Research article

Redox biology and gastric carcinogenesis: the role of *Helicobacter pylori*

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Almost half the world's population is infected by *Helicobacter pylori (H. pylori)*. This bacterium increases the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in human stomach, and this has been reported to impact upon gastric inflammation and carcinogenesis. However, the precise mechanism by which *H. pylori* induces gastric carcinogenesis is presently unclear. Although the main source of ROS/RNS production is possibly the host neutrophil, *H. pylori* itself produces $O_2^{\bullet -}$. Furthermore, its cytotoxin induces ROS production by gastric epithelial cells, which might affect intracellular signal transduction, resulting in gastric carcinogenesis. Excessive ROS production in gastric epithelial cells can cause DNA damage and thus might be involved in gastric carcinogenesis. Understanding the molecular mechanism of *H. pylori*-induced carcinogenesis is important for developing new strategies against gastric cancer.

Keywords: Carcinogenesis, Gastric epithelial cells, *Helicobacter pylori*, Inflammatory cells

Introduction

No one had been able to imagine that a small infectious organism could live under acidic conditions in the human stomach and, still more, cause gastric cancer until 1983, when two researchers, Dr Barry J. Marshall and Dr J. Robin Warren, isolated *Helicobacter pylori* (*H. pylori*) from human gastric mucosa and unravelled the role of the pathogen in inflammation and ulceration of the stomach and duodenum.[1] The discovery of this small bacterium (3.5 mm × 0.5 mm) dramatically changed our perception of gastrointestinal pathogenesis. *H. pylori* is a Gram-negative, helical, microaerophilic bacterium that selectively colonizes the antrum of the human stomach and induces chronic gastritis with varying severity, which in around 10–15% of cases progresses to peptic ulcer and in 1–2% of cases ultimately results in mucosa associated lymphoid tissue lymphoma or gastric adenocarcinoma.[2–4] Consequently, in 1994, *H. pylori* was defined as a group 1 (i.e. a definite) carcinogen by the International Agency for Research on Cancer, a part of the World Health Organization.[5] However, the mechanism through which *H. pylori* damages the gastric mucosa and leads to gastric carcinogenesis is not yet clear.

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The most accepted hypothetical mechanism of *H. pylori*-induced gastric carcinogenesis is that of Correa *et al.*,[6] in which they proposed that gastritis could progress to gastric cancer through multiple 'hits', including oxidative stress and environmental toxins, that increase DNA mutation rates.

In this review, we summarize the reported mechanisms by which reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced in the *H. pylori*-infected stomach, and the possible mechanism by which *H. pylori* induces gastric carcinogenesis.

Redox biology of *H. pylori*

Among the factors that influence gastro-duodenal changes in *H. pylori*-infected patients are ROS and RNS. Excessive ROS/RNS production has been reported in *H. pylori*-infected human gastric mucosa,[7] and correlates well with histological mucosal damage[8] and with bacterial load.[9]

H. pylori itself has been reported to produce a large amount of superoxide anion ($O_2^{\bullet -}$) in order to inhibit the bactericidal effects of nitric oxide (NO) synthesized by inflammatory cells.[10] On the other hand, $O_2^{\bullet -}$ might be passively produced by electrons leaking from the mitochondrial respiratory chain of *H. pylori*, since $O_2^{\bullet -}$ production by *H. pylori* can be suppressed by cyanide (CN^-). The cytotoxicity of $O_2^{\bullet -}$ is moderate, but the cytotoxicity of hydroxyl radicals ($^{\bullet}OH$) produced via Fenton's reaction with

