Helicobacter pylori infection induces oxidative stress and programmed cell death

in human gastric epithelial cells

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Abstract

*Helicobacter pylori* infection is associated with altered gastric epithelial cell turnover. To evaluate the role of oxidative stress in cell death, gastric epithelial cells were exposed to various strains of *H. pylori*, inflammatory cytokines and hydrogen peroxide in the absence or presence of antioxidant agents. Increased intracellular reactive oxygen species (ROS) were detected using a redox-sensitive fluorescent dye, a cytochrome c reduction assay and measurements of glutathione. Apoptosis was evaluated by detecting DNA fragmentation and caspase activation. Infection with *H. pylori* or exposure of epithelial cells to hydrogen peroxide resulted in apoptosis and a dose-dependent increase in ROS generation that was enhanced by pre-treatment with inflammatory cytokines. Basal levels of ROS were greater in epithelial cells isolated from gastric mucosal biopsies from *H. pylori* infected subjects compared to cells from uninfected individuals. *H. pylori* strains bearing the *cag* pathogenicity island (PAI) induced higher levels of intracellular oxygen metabolites than isogenic *cag* PAI-deficient mutants. *H. pylori* infection and hydrogen peroxide exposure resulted in similar patterns of caspase 3 and 8 activation. Antioxidants inhibited both ROS generation and DNA fragmentation by *H. pylori*. These results indicate that bacterial factors and the host inflammatory response confer oxidative stress to the gastric epithelium during *H. pylori* infection that may lead to apoptosis.
Introduction:

*Helicobacter pylori* infection has been implicated in the pathogenesis of gastritis, peptic ulcer disease, gastric carcinoma, and gastric lymphoma (10,18) but the mechanisms leading from chronic active gastritis to other disease manifestations remain unclear. Various bacterial factors as well as the host response are believed to contribute to the outcome of infection with *H. pylori* (25). Strains bearing the *cag* pathogenicity island (PAI) (15), which includes the *cagA* gene, have been shown to be associated with increased gastric inflammation (57), increased bacterial load, and both peptic ulcer disease and gastric cancer (11). Increased induction of gastric epithelial cytokines that recruit and activate immune/inflammatory cells is also observed with these strains (17,41,54,67). Although the full function of genes encoded by *cag* PAI remain unclear, it is known that *cag* PAI-positive strains activate specific transcription factors and cell signaling pathways (37,41,52,54,66).

Bacterial and host factors can damage the gastric mucosal barrier and lead to alterations of epithelial cell growth and differentiation. *H. pylori* infection is associated with increased cellular proliferation *in vivo* (13,43) although most *in vitro* studies demonstrate bacterial inhibition of cell growth (76), suggesting that factors other than *H. pylori* regulate cell growth in the complex milieu of the infected gastric mucosa. Increased numbers of apoptotic cells are found in the gastric epithelium of infected patients (36,45,50,58), suggesting that induction of apoptosis may be a common method of cell growth regulation for *H. pylori* (24). In agreement with these findings, we and others have demonstrated that *H. pylori* induce programmed cell death in cultured gastric epithelial cells, as do pro-inflammatory cytokines that are released during infection (3,4,26,44,68,76). Proliferative (13,43) and apoptotic rates (24,36,50) have both been shown to return to control levels after eradication of infection.
There is increasing evidence that microbial pathogens induce oxidative stress in infected host cells (29,65,69) and this may represent an important mechanism leading to epithelial injury in *H. pylori* infection (70). It is known from other cell systems that oxidative stress regulates cell cycle events via multiple pathways, with net responses that include aberrant proliferation, adaptation cytotoxicity, and cell death (35). Oxidative stress could well play a role in the altered epithelial proliferation, increased apoptosis, (16,34) and increased oxidative DNA damage (7,16,27) associated with *H. pylori* infection. Evidence for this includes increased levels of reactive oxygen species (ROS) measured in the mucosa of infected patients (16,21,23). While activated, ROS-releasing phagocytic leukocytes recruited to the gastric mucosa during infection represent one obvious source of oxidative stress (21,79), other studies demonstrate that *H. pylori* themselves also generate ROS (51) and that ROS accumulate in gastric epithelial cells (5,6,73). In addition, pro-inflammatory cytokines induce ROS in various cell types (47,48,61), and the decreased levels of ascorbic acid that are associated with *H. pylori* infection (64,71) also contribute to a pro-oxidative environment. *H. pylori* infection has been shown to increase expression and activity of spermine oxidase, which oxidizes polyamines that are abundant in epithelial cells to release hydrogen peroxide (77), suggesting another mechanism by which *H. pylori* induce oxidative stress.

To examine oxidative stress that may occur during *H. pylori* infection, measurements of intracellular ROS were made in cultured and native gastric epithelial cells after exposure to *H. pylori* or hydrogen peroxide (H$_2$O$_2$), either alone or in combination with cytokines that are increased in infection. Antioxidants were used to evaluate the role of oxidative stress in the induction of apoptosis by these stimuli. To determine the role of the *cag* PAI in the induction of oxidative stress, *cag* PAI-bearing *H. pylori* strains and their isogenic mutants deficient in the *cag* PAI were compared for their ability to generate ROS in gastric epithelial cells.
Methods:

Cell lines. Gastric epithelial cell lines, Kato III, NCI-N87 and AGS (American Type Culture Collection, Rockville, MD), were grown in standard conditions according to previously published methods (19,26,78). Briefly, cells were cultured in flasks containing RPMI 1640 medium supplemented with 10% fetal calf serum at 37°C in a humidified 5% CO2 incubator. To ensure the cells were in a comparable stage of growth at the time of stimulation, cultured gastric epithelial cells were seeded at an average density of 1.2 x 10^6 cells/cm^2 24 hours before stimulation. Cell viability was assessed by trypan blue exclusion.

