

Spermine Oxidation Induced by *Helicobacter pylori* Results in Apoptosis and DNA Damage: Implications for Gastric Carcinogenesis

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Abstract

Oxidative stress is linked to carcinogenesis due to its ability to damage DNA. The human gastric pathogen *Helicobacter pylori* exerts much of its pathogenicity by inducing apoptosis and DNA damage in host gastric epithelial cells. Polyamines are abundant in epithelial cells, and when oxidized by the inducible spermine oxidase SMO(PAOH1) H₂O₂ is generated. Here, we report that *H. pylori* up-regulates mRNA expression, promoter activity, and enzyme activity of SMO(PAOH1) in human gastric epithelial cells, resulting in DNA damage and apoptosis. *H. pylori*-induced H₂O₂ generation and apoptosis in these cells was equally attenuated by an inhibitor of SMO(PAOH1), by catalase, and by transient transfection with small interfering RNA targeting *SMO(PAOH1)*. Conversely, *SMO(PAOH1)* overexpression induced apoptosis to the same levels as caused by *H. pylori*. Importantly, in *H. pylori*-infected tissues, there was increased expression of *SMO(PAOH1)* in both human and mouse gastritis. Laser capture microdissection of human gastric epithelial cells demonstrated expression of *SMO(PAOH1)* that was significantly attenuated by *H. pylori* eradication. These results identify a pathway for oxidative stress-induced epithelial cell apoptosis and DNA damage due to SMO(PAOH1) activation by *H. pylori* that may contribute to the pathogenesis of the infection and development of gastric cancer.

Introduction

Helicobacter pylori is a Gram-negative microaerophilic bacterium that selectively colonizes the human stomach and causes chronic gastritis, peptic ulcers, and gastric cancer. Despite inciting substantial acute and chronic immune and inflammatory responses, *H. pylori* infection generally persists for the life of the host, in part due to its ability to evade the antimicrobial effects of the immune response (1). We have recently reported that one potential cause of the ineffective immune response is the induction of apoptosis in macrophages caused by oxidation of polyamines resulting in generation of H₂O₂ (2). Apoptosis of gastric epithelial cells in *H. pylori* infection has been an area of focus in both *in vivo* (3) and *in vitro* studies (4). It may be an important contributing factor in the increased epithelial permeability and mucosal damage, and it has been associated with compensatory cell proliferation (5), all of which contribute to both the inflammation and risk for carcinogenesis. Additionally, DNA damage has been

reported in *H. pylori*-infected gastric epithelial cells *in vitro* (6) and *in vivo* (7). We now report that apoptosis and DNA damage in gastric epithelial cells infected with *H. pylori* are mediated by spermine oxidase [SMO(PAOH1); refs. 8 and 9]. SMO(PAOH1) expression and activity are induced by *H. pylori*; the resulting H₂O₂ generation, apoptosis, and DNA damage are blocked by inhibition of polyamine oxidation; and silencing of *SMO(PAOH1)* expression prevents apoptosis and DNA damage. Our data are the first to demonstrate the induction of polyamine oxidation by a microbial pathogen and to link oxidative stress by this pathway to apoptosis, DNA damage, and potentially carcinogenesis.

Materials and Methods

Bacteria and Cells. *H. pylori* strains 60190 and SS1 were grown under microaerobic conditions as described previously (10). The human gastric epithelial cell line AGS was grown in F12 medium (11). Experiments were performed in antibiotic-free medium with 10% fetal bovine serum.

Reverse Transcription-Polymerase Chain Reaction and Real-Time Polymerase Chain Reaction. Total RNA was isolated from AGS cells and cDNA synthesized, and primer sequences and polymerase chain reaction (PCR) product sizes for β -actin and *PAOI* [mouse homologue of *SMO(PAOH1)*] and PCR conditions for the multiplex reactions were as reported (2). Primer sequences for human *SMO(PAOH1)* were: sense, 5'-GAC-CACAATCAGCAGACTGG-3', and antisense, 5'-TTAGCACACCTAGC-GACACG-3', yielding a 160-bp product. Real-time PCR was performed using SYBR Green (2). For AGS experiments, relative expression of *SMO(PAOH1)* was determined using β -actin as the internal control (2). In tissue studies, *SMO(PAOH1)* expression was normalized to 18S rRNA.