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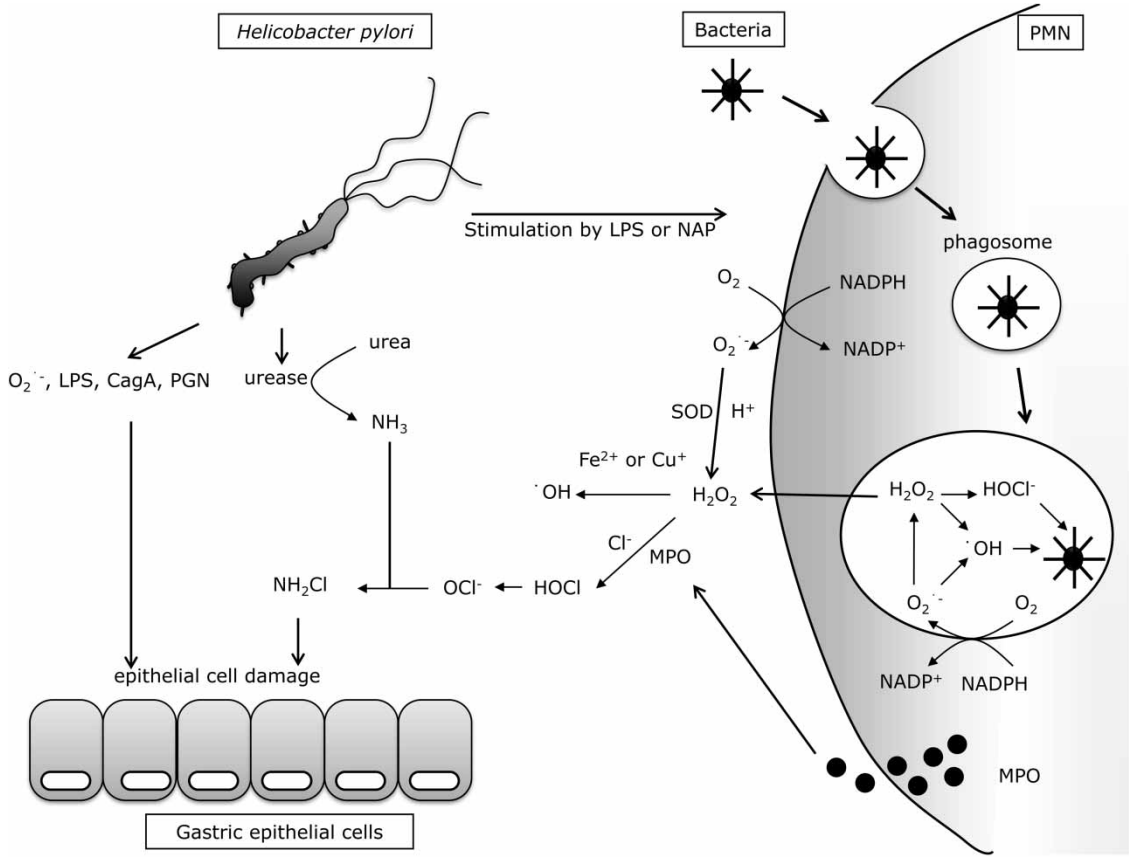

Figure 1 ***H. pylori*-induced oxidative stress in stomach. LPS: lipopolysaccharide; NAP: neutrophil-activating protein; PMN: polymorphonuclear neutrophil; CagA: cytotoxin-associated gene A; PGN: peptidoglycan; MPO: myeloperoxidase; H_2O_2: hydrogen peroxide; NH_2Cl: monocloramine; OCl^-: hypochlorite ion; $NADP^+$/NADPH: nicotinamide adenine dinucleotide phosphate/reduced form of $NADP^+$.**

metals and hydrogen peroxide (H_2O_2) is much higher, and therefore *H. pylori*-produced $O_2^{\bullet -}$ might indirectly induce gastric epithelial cell injury (Fig. 1).

It is well known that *H. pylori* changes from its normal helical bacillary morphology to a coccoid morphology under unfavorable conditions.[11] Since the coccoid form cannot be cultured *in vitro*, it has been speculated that it is a dormant form involved in the transmission of *H. pylori*, and in *H. pylori* reinfection after antibiotic therapy.[12] Interestingly, the coccoid form of *H. pylori* produces more $^{\bullet}OH$ than the helical form.[13] The precise role of $^{\bullet}OH$ in the pathogenesis of the *H. pylori*-infected stomach is unknown.