Isolation of native epithelial cells. Using a protocol approved by our Institutional Review Boards, 4-6 pinch biopsy specimens were collected from the antral gastric mucosa of consenting adult subjects undergoing medically indicated esophagogastroduodenoscopy. Subjects were considered infected with H. pylori if one or more of the following tissue based diagnostic tests were positive; rapid urease testing, routine histopathology or immunostaining (42). Biopsy samples were transported to the lab in cold, sterile collection media (calcium- and magnesium-free Hank’s buffered salt solution with 5% fetal calf serum and penicillin-streptomycin). The tissues were rinsed, gently teased apart and added to media containing 1mM dithiothreitol (Sigma Chemical Co., St. Louis, MO) and 1 mM EDTA (Sigma Chemical Co.) (26,78). After gentle agitation at 37°C for 1 hour, the resulting cell suspension was washed and stained with trypan blue to assess cell viability. Only preparations with greater than 80% viability were used for subsequent experiments. Purity was assessed by labeling the cells with FITC-conjugated monoclonal antibodies to epithelial specific antigen (clone Ber-EP4; Dakopatts A/S, Glostrup, Denmark) and measuring the staining by flow cytometry as previously reported (26,78).
Bacteria. *H. pylori* strains were maintained on blood agar plates under microaerophilic conditions as reported previously (19). Bacteria were cultured overnight in brucella broth supplemented with 10% fetal calf serum before centrifugation at 2500g for 15 min and resuspension in PBS. The strains used included CagA+ LC-11, originally isolated from a child with duodenal ulcer disease (19), two strains bearing the *cag* pathogenicity island (PAI), 26695 and 84-183, and their isogenic *cag* PAI negative mutants, 8-1 and 2-1 respectively (kindly provided by Dr. Doug Berg, Washington University, St. Louis, MO) (1,41,75)(41). Formalin-killed bacteria were prepared as previously reported (19,26), by centrifugation of an overnight culture, suspended in 0.5% formalin and stored at 4°C. On the day of the experiment, the formalin-killed bacteria were washed three times with PBS and resuspended in PBS to give a concentration comparable to the suspension containing viable bacteria.

Stimulation of epithelial cells. Various strains of *H. pylori* were added to epithelial cells at bacteria:epithelial cell ratios ranging from 1:1 to 1000:1 to assess strain-specificity and dose-relationships of epithelial cell responses. Most experiments were conducted using ratios of 300:1. Bacterial numbers were determined as previously reported (26) by measuring absorbance at 530 nm using a spectrophotometer (DU-65, Beckman Instruments, Inc., Fullerton, CA), and comparing the value to a standard curve generated by quantifying viable organisms from aliquots of bacteria at varying concentrations that were also assessed for absorbance. Bacterial motility was confirmed by phase-contrast microscopy before experimental use. In some experiments, formalin-killed bacteria, cell-free bacterial culture supernatants or control culture media were tested. H$_2$O$_2$ (Sigma Chemical Co.) was added at concentrations varying from 50 to 1000 µM, doses similar to those used in other studies examining the effects of ROS on gastrointestinal epithelial cells (30,30,32). Human recombinant tumor necrosis factor-alpha (TNF-α, interferon-gamma (IFN-γ) and interleukin-1 beta
(IL-1β) (R&D Systems, Minneapolis, MN), were used at doses of 10 ng/ml, 100 U/ml, and 10 ng/ml, respectively. We have previously shown that these doses of cytokine stimulate apoptosis (26) and induce IL-8 (19) in gastric epithelial cells. Assays to detect ROS and DNA degradation were performed at varying times after stimulation as described below.

Detection of intracellular oxygen metabolites. Redox-sensitive dye method. Tubes containing 10^6 cultured or freshly isolated gastric epithelial cells were loaded with 5 µM dichlorofluorescin diacetate (DCFH2-DA) (Molecular Probes, Eugene, OR) according to standard methods (14,63,80). After incubation at 37° C for 15 minutes, the cells were washed and resuspended before being examined by flow cytometry (FACs Scan, Becton Dickinson, San Jose, CA). Measurements were made at baseline and at five-minute intervals after adding the stimulants described above. For experiments to evaluate dose-responses and different bacterial strains, this technique was adapted for use in a 96 well plate (2,62). Briefly, 10^5 epithelial cells/well were cultured overnight in media with or without cytokines and loaded with 10 µM DCFH2-DA before washing and adding the stimulants described above. Measurements of fluorescence were made using a FluoroCounter (Packard, Downer Grove, IL). DCFH2-DA freely enters the cell and is cleaved by cellular esterases to the non-fluorescent DCFH that remains intracellular. Oxidants including hydroxyl radical, hydrogen peroxide and peroxynitrite, but not superoxide anion, have been shown to oxidize DCFH to fluorescent dichlorofluorescein (DCF) (39,46,80). Production of these oxidative species has been shown to be proportional to the fluorescence detected in many cell systems using this redox-sensitive dye (14,63,80).

Cytochrome c assay. Using previously reported methods (5,73) 10^5 gastric epithelial cells/well were cultured overnight in 96-well culture plates and washed with PBS before adding 1 ml of HBSS containing 80 µM cytochrome c (Sigma) to each well. Superoxide dismutase (SOD)
(Sigma) was added to the reference wells at a concentration of 40 µg/ml. At various times after stimulation spectrophotometric determinations were carried out at 550 nm. The amount of superoxide anion released was measured by the SOD-inhibitable reduction of cytochrome c and expressed as nmol/1x10^5 cells/unit of time.

