bacteria not physically separated from the cells, previously shown in Fig. 3. Thus, contact with \( H. pylori \) is not required to induce macrophage apoptosis. In addition, \( H. pylori \) above the Transwell filter also stimulated apoptosis by an arginase- and ODC-dependent mechanism, because both norNOHA and DFMO significantly inhibited \( H. pylori \)-stimulated macrophage apoptosis (Fig. 8B).

**Up-regulation of arginase II in \( H. pylori \) gastritis**

To confirm the in vivo relevance of the \( H. pylori \)-stimulated arginase II expression that we have demonstrated in vitro, we assessed arginase II levels in both mouse and human \( H. pylori \) gastritis tissues. As shown in Fig. 9A, in mouse antral tissues assessed 4 mo after inoculation with \( H. pylori \) SS1, there was a marked and consistent increase in arginase II mRNA levels compared with control tissues from mice inoculated with nonpathogenic \( E. coli \). Additionally, there was basal expression of arginase I in control tissues, which was actually decreased in the \( H. pylori \)-infected tissues. We confirmed the arginase II up-regulation at the protein level by...
Apoptosis was then measured by ELISA. Values are expressed as the mean ± SEM of four experiments performed in duplicate. **, p < 0.01 (vs control macrophages); §§, p < 0.01 (vs H. pylori-stimulated macrophages); ###, p < 0.01 (vs macrophages stimulated with H. pylori in the presence of DFMO). 

FIGURE 6. ODC activity and polyamines enhance macrophage apoptosis. A, H. pylori-stimulated macrophage apoptosis is ODC dependent. RAW 264.7 cells were cocultured with H. pylori, with or without DFMO (5 mM) and 5 μM putrescine (put), spermidine (spd), or spermine (sp). After 24 h macrophage apoptosis levels were determined by ELISA. Data are the mean ± SEM of four experiments performed in duplicate. *, p < 0.05; **, p < 0.01 (vs control macrophages). 

Western blot analysis (Fig. 9B). We also assessed arginase mRNA expression in human tissues (Fig. 9C) and found that arginase II was absent in normal tissues and increased in H. pylori gastritis, while arginase I expression was generally very low, with 40 PCR cycles required to detect transcripts and no significant increase compared with control tissues.

Discussion

We speculated that arginase would participate in macrophage apoptosis, either by reducing effects of NO or by increasing the concentration of L-ornithine available for polyamine formation by ODC. We found that, in response to H. pylori, expression of arginase II mRNA, but not that of arginase I, was up-regulated in macrophages and correlated with enhancement of arginase activity. Inducible NOS was similarly induced at the transcriptional level, as with other strains of H. pylori (4), while ODC was up-regulated mainly at the post-transcriptional level. The activities of arginase and ODC, but not iNOS, were associated with macrophage apoptosis.

It is important to realize that generation of L-ornithine as a substrate for ODC in our studies occurs mainly through the arginase metabolic pathway, because l-ornithine is not present in DMEM culture medium. Our data show that both arginase and ODC activities are necessary to observe polyamine-dependent macrophage apoptosis. Although ODC activity is also increased by H. pylori, our data suggest that arginase II expression is rate limiting in the H. pylori-induced apoptosis, because inhibition of arginase, which is upstream of ODC in the metabolic pathway, effectively abolishes the apoptosis. In addition, we have observed that DFMO inhibits basal macrophage apoptosis, while arginase inhibition had no such effect, indicating that constitutive ODC expression and activity are sufficient to facilitate apoptosis once L-ornithine levels are increased by induction of arginase II. The primary importance of arginase in polyamine synthesis has been previously reported in macrophages (29) and rat smooth muscle cells (30). It should be noted that while arginase I is located in the cell cytosol, where ODC is also located, facilitating polyamine synthesis from ornithine, arginase II is a mitochondrial enzyme that could preferentially enhance proline or glutamate synthesis from ornithine, because ornithine aminotransferase is also located in the mitochondria (31). However, it has been shown that proline can be converted to ornithine (31) and that ornithine can be transported from the mitochondria to the cytosol (32).