Besides producing $O_2^{\bullet -}$ or $^{\bullet}OH$, *H. pylori* has various defense mechanisms against external ROS/RNS attack to protect it from elimination. Upon infection, *H. pylori* causes strong inflammation in the gastric mucosa. However, the human immune system cannot eliminate *H. pylori*, and the bacteria can survive in a neutrophil-rich environment in the stomach and induce chronic inflammation with a marked infiltration of polymorphonuclear neutrophils (PMN) that is unusual for chronic infectious diseases, although it is still unclear how these organisms evade phagocytic killing.[14] One of the reasons for this phenomenon is that *H. pylori* is rather non-invasive (although a very few *H. pylori* have been reported to invade the gastric mucosa[15]), and therefore the host immune system-induced inflammatory reaction does not always recognize the bacterium. In addition, *H. pylori* itself causes immune tolerance by selecting *H. pylori* non-reactive T cells via induction of T-cell apoptosis.[16]

Another characteristic defence strategy of *H. pylori* is its strong urease activity, which catalyzes the conversion of urea in the stomach to produce ammonia (NH_3). By this mechanism, *H. pylori* neutralizes the surrounding gastric acid and protects itself from the strong acidity of the stomach. This NH_3 in turn reacts with hypochlorite ions (OCl^-) produced by activated neutrophils to form highly toxic monochloramine (NH_2Cl) in the stomach, a hallmark of *H. pylori* infection[17] and capable of eventually injuring gastric epithelial cells. By using urease activity, *H. pylori* indirectly quenches external NO attack. *H. pylori* induces the expression of inducible NO synthetase (iNOS) in gastric mucosal epithelial cells, vascular endothelial cells, or infiltrating inflammatory cells.[18] Among these cells, macrophages are the main source of NO induced by iNOS using L-arginine as a substrate.[19] Although NO produced by macrophages can kill *H. pylori in vitro*,[20] it cannot eliminate

H. pylori in the human stomach and therefore chronic inflammation persists in the gastric mucosa. NO produced in the *H. pylori*-infected stomach rapidly reacts with $O_2^{\bullet-}$ to produce highly toxic peroxynitrite ($ONOO^-$). Using carbon dioxide (CO_2) produced by urease catalysis, *H. pylori* converts this $ONOO^-$ into non-toxic, short-lived $ONOOCO_2^-$. In addition, *H. pylori* synthesizes alkyl-hydroperoxide reductase, which can quench $ONOO^-$.[21] *H. pylori* also suppresses NO production by inducing apoptosis of macrophages. Briefly, lipopolysaccharides (LPS) of *H. pylori* stimulates macrophages to produce polyamines by inducing arginase and ornithine decarboxylase, and the polyamines induce apoptosis of macrophages[22] and suppress their iNOS expression.[23] *H. pylori* has other strategies for counteracting NO. Since the bacterium synthesizes arginase,[23,24] it can produce urea using L-arginine as a substrate, and use urease to convert the urea to NH_3. Since L-arginine is the substrate for both arginase and iNOS, it is possible that the production of arginase by *H. pylori* is a competitive strategy against NO production by host cells.

H. pylori also removes H_2O_2 produced by neutrophils by its catalase (KatA) and KatA-associated protein (KapA).[25] Some studies have reported that the amounts of catalase and superoxide dismutase (SOD) released by *H. pylori* are likely insufficient to clear excess extracellular oxidants,[26] as these enzymes primarily play a role in the elimination of ROS generated by the bacterium itself. This issue should be examined in more detail.

In summary, with various strategies, *H. pylori* quenches ROS/RNS that are potentially harmful to it, in order to survive the stressful conditions found in the stomach.