**Measurement of glutathione levels.** Glutathione (gamma-glutamylcysteinylglycine (GSH)) levels were measured using a commercially available colorimetric assay (OXIS International, Inc., Portland, OR) according to the manufacturer’s instructions. Briefly, at varying times after stimulation, cells were homogenized, centrifuged at 3000 g and supernatants treated with reagents to generate chromophore thiones before measuring absorbance at 400 nm. 100 µM diethylmaleate (Sigma), which irreversibly depletes intracellular GSH (22) was used as a positive control.

**Antioxidants.** Inhibition of the effects of oxidative stress were performed by the addition of the glutathione precursor, N-acetylcysteine (NAC), to cultures of epithelial cells grown in media alone or stimulated with H_2O_2 or *H. pylori*. 10 mM NAC was added 1 hour before stimulation in all experiments since similar concentrations have been used in other studies of oxidative injury (60), and our initial dose response experiments demonstrated this to be an optimum dose to inhibit DCF fluorescence after stimulation with 400 µM H_2O_2.

Other antioxidants used to inhibit ROS generation included sodium azide, diphenyleneiodinium chloride, and allopurinol, at doses previously shown to inhibit oxidant-induced effects in guinea pig gastric epithelial cells (73). Glutathione (GSH), superoxide dismutase (SOD), catalase, hydroxyl radical scavengers, dimethylthiourea (DMTU), and mannitol, as well as iron-chelating agents, desferrioxamine (DESF) and diethyltriaminepentaacetic acid (DTPA), were also examined for their ability to inhibit ROS generation. The doses employed were based on those used in
previously published studies of ROS generation in gastric epithelial cells (5,73) and other cell types (49,60). All antioxidants were obtained from the Sigma Chemical Co.

Assays of apoptosis. Apoptosis ELISA. Apoptosis was assessed using a sensitive ELISA (Boehringer Mannheim Biochemicals, Indianapolis, IN) that detects endonucleosomes exposed during DNA fragmentation that occurs in apoptosis but not necrosis. Previous studies demonstrated that this assay yielded similar results to other assays of apoptosis but with increased sensitivity since apoptosis could be detected in as few as 10^2 cells (26). Briefly, absorbance was measured at 405 nm by Multiskan (model MCC/340; Titertek Instruments, Inc., Irvine, CA) and compared with substrate solution as a blank. The apoptotic index was calculated by dividing the absorbance of stimulated cells by the absorbance for control cells. Cells treated with 800 U/ml IFN-γ for 6 hours followed by 100 µg/ml anti-Fas antibody (CH 11, Kamiya Biomedical Company, Thousand Oaks, CA) were used as a positive control in some experiments since this treatment was previously shown to induce maximal levels of apoptosis in gastric epithelial cells.

Caspase activation assays. Caspase activity was determined using modifications of previously published methods (28,31,31) using specific synthetic fluorogenic substrates, 0.5 x 10^6 Kato III cells stimulated with H. pylori or 400 µM H_2O_2, were harvested and centrifuged at 1,500 rpm for 5 minutes. The cell pellets were lysed in 0.1 ml buffer (50 mM Hepes buffer, pH 7.5, 10% Sucrose, and 0.1% Triton X-100) for 20 minutes on ice. After centrifugation at 10,000 g for 10 minutes at 4°C, 100 µl of supernatant was transferred to a fresh tube containing 1 µl of 1M dithiothreitol. After placing on ice for 15 minutes, specific caspase substrates were added to a final concentration of 50 mM, incubated at room temperature for 1 hour, then diluted to 1 ml with PBS with fluorescence measured using a spectrofluorophotometer (excitation-400 nm, emission-505 nm). The substrates used for various caspase activity determination were as follows: Caspase-2...
(Nedd2) [Z-VDVAD-AFC (CBZ-Val-Asp-Val-Ala-Asp-AFC)]; Caspase-3 (CPP32) [Z-DEVD-AFC (CBZ-Asp-Glu-Val-Asp-AFC)]; and Caspase-8 (Flice) [Z-IETD-AFC (CBZ-Ile-Glu-Thr-Asp-AFC)] (Enzyme System, Livermore, CA). Each sample was analyzed in duplicate. The 7-amino-4-trifluoromethyl coumarin (AFC) fluorescence units versus concentration of AFC were graphed and the slope was used to convert fluorescence units generated by the enzyme to activity.

To determine specificity of the responses, the general caspase inhibitor, Z-VAD (Bachem, Torrance, CA) as well as specific caspase inhibitors, Z-VDVAD-CH$_2$F, Z-DVED-CH$_2$F, and Z-IETD-CH$_2$F (Enzyme Systems Products, Livermore, CA) for caspases 2, 3 and 8 respectively, were used at 20 and 100 µM before stimulation with _H. pylori_ (28,31,31).

Statistical analysis. Results are expressed as the mean ± SEM. Data were compared by the Student’s _t_-test (unpaired unless otherwise noted) or ANOVA and results were considered significant if _P_ values were less than 0.05.