***SMO(PAOH1)* Promoter Activity.** A genomic DNA plasmid library was constructed from human A549 adenocarcinoma cell DNA in the pBluescript SK(-) plasmid and screened with a cDNA probe homologous to exon 1 of *SMO(PAOH1)*. From this library, a clone containing -4479 bp to the transcriptional start site was identified. Deletion constructs were generated by restriction enzyme digestion and subcloned into pGL-2 basic. AGS cells were transiently transfected (2) with 200 ng of the above constructs and luciferase activity performed according to the manufacturer's instructions (Promega, Madison, WI).

***SMO(PAOH1)* Activity.** Lysates of AGS cells were analyzed by a chemiluminescence assay as described previously (2, 12), and the activity was expressed as nanomoles of H₂O₂ per minute per milligram protein.

Measurement of H₂O₂. AGS cells were incubated with 10 μ mol/L CM-H₂DCFDA and intracellular H₂O₂ detected by flow cytometry as described previously (2). For measurement of H₂O₂ in supernatants, 5 \times 10⁵ cells were plated in 24-well plates. After stimulation, cells were washed and incubated with 50 μ mol/L Amplex Red reagent (Molecular Probes, Eugene OR) and 0.1 unit/mL horseradish peroxidase for 30 minutes at 37°C. Plates were read using a microplate reader at 560 nm, and a standard curve with varying dilutions of H₂O₂ was used (2).

Assessment of Apoptosis. Apoptosis was assayed using an annexin V-fluorescein isothiocyanate apoptosis detection kit (Oncogene Research Products, San Diego, CA) according to the manufacturer's instructions. Cells

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(1×10^4) were analyzed by flow cytometry (2). Apoptosis was also assessed by enzyme-linked immunosorbent assay of cytoplasmic histone-associated DNA fragments (13).

Transient Transfection of *SMO(PAOh1)*. AGS cells were transfected with 200 ng of pcDNA3.1-*SMO(PAOh1)* using LipofectAMINE PLUS and optiMEM medium (2). Transfection efficiency was determined by fluorescence in cells transfected with 400 ng of pIRES2-EGFP (Clontech, Palo Alto, CA).

Transient Transfection of *SMO(PAOh1)* Small Interfering RNA. Small interfering RNA duplexes were used that targeted *SMO(PAOh1)* nucleotides 468 to 486, numbered from the start codon (sense, 5'-GGACGUGGUUGAG-GAAUUC-3'; antisense, 5'-CCUGCACCAACUCCUUUAG-3'). Scrambled small interfering RNA with no sequence homology to any known genes was used as the control. Transfection conditions were as described previously (2).

DNA Damage Assays. DNA damage was assessed by the alkaline single-cell gel electrophoresis (comet assay) method (14). AGS cells were stimulated, trypsinized, and embedded into 0.5% low-melting agarose on glass microscope slides. After treatment with alkaline lysis buffer, slides were electrophoresed, stained with propidium iodide, and analyzed by epifluorescence microscopy. DNA damage was measured by the tail moment, defined as product of the length of the tail (in micrometers), which is DNA migrated from the nucleus, and the percentage of DNA in the tail (14).

DNA damage was also determined by 8-oxoguanosine binding. In brief, after fixation and permeabilization, cells were washed, blocked, and incubated with 8-oxoguanosine-fluorescein isothiocyanate conjugate (Kamiya Biomedical, Seattle, WA) for 1 hour in the dark. Cells were resuspended in PBS and analyzed by flow cytometry for fluorescence.

***H. pylori* Gastritis Tissues.** C57BL/6 mice were infected with *H. pylori* SS1 and gastric tissues harvested 4 months later (13). Human gastritis samples were obtained from patients at the Baltimore Veterans Affairs Medical Center, with *H. pylori* status determined as described previously (15). Antral biopsies from patients with *H. pylori* infection from Uijongbu St. Mary Hospital (Uijongbu, Korea) were evaluated before and 2 months after confirmed *H. pylori* eradication. Histologic analysis after eradication demonstrated complete resolution of acute inflammation in all cases and significant reduction of chronic inflammation. Laser capture microdissection of approximately 5000 gastric epithelial cells from formalin-fixed, paraffin-embedded endoscopic gastric biopsies was performed using the Autopix automated LCM system (Arcturus, Mountain View, CA). RNA was extracted, and *SMO(PAOh1)* mRNA was quantified by real-time PCR and normalized for 18S rRNA.