Redox biology of inflammatory cells

In *H. pylori*-infected gastric mucosa, neutrophils are believed to be the main source of ROS/RNS.[27] Many investigators have reported that in gastric mucosa with *H. pylori* infection, there is a remarkable infiltration of PMN and a significant increase in mucosal interleukin-8 (IL-8), a potent PMN chemotactic stimulant.[28] After successful *H. pylori* eradication therapy, the number of infiltrating PMN and level of mucosal IL-8 return to normal.[29] It is thus clear that PMN, transmigrated and localized into the gastric mucosa by IL-8 stimulation, plays an important role in the pathogenesis of *H. pylori*-induced gastric mucosal inflammation.[30]

In neutrophils, nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase; Nox) on the cell membrane catalyzes ROS production.[31] Upon recognition of pathogenic bacteria, neutrophils immediately engulf the bacteria (phagocytosis) to form phagosomes, in which the invaginated pathogens are killed by actively produced ROS derived from Nox catalysis. During phagocytosis, gp91phox, the catalytic subunit of the phagocytic Nox, becomes activated and receives an electron from cytoplasmic NADPH. Subsequently, phagocytic Nox donates this electron to molecular oxygen in the phagosome or outside the phagocyte to produce $O_2^{\bullet-}$, a precursor of microbicidal oxidants. This $O_2^{\bullet-}$ is converted to H_2O_2 by SOD catalysis, or by non-enzymatic dismutation in the phagosome. Since H_2O_2 can passively permeate cell membranes, it is converted to hypochlorous acid (HOCl), which is 100 times more toxic than H_2O_2. This conversion is mediated by myeloperoxidases released by Azure granules in phagocytes in the presence of chloride ions (Cl^-). H_2O_2 also reacts non-enzymatically with $O_2^{\bullet-}$ to form $^{\bullet}OH$ in the presence of ferrous (Fe^{2+}) or cuprous (Cu^+) ions. In general, the phagocytes use these highly reactive ROS (i.e. HOCl and $^{\bullet}OH$) to kill pathogenic bacteria. However, in the *H. pylori*-infected gastric mucosa, these ROS cannot eradicate the bacteria; therefore, the phagocytes produce more ROS. This excessive production of ROS is believed to be a major cause of gastric mucosal damage (Fig. 1).

Redox biology of gastric epithlial cells

Gastric epithelial cells are one source of ROS in the *H. pylori*-infected stomach. Upon stimulation by bacterial cytotoxic factors of *H. pylori* or cytokines,[32] gastric epithelial cells passively produce ROS as by-products of increased mitochondrial respiration rather than as active products. Recently, not only phagocytic cells but also non-phagocytic epithelial cells of the alimentary tract have been reported to express Nox.[33] *H. pylori* LPS not only activate neutrophils to produce $O_2^{\bullet-}$, but also increase Nox on gastric epithetial cells and Toll-like receptor 4 expression on gastric epithelial cells leading to further production of $O_2^{\bullet-}$.[34]

Bacterial cytotoxic factors of *H. pylori*, such as vacuolating cytotoxin, cytotoxin associated gene product (CagA), and peptidoglycan, also stimulate gastric epithelial cells to cause oxidative stress. CagA and peptidoglycan have been reported to be injected into gastric epithelial cells via the needle-like structure of *H. pylori*, a type IV secretion system. By transfecting the *cagA* gene into gastric epithelial cells, we found that a fraction of the expressed CagA protein localizes to mitochondria and induces formation of a significant amount of ROS in the cells. In addition, increased ROS production might be involved in acceleration of the cell cycle and subsequent cell proliferation.[35] The mechanism by which CagA induces ROS production in gastric epithelial cells is not known. However, CagA localized to mitochondria may deregulate the function of the mitochondrial

electron transport chain so that it produces primarily $O_2^{\bullet -}$. To clarify the importance of ROS production in CagA-expressing gastric epithelial cells, an *in vitro* study using CagA-transfected normal rat gastric epithelial cells (RGM1) is currently under way in our laboratory.