Consistent with our findings for H. pylori stimulation, up-regulation of arginase II has been reported for RAW 264.7 cells activated by LPS (33) or cAMP (34) and in peritoneal macrophages stimulated by LPS (35). Arginase I expression was not increased in macrophages stimulated by H. pylori, even though this gene has been shown to be induced in macrophages by a cAMP-related pathway (34) involving activation of PKA (36). The signal transduction mechanisms leading to arginase II expression are less well understood. Induction of arginase II by LPS in rat alveolar macrophages was prevented by two NF-κB inhibitors, including pyrrolidine dithiocarbamate (PDTC) (37). However, arginase II expression has also been reported to be unaffected by PDTC in RAW 264.7 macrophages stimulated with LPS (12). In our experiments H. pylori-stimulated arginase II mRNA levels were decreased by 25% when 20 μM PDTC was used (data not shown), but a complete inhibition of arginase II expression occurred with the more specific NF-κB inhibitor, MG-132. These findings were specific, because inhibitors of PKA and PKC had no effect. Consistent with reported activation of NF-κB by H. pylori (38), our data suggest that macrophage arginase II expression in response to H. pylori involves NF-κB activation.

Numerous reports have investigated the involvement of NO in apoptosis. The type of cells, the time of exposure to NO or to its different derivatives, and the type of stimulus is a nonexhaustive list of the parameters that can modulate apoptotic events (39). NO can have paradoxical effects on programmed cell death. It has been reported to have proapoptotic effects on epithelial cells (40) and macrophages (11, 41). It also has been shown to have antiapoptotic activity by inactivating enzymes that mediate apoptosis, such as IL-1β-converting enzyme, cysteine protease protein (42), caspas, and tissue-transglutaminase (43), or by inhibiting ceramide formation (44). In murine macrophages stimulated by LPS alone, induction of both iNOS and arginase II has been reported (12), and apoptosis is NO independent under these conditions (45). However, when arginase was inhibited by addition of IFN-γ to
LPS (12), macrophage apoptosis was NO dependent (11, 41). From our experiments three findings support NO-independent macrophage apoptosis in response to H. pylori: 1) apoptosis was not affected by iNOS inhibition; 2) apoptosis occurred at the same levels in macrophages from iNOS-deficient mice as in those from WT mice; and 3) apoptosis was increased by spermidine and spermine, polyamines that inhibited iNOS-derived NO production by H. pylori. Consistent with our finding that polyamines inhibited iNOS, potentiation of LPS-induced iNOS activity by DFMO has been described (46). It is possible that the viability of peritoneal macrophages is decreased by H. pylori-induced NO production, but this would not affect our results, because the apoptosis ELISA we used includes all cellular material in the culture wells from both viable and nonviable cells.

Although the role of NO in apoptosis has been extensively studied, the effect of arginase in this process remains elusive. In agreement with our findings of a proapoptotic effect of arginase activity, it was recently reported that transfection and overexpression of p53 in bladder carcinoma cells resulted in up-regulation of arginase II that paralleled the induction of apoptosis (47). In contrast, it has also been described that long-term inhibition of arginase induces apoptosis of human breast cancer lines expressing high basal levels of arginase (48). In addition, overexpression of arginase in RAW 264.7 cells by transfection or stimulation with dexamethasone and cAMP decreased NO generation and related apoptosis in cells stimulated with IFN-γ plus LPS (16). In our studies induces apoptosis of human breast cancer lines expressing high basal levels of arginase (48). In addition, overexpression of arginase in RAW 264.7 cells by transfection or stimulation with dexamethasone and cAMP decreased NO generation and related apoptosis in cells stimulated with IFN-γ plus LPS (16). In our studies

FIGURE 7. Confirmation of arginase- and ODC-dependent H. pylori-stimulated macrophage apoptosis by TUNEL assay. RAW 264.7 macrophages were treated for 24 h as follows: A, unstimulated control; B, coculture with H. pylori SS1 alone; C, H. pylori plus norNOHA (100 μM); or D, H. pylori plus DFMO (5 mM). TUNEL positivity was determined by staining of nuclei with 3,3′-diaminobenzidine (magnification, ×600). The percentage of apoptotic macrophages (E) represents the mean ± SEM of 10 high power fields in each of two separate experiments. **, p < 0.01 (vs control macrophages); §§, p < 0.01 (vs H. pylori-stimulated macrophages).