Statistical Analysis. For quantitative data, values represent the mean \pm SE. 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

Parallel Induction of *SMO(PAOh1)* and Apoptosis in *H. pylori*-Stimulated Human Gastric Epithelial Cells. *H. pylori* infection has been linked to DNA damage and apoptosis in gastric epithelial cells (3, 4, 6, 7). However, the origin of the damaging insult has not previously been elucidated. Therefore, based on our previous results demonstrating that *H. pylori* induces *SMO(PAOh1)* in macrophages (2), we sought to determine its effects in gastric epithelial cells. *H. pylori* (strain 60190) induced a significant increase in *SMO(PAOh1)* mRNA expression in AGS cells as determined by real-time PCR analysis (Fig. 1A). Stimulation with *H. pylori* also resulted in a significant increase in *SMO(PAOh1)* promoter activity with the -1117-bp construct (Fig. 1B), indicating that the observed increase in *SMO(PAOh1)* mRNA is due to infection-induced transcription. There was a time-dependent increase in *SMO(PAOh1)* enzyme activity (Fig. 1C) that peaked at 12 hours after *H. pylori* stimulation. In contrast, there was no induction of activity of the acetyl PAO (ref. 16; data not shown) when assessed by a specific assay (2). To determine whether this increase in oxidase activity was accompanied by apoptosis, the sensitive technique of annexin V and propidium iodide staining of live cells was used to measure apoptosis in a highly quantitative manner.

As shown in Fig. 1C, the time course of the induction of apoptosis closely paralleled that of *SMO(PAOh1)* activity.

***H. pylori*-Induced Spermine Oxidation Results in H_2O_2 -Mediated Apoptosis.** To confirm that the *SMO(PAOh1)*-produced H_2O_2 was causally linked to the observed apoptosis, the effects of an oxidase inhibitor (MDL 72527), H_2O_2 detoxifying agent (catalase), and an *SMO(PAOh1)*-specific small interfering RNA were examined. *H. pylori* induced a significant increase in intracellular H_2O_2 (Fig. 1D) that was prevented by the inhibition of *SMO(PAOh1)* by MDL 72527 or by the addition of catalase. We also used the Amplex Red assay, specific for H_2O_2 in the medium, to demonstrate that *H. pylori* induced a significant, 2.7 ± 0.1 -fold increase in extracellular H_2O_2 that was inhibited by $79.6 \pm 7.1\%$ with MDL 72527 and $100.4 \pm 7.8\%$ with catalase ($P < 0.01$ for *H. pylori* versus control and for *H. pylori* + inhibitors versus *H. pylori* alone; data not shown). Consistent with the H_2O_2 data, apoptosis induced by *H. pylori* was significantly attenuated by MDL 72527 and catalase, as shown in Fig. 1E and F. We also confirmed these findings by analysis of apoptosis by DNA fragmentation enzyme-linked immunosorbent assay (data not shown).

Because MDL 72527 inhibits both PAO and *SMO(PAOh1)* (8, 9, 16), to determine whether the spermine oxidation-mediated apoptosis was specifically due to *SMO(PAOh1)*, we transiently transfected AGS cells with a duplex small interfering RNA specific for *SMO(PAOh1)*. This treatment significantly inhibited *H. pylori*-stimulated *SMO(PAOh1)* mRNA expression (Fig. 2A) and produced a $79.1 \pm 8.1\%$ inhibition of *SMO(PAOh1)* enzyme activity (Fig. 2B). This knockdown of *SMO(PAOh1)* was associated with a $71.6 \pm 5.8\%$ inhibition of apoptosis (Fig. 2C).

To confirm that *SMO(PAOh1)* has a causal role in gastric epithelial cell apoptosis, we transiently transfected AGS cells with a full-length cDNA for *SMO(PAOh1)*. There was a significant increase in apoptosis with *SMO(PAOh1)* transfection that was similar to the level of increase with *H. pylori* stimulation in mock-transfected cells (Fig. 2D).