Gastric epithelial cells protect themselves from oxidative stress by activating antioxidant defense mechanisms, including oxygen scavenger enzymes such as SOD, catalase and glutathione peroxidase,[36] as well as vitamins (Vit) E and C.[37] Those cells infected by *H. pylori* being predominantly on the antrum of the stomach, Vit E levels in the gastric body are known to decrease, which may reflect a mobilization of antioxidant defense mechanisms to maximally inflamed sites.[38] Another study in Mongolian gerbils concluded that Vit C or Vit E supplementation protects from *H. pylori*-induced gastritis in the short term, its effects seemingly declining during persistent infection.[39] Thus, although antioxidant systems seem to be involved in infection to counteract the increased ROS,[40] when *H. pylori*-induced excessive ROS generation on the epithelium is augmented by reduced effectiveness of antioxidant defenses, the risk of cytoxicity from oxidation, and DNA damage is potentially increased.[41] An impaired redox balance may then result in cell death, which would increase the proliferation rate of the remaining cells and thereby increase the chance of mutations leading to increased oncogene expression, which ultimately results in gastric cancer.[42]

To summarize the findings described above, the role of *H. pylori*-induced oxidative stress on the gastric mucosa is multi-functional. For neutrophils, it is the result of excessive defense reactions of the human body against *H. pylori* intrusion, and for *H. pylori*, it might be a convenient tool for invading the human gastric mucosa. Although the role of *H. pylori*-induced oxidative stress in gastric epithelial cells is not clear, it might be involved in signal transduction in epithelial cells,[32,43] or in gastric carcinogenesis. Further study is required to elucidate the effects of *H. pylori*-induced oxidative stress on gastric epithelial cells and its role in gastric carcinogenesis.

Redox biology of gastric carcinogenesis

In patients with *H. pylori* infection, chronic gastritis sometimes results in glandular loss, which triggers multifocal atrophic gastritis and intestinal metaplasia,[44] risk factors for the progression to gastric carcinoma.[45] Determinants and mechanisms involved in the occurrence of such divergent pathways are unknown and represent a primary focus of research.

One mechanism of *H. pylori*-induced carcinogenesis is now believed to be dependent on cumulative oxidative DNA damage.[46] This concept is supported by several reports of the production of 8-hydroxydeoxyguanosine (8-OHdG), the main oxidatively modified product of DNA. In gastric carcinoma patients, significantly higher levels of 8-OHdG were reported in tumor-adjacent tissues and tumor tissues than in normal tissues.[47] Although DNA damage induced by oxidative stress is made good by several repair genes, accumulated oxidative DNA damage such as 8-OHdG can be only partially repaired through enzyme pathways that may, in turn, cause further DNA damage[48] that induces DNA mutation and ultimately gastric carcinogenesis. Also, only a small proportion of the *H. pylori*-infected patients show clear reduction in the levels of the damaged DNA.[49] Moreover, repair genes damaged by oxidative stress may not able to repair damaged DNA and therefore may cause gastric carcinogenesis. Therefore, in younger patients with *cagA*-positive *H. pylori*, DNA oxidative damage in the gastric mucosa accumulates in earlier stages of their life, and may cause more extensive gastric mucosal derangement.[49,50]

In addition to 8-OHdG production, an accumulation of intracellular ROS/RNS can induce point mutation in the DNA, thus disrupting the expression and function of several tumor-suppressing genes such as p53, which might contribute to the pathogenesis of gastric cancer.[51] The importance of NO in *H. pylori*-induced gastric carcinogenesis has been shown in experiments using $iNOS^{-/-}$ mice, in which gastric carcinogenesis was induced by administration of *N*-methyl-*N*-nitrosourea. The overall incidence of gastric cancer after 50 weeks was significantly lower in $iNOS^{-/-}$ mice compared with their wild-type counterparts.[52] In gastric cancer, the most frequent gene mutation observed in the tumor suppressive gene, p53, is G:C to A:T conversion. Methylated cytosine (C) is deaminated in the presence of NO to form thymine (T) (G:methylated C to G:T). Since thymine is a usual nucleobase in DNA, the G:T mis-pair is rarely repaired, and therefore G:C to A:T transversion is fixed after DNA replication.[53,54] This concept is supported by a report that human gastric cancer has a mutation with a high prevalence for G:C to A:T transversion.[55]