Results:

Induction of intracellular ROS in response to _H. pylori_. Infection of DCFH-DA treated gastric epithelial cell lines with _H. pylori_ was associated with a rapid increase in fluorescence compared to levels of fluorescence measured in uninfected control cells, indicating increased accumulation of intracellular ROS in infected cell lines (Figure 1A). No increase in DCF fluorescence was detected when DCFH-DA treated bacteria were assayed by flow cytometry in the absence of epithelial cells. Since superoxide anion production is not thought to directly induce DCF fluorescence (80), the cytochrome c reduction assay was used to demonstrate a dose-dependent increase in superoxide anion in _H. pylori_-infected Kato III cells (Figure 1B). The results of our cytochrome c reduction assay confirm the findings of Nagata _et al_ (51) and show evidence of
superoxide anion generation by bacteria alone, although additional superoxide anion is measured over time when epithelial cells are present with the bacteria (Figure 1B). As shown in Figure 1C, glutathione levels were decreased in both Kato III at 24 hours and N87 cells at 6 and 24 hours after infection, providing evidence of a more sustained effect of infection through increased intracellular ROS. Taken together, these data indicate that \textit{H. pylori} organisms release superoxide anion and demonstrate that additional ROS are generated in host cells through bacterial interaction with epithelial cells.

Bacteria prepared directly from blood agar plates did not consistently induce fluorescence in DCFH-DA treated gastric epithelial cells in contrast to preparations made from overnight brucella broth cultures. There was no early effect of cell-free bacterial culture supernatants or formalin-killed \textit{H. pylori}, but formalin-killed bacteria increased levels of fluorescence in DCFH-DA treated Kato III cells at later time points (116 $\pm$ 2 as percent of control, mean $\pm$ SEM, n=3, $p < 0.05$ compared to control at 30 minutes). Formalin-killed bacteria also decreased levels of glutathione (54.6 $\pm$ 9.4 as percent of control, mean $\pm$ SEM, n=3, $p < 0.05$ compared to control at 24 hours). Together, these results suggest that viable motile bacteria are necessary for early generation of oxidative stress seen in cultured human gastric epithelial cells whereas oxidative stress induced by killed bacteria is more delayed.

ROS induction in native human gastric epithelial cells. In order to validate the use of gastric epithelial cell lines in studies of human disease pathogenesis it is important that findings observed in cultured cells can be demonstrated in native cells either \textit{in situ} or in isolated cell preparations. Using an approach that we have employed to demonstrate the expression of various immune adhesion or accessory molecules, including class II MHC (26) and B7 (78), in native gastric
epithelial cells, our data indicate that intracellular ROS can be detected in freshly isolated gastric epithelial cells. DCF fluorescence levels are increased in cells isolated from *H. pylori*-infected subjects compared to cells from uninfected individuals (Figures 2A and 2B). Moreover, native cells exhibit a similar accumulation of ROS in response to treatment with exogenous oxidative metabolites as observed in cell lines (Figure 2C).

**Induction of intracellular ROS in response to exogenous oxidative metabolites or inflammatory cytokines.** Treatment of Kato III, AGS and NCI-N87 cells with increasing concentrations of H$_2$O$_2$ resulted in a time- and dose-dependent increase in levels of fluorescence in DCFH-DA treated epithelial cells indicating accumulation of intracellular ROS (Figure 1A). The pattern of the response was similar in the three cell lines tested. These results confirm that gastric epithelial cells respond to exogenous oxidative metabolites with an increase in intracellular ROS analogous to many other cell types (2,14,62,63,80). As certain cytokines that are increased during *H. pylori* infection, including IFN-γ TNF-α and IL-1β (53), can induce apoptosis (26,76) and are reported to induce oxidative stress (47,48,61), we examined these cytokines for their ability to generate ROS in gastric epithelial cells. Although TNF-α has been shown to induce a transient surge of ROS in some cell systems (48,61), no increase in fluorescence was detected by flow cytometry up to 20 minutes after stimulating DCFH-DA treated Kato III or NCI-N87 cells with 10 ng/ml TNF-α or 100 U/ml IFN-γ. However, longer term (overnight) exposure to these cytokines, as well as 10 ng/ml IL-1β, resulted in increased basal levels of fluorescence and enhanced DCF fluorescence responses to bacteria and H$_2$O$_2$ (Figure 3). The results demonstrating increased basal levels of ROS after overnight cytokine treatment are consistent with the reduced levels of glutathione measured 24 hours after treatment with IFN-γ or TNF-α (data not shown). These experiments indicate that
factors generated through the host response to infection can also contribute to oxidative stress in the gastric mucosa.

**Effect of cag PAI on ROS accumulation in gastric epithelial cells.** Since *H. pylori* strains bearing the *cag* PAI are known to induce more inflammation and are associated with the more significant disease manifestations of chronic *H. pylori* infection (25), we examined bacteria with and without the *cag* PAI for their effect on ROS accumulation in Kato III and NCI-N87 cells. As shown in Figure 4A, both the 26695 and 84-183 strains, which contain the *cag* PAI, induced intracellular fluorescence while the corresponding *cag* PAI-deficient isogenic mutants, 8-1 and 2-1, had a more limited effect. A similar difference in superoxide generation was noted at 30 and 60 minutes after stimulation with strain 26695 and its isogenic mutant, 8-1 (Figure 4B). These data suggest that bacterial genetic factors may play a role in the generation of oxidative stress. It is not clear why *cag* PAI negative strains did not induce DCF fluorescence at levels over those in uninfected control cells while *cag* PAI negative strains were capable of inducing superoxide at greater levels than in control cells. This may reflect the different rates at which superoxide is generated compared to other ROS.