***H. pylori*-Induced Deoxyribonucleic Acid Damage in Gastric Epithelial Cells Is Mediated by *SMO(PAOh1)*.** The comet assay was used to directly visualize DNA damage morphologically. There was a marked increase in the size and intensity of the tail of the DNA fluorescence of the cells in the *H. pylori*-treated (Fig. 3B) versus untreated cells (Fig. 3A). Treatment of AGS cells with MDL 72527 (Fig. 3C) or catalase (Fig. 3D) resulted in reduction of damage. We quantitated the tail moment in >270 cells for each condition and found that there was a 4.4 ± 0.1 -fold increase with *H. pylori* ($P < 0.01$ versus control) that was inhibited by $90.3 \pm 4.1\%$ with MDL 72527 and $87.6 \pm 4.2\%$ with catalase ($P < 0.01$ for both inhibitors versus *H. pylori* alone). When we assessed 8-oxoguanosine binding by flow cytometry as an indicator of oxidatively damaged DNA, we found that stimulation with *H. pylori* resulted in increased fluorescence that was significantly attenuated with MDL 72527 or catalase (Fig. 3E) or transfection with *SMO(PAOh1)* small interfering RNA (Fig. 3F).

***SMO(PAOh1)* Is Up-Regulated in *H. pylori* Gastritis Tissues and Down-Regulated with *H. pylori* Eradication.** To determine whether the *in vitro* observations were relevant in an *in vivo* setting, mouse and human tissues from *H. pylori*-induced gastritis were examined. There was a significant increase in mRNA expression of mouse *PAO1* (Fig. 4A) and human *SMO(PAOh1)* (Fig. 4B) in *H. pylori* gastritis tissues. Levels of *SMO(PAOh1)* in human gastritis tissues from *H. pylori*-negative patients (Fig. 4B) were only modestly increased, whereas tissues from *H. pylori*-infected patients exhibited consistently higher levels of expression. Real-time PCR analysis in