As described above, CagA, a cytotoxin of *H. pylori*, induces ROS production in mitochondria. The mitochondrial DNA (mtDNA) is more susceptible to ROS damage than nuclear DNA because of its close proximity to the electron transport chain and its lack of protective histones or DNA-binding proteins.[56] In addition, mtDNA damage is not sufficiently repaired because of the low level of repair enzymes in some cells.[57] As a result, the respiratory enzymes containing the defective mtDNA-encoded protein subunits may exhibit impaired electron transport function and thereby increase the electron leak and ROS production, which in turn elevates oxidative stress and oxidative damage to mitochondria. Consequently,

CagA might induce oxidative stress to the gastric mucosa, and may damage cellular components, including polyunsaturated fatty acids, proteins, and mtDNA, which may enhance nuclear DNA damage, and possibly result in the pathogenesis of gastric carcinogenesis.

Besides inducing DNA damage, *H. pylori*-induced oxidative stress has been shown to modify epithelial cell turnover in the stomach.[51] This notion is supported by studies describing an increase in both epithelial cell proliferation and cell death by apoptosis, in response to infection. Cell death, including apoptosis, is triggered by *H. pylori* as well as various inflammatory mediators. Firstly, activated T cells kill gastric epithelial cells directly, and the host response increases the expression of receptors for *H. pylori* and thus increases bacterial binding and the induction of apoptosis by the bacteria.[51] Next, *H. pylori*-induced epithelial cell death can stimulate the proliferative response of epithelial cell precursors.[51] Recently an interesting finding has been reported with regard to murine double minute 2 (MDM2), an inactivator of the tumor suppressor p53.[58] It was found that MDM2 expression is higher in biopsy specimens of intestinal metaplasia and gastric cancer than those from chronic gastritis in *H. pylori*-infected stomach and that interleukin-16 (IL-16), a T-cell chemoattractant that has been shown to be involved in *H. pylori*-induced gastric inflammation, increased the expression of MDM2 and cell proliferation of a gastric cancer cell line. Since the overexpression of MDM2 has been shown in several human tumors, and increased expression of MDM2 inactivates the apoptotic and cell cycle arrest function of p53, the expression of MDM2 in long-term *H. pylori*-infected gastric mucosa may indicate a risk for carcinogenesis, and IL-16 secretion in *H. pylori*-infected mucosa could well be one of the factors promoting gastric carcinogenesis.

Conclusion

As well as host factors, bacterial factors, and host–bacterial interactions, there are many other factors that might be involved in the pathogenesis of gastric cancer (Fig. 2). For example, ingested food and tobacco smoke directly influence mucosal redox status,[41] as they expose the gastric epithelium to the