FIGURE 8. Arginase- and ODC-dependent macrophage apoptosis does not require contact with H. pylori. A, RAW 264.7 cells were cocultured with H. pylori in the presence or the absence of a Transwell filter for 18 h. Levels of arginase II mRNA were assessed by RT-PCR. B, Apoptosis in RAW 264.7 macrophages cocultured with H. pylori separated from cells by a filter support, with or without norNOHA (100 μM) or DFMO (5 mM). After 24 h macrophage apoptosis was evaluated by ELISA. Data are the mean ± SEM of two experiments performed in duplicate. **, p < 0.01 (vs control macrophages); §§, p < 0.01 (vs H. pylori-stimulated macrophages).

FIGURE 9. Increased expression of arginase II in mouse and human H. pylori gastritis tissues. A, RT-PCR was performed on gastric antrum tissues from WT mice at 4 mo after inoculation. The first three tissues from the left were mice gavaged with nonpathogenic E. coli, with normal histology; the four tissues on the right were from mice gavaged with H. pylori SS1 with moderate gastritis on histology. B, Western blot analysis performed with a polyclonal Ab to mouse arginase II; the arginase II band corresponds to 37 kDa. Equal amounts of protein (50 μg) were loaded per lane. C, RT-PCR was performed on gastric antrum tissues from patients without H. pylori infection and normal histology (first two lanes from the left after marker) and with H. pylori gastritis (last five lanes).
H. pylori

in mucosal inflammation (53) and protection against apoptosis (53, 54). In intestinal epithelial cells, for example, polyamines have been shown to either prevent or induce apoptosis depending on the nature of the death stimulus (53). In the latter report TNF-α-mediated apoptosis was blocked by DFMO and facilitated by spermidine (53), similar to the current findings with H. pylori-stimulated macrophages. The levels of polyamines that we have added to induce apoptosis are similar to those used in previous studies (53). Additionally, the concentrations of polyamines used in Fig. 6 have in vivo relevance. We used a range of 5–50 μM, which converts to 2.5–25 nmol/mg macrophage protein. We have measured polyamine levels in H. pylori-infected murine gastric tissues and found concentrations of spermine and spermidine in the range of 10–12 nmol/mg protein, which is in the same range as the concentrations we used in the in vitro studies herein. Our finding that putrescine lacked the ability of the other biogenic polyamines to induce apoptosis has been previously described in murine leukemia L1210 cells (55). An important link between polyamine levels and apoptosis appears to be the regulation of NF-κB, because polyamine depletion can induce NF-κB activation (56), and NF-κB has been shown to protect against apoptosis in a variety of cell types, including macrophages (57) and lymphocytes (58). However, it should be noted that H. pylori-stimulated gastric epithelial cell apoptosis has been attributed to activation of NF-κB (59).

Our data suggest that analysis of arginase expression and activity in mucosal inflammation may provide new insights into understanding the often ambiguous effects of iNOS-derived NO. In support of the relevance of our in vitro data linking arginase II to H. pylori infection, we have demonstrated that arginase II expression is up-regulated in both murine and human H. pylori gastritis tissues. Therefore, experiments are now underway in our laboratory to elucidate the role of the balance of iNOS and arginase activities in murine H. pylori gastritis in vivo. Intriguingly, H. pylori itself possesses a constitutively expressed arginase (60), which we have shown inhibits macrophage NO production by iNOS under conditions of low arginine availability, thus enhancing H. pylori survival (15). It is highly unlikely, however, that the arginase inhibitors used in the present study decreased macrophage apoptosis by affecting H. pylori arginase, because isogenic mutant strains of H. pylori lacking rocF, the gene encoding for bacterial arginase, have normal viability under conditions of physiologic pH (61), as in the present study. It should be noted that we observed that rocF mutant H. pylori strains lacking bacterial arginase induce macrophage arginase II expression and apoptosis to an equal degree as WT H. pylori strains; in addition, treatment of cocultures of macrophages and rocF mutant strains with arginase inhibitors resulted in the same reversal of macrophage apoptosis as with WT strains (data not shown). These findings indicate that under the experimental conditions in the present study macrophage apoptosis is not altered by potential effects of arginase inhibitors on H. pylori arginase. It should also be recognized that H. pylori can release ornithine (60) and produce polyamines, such as agmatine (62), histamine, and spermidine (63). Thus, H. pylori may modulate the immune response by facilitating host polyamine synthesis or by producing the polyamines directly. In summary, our data strongly implicate arginase II in the pathogenesis of H. pylori infection, because it acts as the rate-limiting enzyme for polyamine synthesis and macrophage apoptosis, providing an important clue to the ineffectual mucosal immune response to this gastric pathogen.

References

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