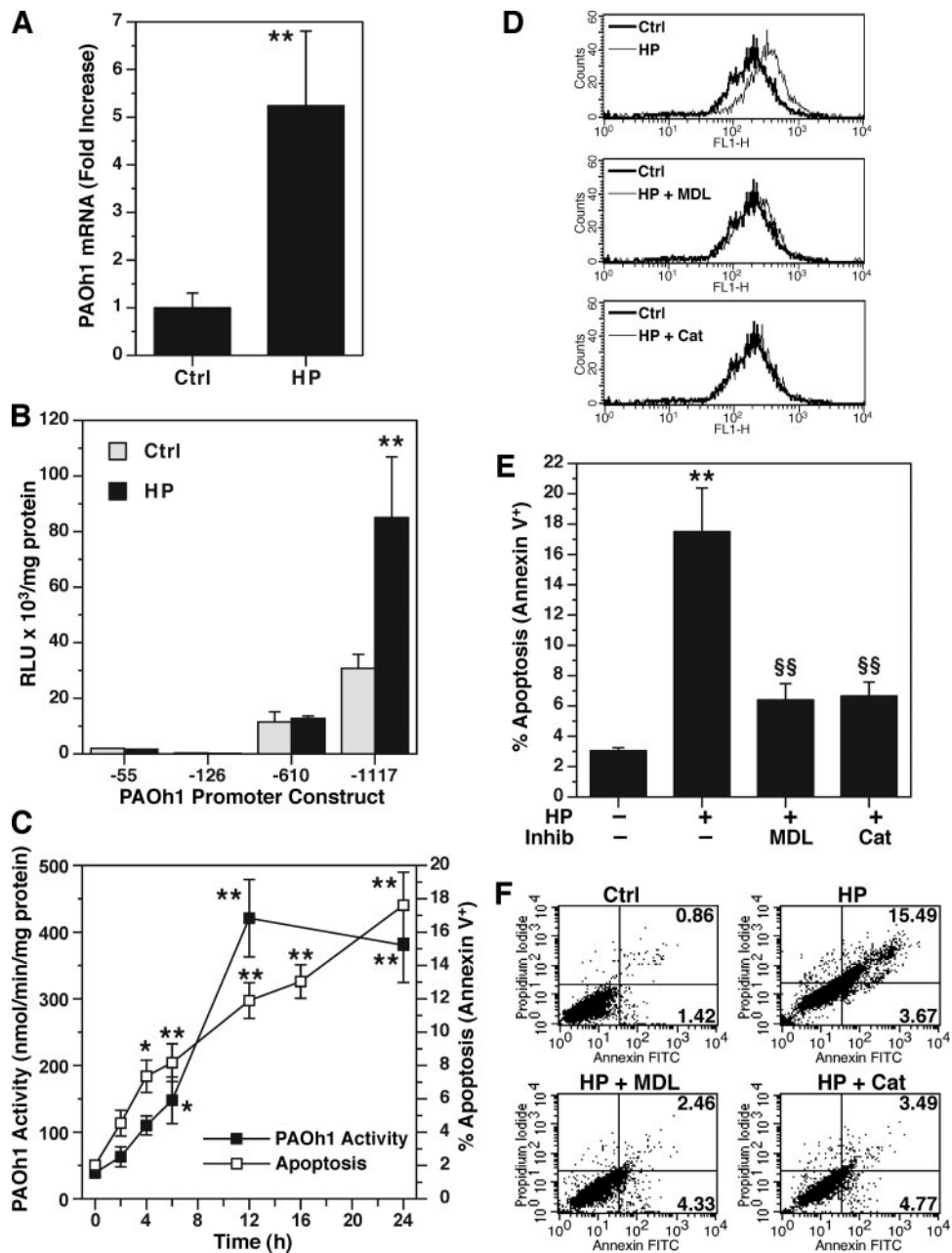


Fig. 1. Induction of SMO(PAOh1) by *H. pylori* results in H_2O_2 production and apoptosis in AGS gastric epithelial cells. *A*, real-time PCR performed after 6 hours of stimulation. *B*, *SMO(PAOh1)* promoter activity as determined by luciferase reporter assay at 24 hours after stimulation. *C*, time course of SMO(PAOh1) enzyme activity and apoptosis after stimulation with *H. pylori*. *D*, representative fluorescence tracings of live cells treated with CM- H_2DCFDA 4 hours after *H. pylori* stimulation, indicative of intracellular H_2O_2 . *E*, total apoptosis (annexin V⁺) summary data. MDL, 250 μ g/mL MDL 72527; Cat, 1,000 units/mL catalase. **, $P < 0.01$ versus unstimulated cells; §§, $P < 0.01$ versus *H. pylori* alone without inhibitor. *F*, representative plots of annexin V versus propidium iodide for the conditions as marked. The values for percentage of positive cells in the annexin V⁺/PI⁺ and annexin V⁺/PI⁻ quadrants are shown. Studies were conducted with a multiplicity of infection of 200. The number of separate experiments in duplicate were as follows: *A*, 5; *B*, 2; *C*, 3; *D*, 4; and *E* and *F*, 3.

the mouse tissues revealed a 3.8 ± 0.4 -fold increase in *H. pylori* gastritis versus uninfected tissues ($P < 0.01$), and in human samples, there was a 2.5 ± 0.4 -fold increase in *H. pylori*-negative gastritis and a 4.9 ± 1.5 -fold increase in *H. pylori*-positive gastritis ($P < 0.05$ versus normal for *H. pylori* positive only). To confirm that *SMO(PAOh1)* was expressed *in vivo* in the gastric epithelium, we used RNA extracted from epithelial cells harvested by laser capture microdissection from *H. pylori*-infected gastric tissues (Fig. 4C). After eradication therapy, *SMO(PAOh1)* mRNA levels determined by real-time PCR decreased in each patient, with an $85.4 \pm 7.5\%$ inhibition compared with levels before treatment ($P < 0.001$).

Discussion

The involvement of oxidative stress in carcinogenesis is well established, both generally and in gastrointestinal cancers (17). However, the origins of the reactive oxygen species leading to DNA damage and cancer, in many cases, have not been identified. The

results presented here describe the pathway that establishes the source of oxidative stress in human gastric epithelial cells as H_2O_2 that is specifically produced from *H. pylori*-induced spermine oxidase activity and results in both apoptosis and DNA damage. These results suggest that one link between *H. pylori* infection and gastric cancer may be SMO(PAOh1)-produced H_2O_2 .