**Inhibition of oxidative stress in gastric epithelial cells treated with antioxidants.** As shown in Figure 5A, 10 mM NAC significantly reduced DCF fluorescence in Kato III cells after stimulation with *H₂O₂* or *H. pylori*. Similar inhibitory effects of NAC were seen in epithelial cells isolated from gastric biopsy specimens obtained from uninfected subjects (Figure 5B) and in NCI-N87 cells (data not shown). Other antioxidants were tested for their ability to inhibit ROS generation after *H. pylori* infection (Figures 6 and 7). As shown in Figure 6, glutathione (GSH), the hydroxyl scavenger,
dimethylthiourea (DMTU), and the iron chelators desferrioxamine (DESF) and diethylenetriaminepenta-acetic acid (DTPA), each significantly inhibited *H. pylori*-induced DCF fluorescence. Other antioxidants including catalase, mannitol, and SOD had no inhibitory effect. In order to determine the cellular source of ROS, sodium azide to inhibit mitochondrial electron transport, allopurinol to inhibit xanthine oxidase, and dipheylene iodinium (DPI), which inhibits NADPH oxidase, were added to cells before *H. pylori* stimulation. Only allopurinol had a significant inhibitory effect on superoxide anion generation after infection (Figure 7). Together, the results of these inhibitor studies suggest that *H. pylori* infection leads to the formation of several species of ROS within gastric epithelial cells including superoxide anion, hydrogen peroxide, hydroxyl radical, and peroxynitrite.

**Role of ROS in apoptosis induced by *H. pylori*.** As we have previously shown that *H. pylori* induces apoptosis in gastric epithelial cells (26), we examined a role for oxidative stress in mediating this response a) by mimicking the effect of *H. pylori* with exogenous oxidative metabolites and b) by inhibiting the response to bacterial infection with antioxidants. As shown in Figure 8A, a dose-dependent decrease in cell viability and a dose-dependent increase in apoptosis were observed when NCI-N87 cells were treated with varying concentrations of H$_2$O$_2$. The degree of apoptosis induced by 400 µM H$_2$O$_2$ approached that induced by a CD 95-activating anti-Fas antibody in IFN-γ treated cells. This treatment was used as a positive control as it was previously shown to induce maximal apoptosis in gastric epithelial cells. *H. pylori* infection was also shown to induce apoptosis (Figure 8B) but to a lesser degree than that induced by 400 µM H$_2$O$_2$. Although we demonstrated greater ROS accumulation in gastric epithelial cells very early during infection with *cag* PAI positive strains, this did not correlate with an increased rate of apoptosis measured at
48 hours after infection. This discrepancy likely reflects the observation that longer term effects on ROS, as measured by glutathione levels, did not differ significantly at 6 or 24 hours of infection between cag PAI positive and negative strains (data not shown). Both H. pylori and H₂O₂ stimulated the activation of caspases 3 and 8, but only H. pylori had a significant effect on caspase 2 activation (Table 1). Inhibition of H. pylori-induced caspase activation by the general caspase inhibitor, Z-VAD, as well as by specific caspase inhibitors, demonstrates specificity of these responses (data not shown). 10 mM N-acetylcysteine significantly reduced the degree of apoptosis induced by H. pylori and by H₂O₂ (Figure 8B). Together, these data indicate that both exogenous ROS and H. pylori induce apoptosis of gastric epithelial cells as evidenced by caspase activation and DNA fragmentation, and implicate ROS in the programmed cell death induced by H. pylori infection.
Discussion:

*Helicobacter pylori* infection is a causal factor in various disorders of the gastric epithelium including ulceration, metaplasia, dysplasia, and carcinoma. Alterations of epithelial cell growth and enhanced programmed cell death may play a role in *H. pylori* disease manifestations (4,44), but the mechanisms responsible for these changes in the epithelium remain unknown. This study demonstrates that increased levels of reactive oxygen are generated in *H. pylori*-infected gastric epithelial cells and that this may be one mechanism leading to apoptosis associated with infection. Our results indicate that both bacterial and host factors contribute to the oxidative stress induced by infection. The finding that antioxidants prevent the generation of reactive oxygen species (ROS) and inhibit *H. pylori*-induced programmed cell death has implications for the prevention and treatment of this common and chronic infectious disease.

It is increasingly recognized that microbial pathogens including *Mycobacterium avium*-intercellular complex, bovine viral diarrhea virus, and human immunodeficiency virus, affect host cells via ROS generation (29,65). Similarly, another species of Helicobacter, *H. hepaticus*, has been shown to induce oxidative DNA damage in hepatocytes of infected mice (69). Superoxide anion was also detected in epithelial cell preparations isolated from guinea pig gastric mucosa after experimental *H. pylori* infection (73). These studies lend support to our results demonstrating that *H. pylori* infection stimulates the accumulation of intracellular ROS in different human gastric epithelial cell lines (5,6). Reports demonstrating decreased levels of glutathione in *H. pylori*-infected HMO2 human gastric epithelial cells (9,55) provide additional evidence that *H. pylori* serves as a stimulus for the accumulation of ROS within gastric epithelial cells. Furthermore, our results confirm the findings of a previous report describing the accumulation of ROS in another
human gastric epithelial cell line, CRL 1739, after infection with *H. pylori* (5). Importantly, our findings indicating that ROS levels are higher in gastric epithelial cells isolated from infected subjects, compared to those from uninfected subjects, demonstrate that *H. pylori*-induced oxidative stress is not an artifact of cultured cells or animal models and does occur in naturally infected, native human epithelial cells. Moreover, we have shown that native human gastric epithelial cells respond in a similar fashion to cultured cells with the generation of ROS after experimental *H. pylori* infection.