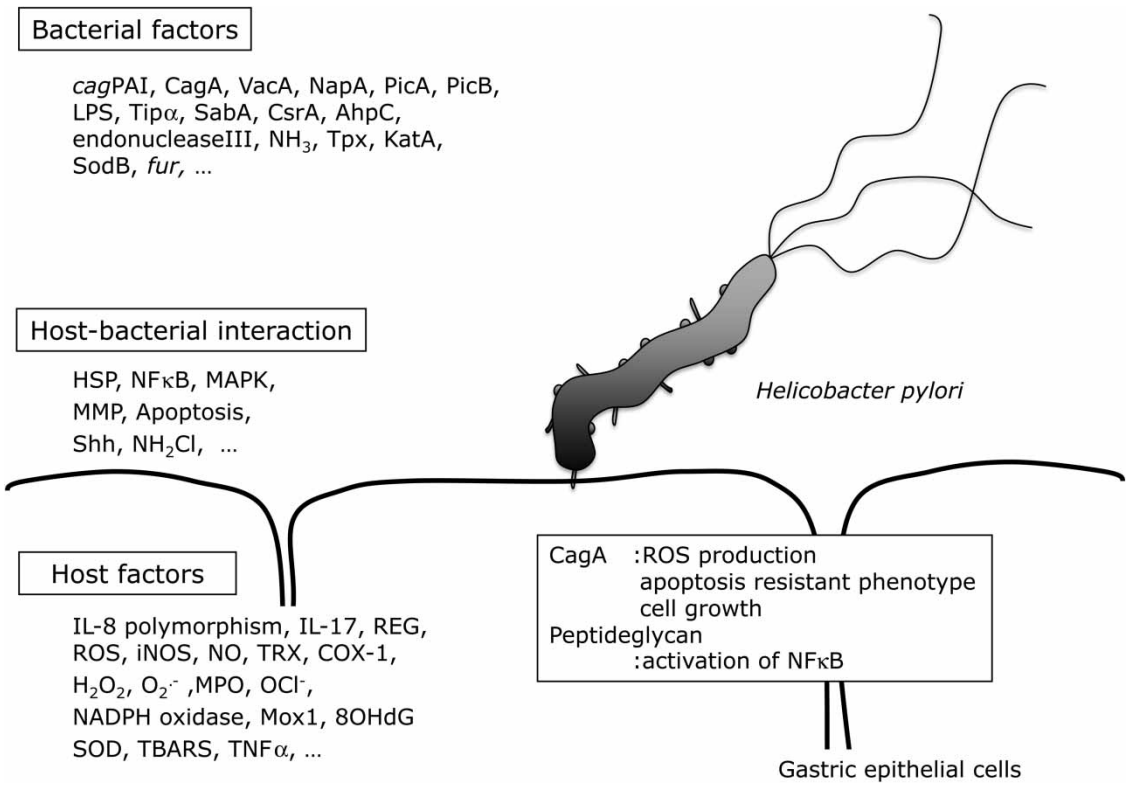

Figure 2 Factors in *H. pylori*-induced gastric inflammation. *cag*PAI: cag pathogenicity island; VacA: vacuolating cytotoxin A; PicA: permit the induction of cytokines A; PicB: permit the induction of cytokines B; Tipα: tumor necrosis factorα inducing protein; SabA: sialic acid-binding adhesin A; CsrA: carbon storage regulator A; AhpC: alkyl hydroperoxide reductase C; Tpx: thiol-peroxidases; KatA: catalase A; SodB: superoxide dismutase B; fur: ferric uptake regulator; HSP: heat shock protein; MAPK: mitogen-activated protein kinase; MMP: matrix metalloproteinase; Shh: sonic hedgehog; IL-8: interleukin-8; IL-17: interleukin-17; iNOS: inducible nitric oxide synthase; NO: nitric oxide; TRX: thioredoxin; COX-1: cyclooxygenase-1; NADPH: nicotinamide adenine dinucleotide phosphate; Mox1: mitogen oxidase 1; 8OHdG: 8-hydroxy deoxyguanosine; SOD: superoxide dismutase; TBARS: thiobarbituric acid reactive substances; TNFα: tumor necrosis factor alpha; ROS: reactive oxygen species; NFκB: nuclear factor kappa B; CagA: cytotoxim associated gene A; NapA: neutrophil-activating protein A; Lps: lipopolysacharide; COX1: cyclokxygenare; NH_2Cl: monochloradine; ROS: reactive oxygen species; H_2P_2: hydrogen peroxide; OCl^-: hypochloride ion.

ROS they generate within the gastric lumen in a sustained manner. This multiplicity of factors makes it difficult to fully understand the mechanism by which *H. pylori*-induces gastric carcinogenesis. Therefore, further research is needed to clarify this, and to identify useful strategies to combat the carcinogenic effects of *H. pylori*.

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