H. pylori infection has been reported to cause production of H_2O_2 in AGS human gastric epithelial cells, and it has been suggested that this may contribute to the carcinogenic process in *H. pylori*-induced gastric cancer (18). However, the source of the H_2O_2 was not determined. Recently, it has been demonstrated that in glutathione peroxidase 1 and 2 (*Gpx1* and *Gpx2*) knockout mice, there is a high incidence of ileocolitis and microflora-associated cancers when mice are raised in conventional housing that includes infection with *Helicobacter* species (19), but when these mice are raised under germ-free conditions, they do not develop tumors. Because *Gpx1* and *Gpx2* are major detoxifying enzymes for H_2O_2 , these results indicate that the

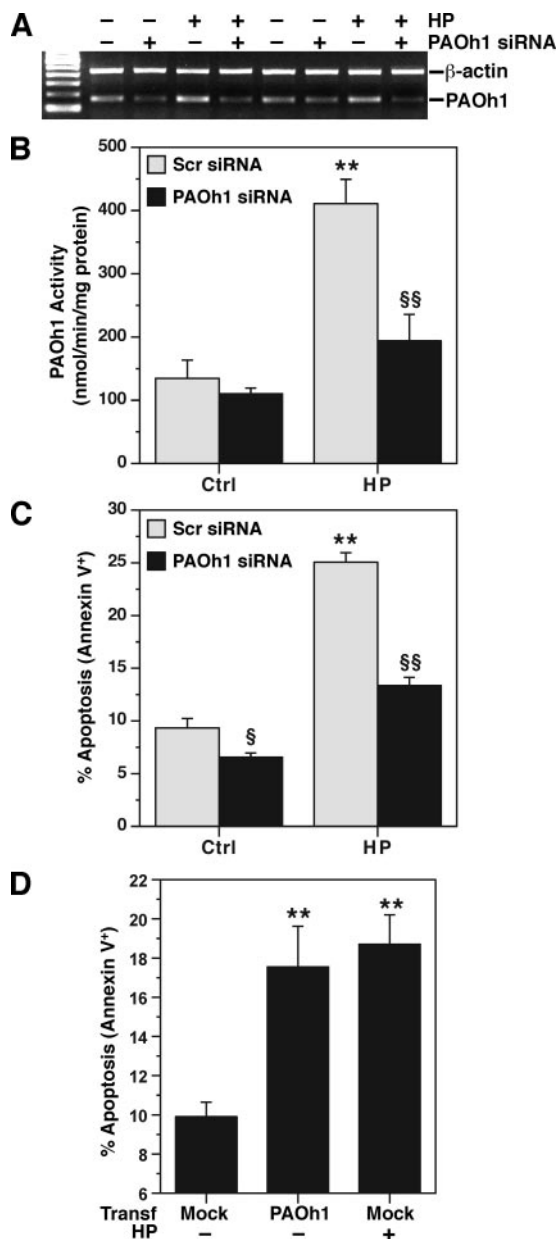


Fig. 2. Transfection with *SMO(PAOh1)* small interfering RNA inhibits *H. pylori*-stimulated apoptosis, whereas transfection of *SMO(PAOh1)* induces apoptosis in gastric epithelial cells. **A**, reverse transcription-PCR analysis of cells transfected with either scrambled small interfering RNA (-) or *SMO(PAOh1)* small interfering RNA (+) in the absence and presence of *H. pylori* for 6 hours. Results from two separate experiments are shown. **B**, *SMO(PAOh1)* enzyme activity measured after 16 hours. **C**, summary data for total apoptosis (annexin V⁺) at 24 hours. In **B** and **C**, **, $P < 0.01$ versus unstimulated cells transfected with scrambled small interfering RNA control; §, $P < 0.05$; §§, $P < 0.01$ versus *H. pylori*-stimulated cells transfected with scrambled small interfering RNA. **D**, AGS cells were transfected with human *SMO(PAOh1)* in the pcDNA3.1 vector. Summary data of apoptosis determined by flow cytometry with total annexin V⁺ cells shown. **, $P < 0.01$ versus unstimulated cells transfected with empty vector (*Mock*). $n = 3$ separate experiments in duplicate.

reactive oxygen species responsible for the inflammation and carcinogenesis is bacterial infection-induced H₂O₂ production.