The present study confirms a report that *H. pylori* release superoxide radicals (51), but since superoxide anion cannot diffuse across the cell membrane, further studies were needed to identify the source and type of intracellular ROS generated in infected gastric epithelial cells. It was assumed that *H. pylori* infection must stimulate accumulation of ROS above the levels generated by epithelial cells through normal cellular metabolism. The results of our studies in which various antioxidants were shown to inhibit the generation of ROS by *H. pylori*, suggest that hydrogen peroxide, hydroxyl radical, and peroxynitrite, as well as superoxide, all contribute to increased net levels of ROS in epithelial cells (5,6,38). Our findings do not exclude a role for other forms of ROS or reactive nitrogen species. Although the major source of ROS in most cells is mitochondrial electron transport, sodium azide did not inhibit ROS generation by *H. pylori* in our studies. Similarly, the NADPH oxidase inhibitor, diphenylene iodonium, was without effect while allopurinol, a xanthine oxidase inhibitor, reduced ROS accumulation by bacterial infection. These findings contrast with the study in guinea pig mucosal cells in which it was shown that *H. pylori* induced superoxide anion in epithelial cells through an NADPH-oxidase-like system (73). Since there are limitations to using pharmacological inhibitors to determine the type and source of ROS, it is not surprising that such differences exist. Substantial variation in the ability of such agents to
inhibit ROS and ROS-mediated events in various cell types is reported in the literature (5,5,48,48,61,61,65,73,73) and differences in mechanisms of ROS generation may vary according to the cell type and species of origin (73).

The current study indicates that host factors also contribute to oxidative stress during *H. pylori* infection. Since activated neutrophils or macrophages are potent sources of ROS, including H$_2$O$_2$ (74), we studied exogenous H$_2$O$_2$ for its effect on gastric epithelial cells. A dose-dependent accumulation of intracellular ROS was observed in both cultured and freshly isolated human gastric epithelial cells. Cytokines that are increased in the gastric mucosa of infected subjects, including TNF-α, IFN-γ, and IL-1β (53), also induced oxidative stress and augmented oxidative responses to both *H. pylori* and H$_2$O$_2$ in our studies. Although cytokines have been reported to induce ROS in other cell types (47,48,48,61,61), our results indicate cytokine-mediated oxidative signaling occurs in gastric epithelial cells.

The role of bacterial genotype has been a focus of recent investigations into *H. pylori* pathogenesis. *H. pylori* have been classified based on their expression of the *cagA* gene and the *cag* pathogenicity island (PAI) (15) as well as the *vacA* genotype. Strains that are *cagA*+ have been shown to be associated with increased gastric inflammation, increased bacterial load, and both peptic ulcer disease and gastric cancer (4,11,57). Strains bearing the PAI induce higher levels of IL-8 (41) and activate transcription factors NF-κB (37,66) and activator protein (AP)-1 (52,54). *cag*PAI status also affects gastric epithelial apoptosis (40) and oxidative DNA damage (20,56). Our results, which indicate that *cag* PAI status influences the ability of *H. pylori* to induce intracellular ROS in gastric epithelial cells, provide further insight into how bacterial genetic factors may play a role in disease pathogenesis. Moreover, since *cag* PAI-positive strains are associated with greater inflammation, the host response may also contribute to enhanced oxidative stress associated with
these strains. The differential induction of ROS shown in the present study may be relevant to the reported associations of the cag PAI and the activation of epithelial cell signaling pathways (41,41,52,54). As the genome sequence of one of the strains used in this study, strain 26695, has been determined (41), the opportunity exists to identify more specific bacterial genes that regulate ROS generation.

We have shown that both H. pylori infection and exogenous ROS treatment induce caspase activation and DNA fragmentation while antioxidant treatment inhibits the induction of apoptosis due to H. pylori infection. Further evidence that oxidative stress may be involved in the alterations of epithelial cell growth in H. pylori infection is found in a study in which decreased epithelial cell apoptosis was observed in gastric tissues from H. pylori-infected patients treated with antioxidant therapy only (45). Although ROS have not been previously shown to play a role in programmed cell death of gastric epithelial cells, ROS have been implicated in apoptosis resulting from various stimuli (12,34) in other cell types. Of particular relevance to our findings is a recent report of ROS involvement in apoptosis induced in host cells by bovine viral diarrhea virus (65). The present study does not address the mechanisms whereby oxidative stress leads to apoptosis, although ROS have been shown to contribute to p53 (59), Fas-Fas ligand (8,33), ceramide (60), and TNF-mediated killing (72). It is known that mammalian cells respond to oxidative stress with the initial generation of ROS and the subsequent activation of redox-sensitive signaling pathways which control the transcription of genes that may regulate cell growth, repair, and death processes. Studies are in progress that will examine redox-dependent pathways leading to epithelial cell death during H. pylori infection.

In summary, we have demonstrated that H. pylori infection, exogenous oxidative metabolites, and inflammatory cytokines induce the generation of intracellular reactive oxygen
species in gastric epithelial cells. These *in vitro* results are corroborated by the higher levels of ROS measured in native epithelial cells from individuals infected with *H. pylori*. Our findings suggest that bacterial genotype may be an important determinant of the level of oxidative stress generated by infection. We conclude that oxidative stress may play a role in the increased programmed cell death that occurs during infection, since antioxidant treatment inhibited *H. pylori*-induced apoptosis. Further studies are necessary to explore how oxidative stress regulates epithelial responses to *H. pylori* infection, as this will provide new insight into the pathogenesis of *H. pylori*-associated conditions.