Here, we demonstrate that *H. pylori* exposure leads to increased H₂O₂ production in AGS cells that can be inhibited by MDL72527, indicating that a polyamine oxidase activity is responsible for the H₂O₂ production. It should also be noted that our results are not restricted to a single cell line, because we have found similar effects of MDL 72527 in MKN-28 gastric epithelial stimulated with *H. pylori* (data not shown). The *SMO(PAOh1)* activity is sufficient to produce

DNA-damaging amounts of H₂O₂, as evidenced by both 8-oxoguanosine production and comet assay. Although generation of reactive oxygen species in response to *H. pylori* has been previously linked to DNA damage (6, 7), our studies provide a new mechanism for generation of reactive oxygen species in epithelial cells and directly demonstrate that the polyamine oxidation causes both apoptosis and DNA damage by this mechanism. The DNA damage and apoptotic cell death produced by H₂O₂ was inhibited by MDL 72527, catalase, and most importantly, *SMO(PAOh1)*-specific small interfering RNA, thus demonstrating that the oxidase in question is, in fact, the spermine oxidase *SMO(PAOh1)* and not the classical N¹-acetyl-polyamine oxidase, PAO (16). These data combined with those from the transient transfection studies demonstrating that *SMO(PAOh1)* produces the same effects as *H. pylori* exposure confirm that the oxidation of spermine by *SMO(PAOh1)* is the source of H₂O₂ in *H.*

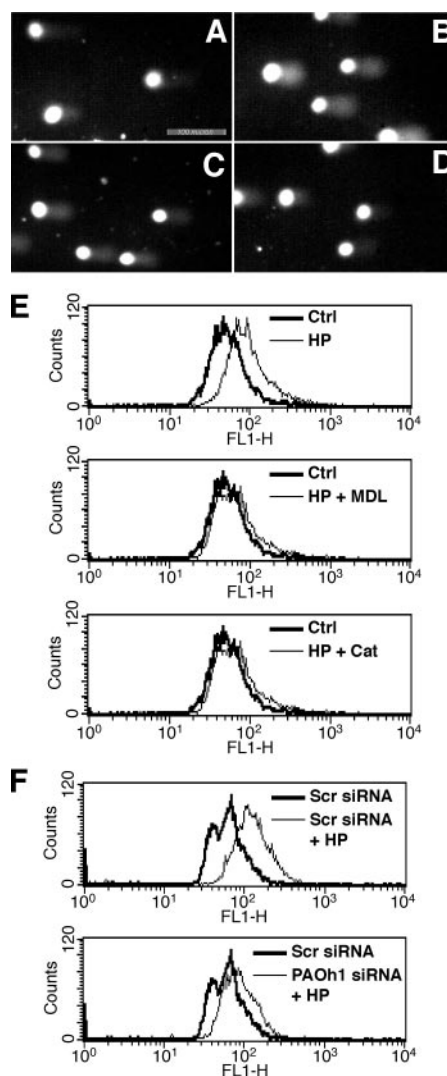


Fig. 3. *H. pylori*-induced DNA damage in gastric epithelial cells is ameliorated by MDL 72527, catalase, or transfection with *SMO(PAOh1)* small interfering RNA. **A–D**, comet assay in AGS cells. Images are photomicrographs of cells after alkaline electrophoresis and propidium iodide staining after 6 hours of stimulation. DNA damage is indicated by the amount of light intensity in the tail portion of the cells. Bar = 100 μm. **A**, control, unstimulated cells; **B**, *H. pylori* at multiplicity of infection of 800; **C**, *H. pylori* + MDL 72527 (250 μmol/L); **D**, *H. pylori* + catalase (1,000 units/mL). **E** and **F**, flow cytometric analysis of 8-oxoguanosine binding at 16 hours after stimulation. Note the shift to the right with *H. pylori* stimulation, indicating more fluorescence intensity, which is prevented by MDL 72527 or catalase in **E** or *SMO(PAOh1)* small interfering RNA in **F**. Data are representative of three separate experiments, each in duplicate, with similar results.