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**Figure Legends:**

**Figure 1. Induction of ROS in gastric epithelial cells after exposure to *H. pylori* or oxygen metabolites.** (A) Dichlorofluorescin diacetate (DCFH₂-DA)-treated Kato III cells were exposed to H₂O₂ at concentrations from 0 to 1000 μM or infected with *H. pylori* (Hp) at a bacteria:epithelial cell ratio of 300:1. Intracellular dichlorofluorescein (DCF) fluorescence was measured by flow cytometry at intervals up to 20 minutes after stimulation. ROS accumulated in proportion to the concentration of H₂O₂ added to the cells, and bacteria stimulated the production of ROS in epithelial cells in a time-dependent manner. A representative experiment is shown. (B) Superoxide anion was measured by the cytochrome c reduction assay method at 0, 0.5, and 1 hour after varying concentrations of *H. pylori* strain 26695 (equivalent to bacteria:epithelial cells ratios of 0:1, 300:1, 600:1, or 1000:1) were added to wells containing media alone (dotted line) or media with Kato III gastric epithelial cells (continuous line). A dose-dependent increase in superoxide anion generation was measured with increasing concentrations of bacteria both with and without epithelial cells. Data represent superoxide anion production in nmol/10⁵ cells, expressed as mean ± SEM (n = 8-12). *, P
< 0.05 compared to bacteria alone; #, P < 0.05 compared to Kato III cells alone. (C) Kato III and N87 gastric epithelial cells were exposed to *H. pylori* (bacteria:epithelial cell ratio of 300:1) and harvested at 6 or 24h post-stimulation and assayed for levels of intracellular glutathione using a colorimetric assay. Oxidative stress generated by *H. pylori* results in a sustained decrease in glutathione levels. The data are mean ± SEM, expressed as percent of control levels. *, P < 0.05 compared to control (n=5-7).

**Figure 2. Detection of ROS in freshly isolated human gastric epithelial cells.** Gastric epithelial cells were isolated from gastric biopsy specimens, stained with DCFH-DA, and analyzed via flow cytometry. The y axes of panels A and B reflect the frequency of events on a linear scale while the x axis indicates increasing levels of ROS as estimated by the DCF fluorescence on a logarithmic scale. (A) A representative experiment in which ROS levels were measured in cells from a subject infected with *H. pylori* and from an uninfected subject is depicted. (B) In another set of experiments, cells isolated from an uninfected subject were assayed for ROS before (resting) or after exposure to 400 mM H2O2 (stimulated). Treatment with H2O2 markedly increased intracellular ROS. (C) Mean levels of fluorescence ± SEM measured by fluorimeter in DCFH2-DA treated cells isolated from 3 uninfected and 3 infected subjects (*, P < 0.05 compared to uninfected).

**Figure 3. Effect of pro-inflammatory cytokines on ROS generation.** Kato III cells were treated overnight with media alone (no cytokine) or media containing 10 ng/ml TNF-α, 100 U/ml IFN-γ, or 10 ng/ml IL-1β before stimulation with media alone (control), 400 µM H2O2, or *H. pylori* at a bacteria:epithelial cell ratio of 300:1. Peak increases in ROS levels (measured as increase in DCF fluorescence) are depicted as mean ± SEM (n=3-6). All three cytokines increased basal levels of
fluorescence and ROS responses to *H. pylori* while IFN-γ and IL-1β also increased ROS generation after H₂O₂ (*, P < 0.05 compared to cells without cytokine treatment).

**Figure 4. Induction of ROS by cag PAI-bearing strains in gastric epithelial cells.** (A) Kato III cells were infected with *cag* PAI-positive strains 26695 and 84-183 (solid bars) or their isogenic *cag* PAI negative mutants, 8-1 and 2-1, respectively (open bars) at comparable concentrations. Peak increases in DCF fluorescence occurring within 40 minutes of infection are expressed as percent of levels in uninfected control cells and depicted as mean ± SEM (n=5-7). (*, P < 0.05 PAI+ strains compared to their PAI- counterparts and to control levels). (B) Kato III cells were infected with the *cag* PAI positive strain 26695 (solid bars) or its isogenic mutant, 8-1 (open bars), at comparable concentrations. The amount of superoxide anion released was measured by the cytochrome c assay. Values in nmol/10⁵ cells/30 min at 30 and 60 minutes after infection are depicted as mean ± SEM (n=6, 3 replicates each). ***, P < 0.0001, *, P < 0.05 compared to control levels for both the PAI+ strain and its PAI- counterpart, #, P < 0.01 cells infected with the PAI+ strain compared to the PAI- strain at both time points.

**Figure 5. Inhibition of ROS induction by N-acetylcysteine.** (A) DCFH₂-DA loaded Kato III cells were treated with 10 mM N-acetylcysteine (NAC) or media alone 1 h before stimulation with media, 400μM H₂O₂, or *H. pylori* at a ratio of bacteria to epithelial cells of 300:1. Data are depicted as mean levels of maximal DCF fluorescence within 20 minutes of stimulation in NAC-treated cells expressed as a percentage of fluorescence in cells without NAC (mean ± SEM, n=4-6 experiments). (*, P < 0.05 compared to cells without NAC pretreatment). (B) Identical experiments performed in
epithelial cells isolated from subjects without *H. pylori* infection (n=3) (*, P < 0.05 compared to cells without NAC pretreatment).

**Figure 6. Effects of antioxidants on *H. pylori*-induced ROS production in Kato-III cells.**

DCFH$_2$-DA loaded Kato III cells were treated with various antioxidants or media alone 1 h before stimulation with *H. pylori*, strain 26695, at bacteria:epithelial cell ratios of 300:1. Antioxidants tested in these experiments were 10 mM glutathione (GSH), 50 mM dimethylthiourea (DMTU), 5 mM desferroxamine (DESF) and diethylenetriaminepentaacetic acid (DTPA). Data are depicted as mean levels of DCF fluorescence 30 minutes after stimulation with *H. pylori* in untreated cells (open bars) or in antioxidant treated cells (dark bars), as a percentage of uninfected, untreated control cells ± SEM (n=3-4 experiments). The effects of drugs alone are shown by hatched bars. (*, P < 0.05, **, P < 0.01 compared to *H. pylori* alone using paired Student’s *t*-test).

**Figure 7. Effects of the xanthine oxidase inhibitor, allopurinol, on *H. pylori*-induced superoxide anion in Kato-III cells.** Cells were incubated with varying concentrations of allopurinol (10-100 µM) for 1 hour before stimulation with *H. pylori*, strain 26695, at bacteria:epithelial cell ratios of 300:1. Superoxide anion was assessed by cytochrome c assay 30 minutes after stimulation. Each experiment was performed in triplicate. Values are mean ± SEM of 3 separate experiments. (*, P < 0.05 compared to control cells without allopurinol using paired Student’s *t*-test).

**Figure 8. Induction of apoptosis in gastric epithelial cells and its inhibition by antioxidants.**

(A) N87 cells were exposed to H$_2$O$_2$ at concentrations from 0 to 400 µM with cell viability
(expressed as % of control, solid bars) measured by trypan blue exclusion and apoptosis (shown as apoptotic index [A.I., hatched bars] determined by detection of endonucleosomes via ELISA. Cells exposed to 800 U/ml IFN-$\gamma$ for 6 hours followed by 100 $\mu$g/ml anti-Fas antibody were used as a positive control. Dose-dependent changes were observed with decreases in cell viability and increases in apoptosis significantly different from those of control cells at doses of 100-400 $\mu$M H$_2$O$_2$ ($P < 0.05$). Data shown as mean $\pm$ SEM, $n=3-5$. (B) Kato cells were treated with $H. pylori$ (300 bacteria: 1 epithelial cell), 400 $\mu$M H$_2$O$_2$, or media alone (control) for 48 hours in the presence or absence of 10 mM NAC. Apoptosis was assessed using an ELISA assay to detect endonucleosomes exposed by DNA fragmentation. Values depicted are mean $\pm$ SEM, $n=9-15$. *, $P < 0.05$ compared to cells without NAC.

Table 1. $H. pylori$ and H$_2$O$_2$ stimulate caspase activation.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>$H. pylori$ (300:1)</th>
<th>H$_2$O$_2$ (400 $\mu$M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Caspase 2</td>
<td>Caspase 3</td>
</tr>
<tr>
<td>3</td>
<td>95±9</td>
<td>122±3</td>
</tr>
<tr>
<td>6</td>
<td>138±8*</td>
<td>185±12*</td>
</tr>
<tr>
<td>9</td>
<td>138±10*</td>
<td>161±19*</td>
</tr>
<tr>
<td>12</td>
<td>139±10*</td>
<td>187±25*</td>
</tr>
</tbody>
</table>

Caspase activity was determined using specific synthetic fluorogenic substrates in Kato III cells stimulated with $H. pylori$ (bacteria to epithelial cell ratio of 300:1) or 400 $\mu$M H$_2$O$_2$. Each sample
was analyzed in duplicate. Values are shown as caspase activity expressed as mean percent of
caspase activity in unstimulated cells, ± the standard error of the mean (*, \( P < 0.05 \)).
A

B

C

Glutathione Levels (% of control)

6 h 24 h

1000 μM 400 μM 200 μM 100 μM 50 μM 0 μM Hp

DCF Fluorescence (arbitrary units)

1000 μM 400 μM 200 μM 100 μM 50 μM 0 μM Hp

O₂⁻ nmol/10⁵ cells

0 x 10⁷ Hp/Kato 3 x 10⁷ Hp 3 x 10⁷ Hp Kato 6 x 10⁷ Hp 6 x 10⁷ Hp Kato 10 x 10⁷ Hp 10 x 10⁷ Hp Kato

Glutathione Levels (% of control)

Kato III N87

6 h 24 h

0 5 10 15 20 25

Time (min)

Time (h)

0 0.5 1 1.5

0 0.5 1 1.5

0 0.5 1 1.5
A

![Graph showing DCF Fluorescence (% of uninfected control) for H. pylori strains 26695/8-1 and 84-183/2-1. The graph compares cag PAI+ and cag PAI- strains, with significant differences indicated by * and **.](https://example.com/graph_a.png)

B

![Graph showing O2 nmol/10^5 cells/30 min for H. pylori strains at 30 and 60 min. The graph compares cag PAI+ and cag PAI- strains, with significant differences indicated by * and **.](https://example.com/graph_b.png)
A.

![Graph A]

- Horizontal axis: Concentration of \( \text{H}_2\text{O}_2 \) (\( \mu \text{M} \))
- Vertical axis: % Viability and Apoptotic Index
- Bars for Control, 26695, 8-1, 84-183, 2-1, \( \text{H}_2\text{O}_2 \) with and without NAC
- IFN-\( \gamma \) + anti-Fas treatment

B.

![Graph B]

- Vertical axis: Apoptotic Index
- Bars for Control, 26695, 8-1, 84-183, 2-1, \( \text{H}_2\text{O}_2 \) with and without NAC
- \( \ast \) indicates significant difference

H. pylori