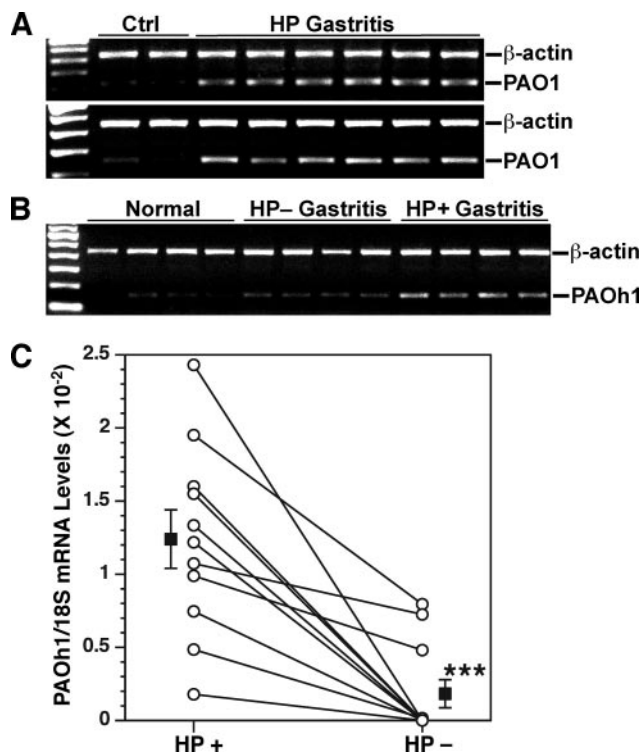


Fig. 4. Increased expression of PAO1 in mouse and human *H. pylori* gastritis. *A*, reverse transcription-PCR in mouse tissues; *top* and *bottom* panels are tissues from two separate experiments, and each lane is a different mouse. *B*, reverse transcription-PCR in human endoscopic biopsy tissues from patients with histologically normal tissue, gastritis without *H. pylori* infection, and gastritis due to *H. pylori* infection. *C*, real-time PCR analysis of RNA from epithelial cells isolated by laser capture microdissection from patients with *H. pylori* gastritis pre-eradication (HP +) and post-eradication with antibiotics (HP -), demonstrating decreased *SMO(PAOh1)* expression after eradication of *H. pylori*. ○, individual patients; ■, mean ± SE for each group; ***, $P < 0.001$ versus HP + before eradication by paired Student's *t* test.

pylori-exposed AGS cells and is directly responsible for the observed downstream effects.

It is important to note the potential that some reactive oxygen species-induced mutations may not be lethal and may lead to growth and/or survival advantages. 8-Hydroxydeoxyguanosine, a common adduct downstream from H₂O₂ production, is known to produce G>T transversion mutations that are commonly found in tumor suppressor genes and oncogenes (20, 21).

The *in vitro* results are consistent with an association between activation of SMO(PAOh1) and *H. pylori*-induced pathogenesis. The results from both the C57BL/6 mouse model of gastritis and human gastritis patients clearly demonstrate an association between *H. pylori* infection and expression of *SMO(PAOh1)* (or its mouse homologue). Importantly, when human *H. pylori* infection is eradicated by antibiotic treatment, there is a significant decrease in *SMO(PAOh1)* mRNA expression in the gastric epithelium. These data demonstrate that *H. pylori*-induced *SMO(PAOh1)* expression extends beyond *in vitro* systems and has *in vivo* relevance.

In summary, the results presented here demonstrate that *H. pylori* infection leads to the increased expression of an important polyamine catabolic enzyme, the spermine oxidase, SMO(PAOh1). This enzyme oxidizes spermine producing the DNA-damaging reactive oxygen species, H₂O₂. Because reactive oxygen species have been directly linked to the etiology of multiple cancers including *H. pylori*-induced gastric cancer, these data are entirely consistent with the hypothesis that *H. pylori*-induced SMO(PAOh1) activity is responsible for the genotoxic insult that results in tumorigenic transformation of affected gastric epithelial cells.

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