Gastric mucosal superoxide dismutases in *Helicobacter pylori* infection

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Abstract

Background—The mucosal pathology of *Helicobacter pylori* infection may in part be due to excessive production of reactive oxygen metabolites (ROMs) by phagocytes. The influence of *H pylori* infection on mucosal superoxide dismutases, some major scavenger enzymes of ROM was investigated. In humans superoxidase dismutase is present in at least two forms – that is, mitochondrial manganese (Mn)-superoxide dismutase and cytoplasmic copper-zinc (CuZn)-superoxide dismutase.

Methods—The amount and activity of both superoxide dismutases were measured, respectively by enzyme linked immunosorbent assay (ELISA) and spectrophotometrical enzyme activity assay, in gastric biopsy homogenates of patients with normal mucosa (n=39) and in patients with *H pylori* related gastritis (n=71). Infection and gastritis were confirmed by a combination of culture, serology, and histology.

Results—The amount ($p<0.001$) and activity ($p\leq 0.05$) of Mn-superoxide dismutase were increased by about twofold to threefold, whereas the amount and activity of CuZn-superoxide dismutase showed a slight decrease in gastric mucosa of patients with *H pylori* gastritis, in both antrum and corpus, compared with normal mucosa of patients without *H pylori* infection. Mn-superoxide dismutase concentrations in biopsy specimens of histologically normal corpus from patients with an inflamed antrum were significantly higher ($p<0.01$) than that of patients with a histologically normal antrum.

Conclusion—*H pylori* infection has a differential effect on mitochondrial and cytoplasmic superoxide dismutase in the gastric mucosa, reflected by a pronounced increase in the cytokine inducible Mn-superoxide dismutase and a marginal decrease in the constitutive CuZn-superoxide dismutase.

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cause of gastritis.² Almost 100% of people infected with *H pylori* have gastritis, while over 90% of patients with gastritis have *H pylori* infection.^{3,4} The prevalence of gastritis as well as *H pylori* infection increases with advancing age.⁵ Furthermore, infection with *H pylori* is the most important factor in peptic ulcer disease, and it is recognised as a pathogenic factor in gastric carcinogenesis.⁶⁻⁸ Normalisation of the gastric mucosa occurs as a result of successful eradication of *H pylori* through antibacterial treatment. In the rare event of recurrent infection, there may be a relapse of the disease.⁹

Mucosal pathology related to *H pylori* infection was at first explained by locally acting toxic factors such as cytotoxin, urease, and ammonia. There is growing evidence, however, that the mucosal damage is partly caused by attraction and activation of phagocytes, producing excessive amounts of reactive oxygen metabolites (ROMs).^{10,11} In a previous study, for example, a positive correlation was found between ROM production and *H pylori* status of patients.¹² There was even a positive association between mucosal ROM production and quantitative histological and microbiological *H pylori* assessments.¹³ These ROMs are highly toxic and can cause damage to all cellular components, including structural and regulatory proteins, carbohydrates, and DNA.¹⁴ Phagocytic cells produce large amounts of ROMs to facilitate killing of micro-organisms.¹⁵ Excessive ROM production seems to play a part in a number of diseases, including disorders of the gastrointestinal tract, as is shown in animal models and in some human studies.^{16,17}

Organisms possess a series of defence mechanisms, so called antioxidants, to terminate or reduce the toxicity of ROM reactions.¹⁸ One of the situations in which ROM production may exceed the natural defence system is that of phagocyte activation. Activated phagocytes produce superoxide anion (O_2^-) via a membrane bound NADPH oxidase.¹⁵ Myeloperoxidase, released from phagocytic granules of neutrophils, increases toxicity by catalysing the formation of hypochlorite. *H pylori* can activate phagocytes in vitro with production of ROMs¹⁹⁻²¹ but *H pylori* itself seems to be resistant to ROM toxicity because of either spatial isolation or to production of antioxidants.¹³

An enzyme that plays an important part in the defence mechanisms against reactive oxygen species is superoxide dismutase.²² Superoxide dismutase is an O_2^- scavenger and is known in humans to be present in at least two forms – that is, cytoplasmic copper/zinc

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Helicobacter pylori is a small, curved or spiral shaped, Gram negative bacillus that lives in the human stomach and duodenum.¹ *H pylori* infection is one of the most common bacterial infections worldwide and is the most common

(CuZn)-superoxide dismutase and mitochondrial manganese (Mn)-superoxide dismutase. Because Mn-superoxide dismutase is strategically located in the mitochondria, a site of considerable O_2^- production, it is probably an important first line of cellular defence against injury mediated by oxygen metabolites. Our group recently showed that intestinal inflammation in inflammatory bowel disease is accompanied by a dysfunction of superoxide dismutase, thus rendering the tissue vulnerable to ROM damage.²³

The hypothesis for this study was that *H. pylori* infection might affect the amount or activity, or both, of gastric mucosal superoxide dismutases. Considering the excessive ROM production as a result of neutrophil activation, the amount and activity of gastric mucosal superoxide dismutase could modify potential mucosal injury by the bacteria. Thus, the aim of the study was to measure the amount and activity of Mn-superoxide dismutase as well as CuZn-superoxide dismutase in biopsy specimens of *H. pylori* associated inflamed mucosa and of normal gastric mucosa.

Methods

Biopsy material obtained through gastroscopy was initially available from 110 consecutive patients (65 male, 45 female, mean age 51 (13–80) years). Patients who used or had recently used proton pump inhibitors, corticosteroids, non-steroidal anti-inflammatory drugs (NSAIDs), bismuth compounds, sucralfate, or antibiotics were excluded. Use of H_2 receptor antagonists was not considered a reason for exclusion. At endoscopy four biopsy specimens were obtained for histological examination, two from the antrum, 3–5 cm proximal to the pylorus, and two from the corpus, approximately 5 cm above the junction between antrum and corpus. These biopsy specimens were examined in accordance with the guidelines of the Sydney classification, by an experienced pathologist. A single specimen for culture was taken from the antrum and was processed as previously described.²⁴ In 39 cases the stomach was histologically normal, while 48 patients had a pangastritis: both antrum and corpus showed histological signs of inflammation. In the other 23 cases only the antrum was inflamed.

A further two biopsy specimens of antrum and corpus were used for determination of Mn-superoxide dismutase and CuZn-superoxide dismutase concentration and activity. Homogenates were made by adding 100 μ l PBST (0.05% TWEEN-20 in phosphate buffered saline) per mg biopsy material and homogenising on ice in a Potter S (B Braun). The protein concentration in the supernatant was determined by the Lowry assay.²⁵

All cases of gastritis were caused by *H. pylori*. The presence of these bacteria was determined by a positive culture or histological identification, or both, and confirmed by specific IgG-*H. pylori* antibodies.

Enzyme linked immunosorbent assay for CuZn-superoxide dismutase

The CuZn-superoxide dismutase concentration in the tissue homogenates was determined by an ELISA.²⁶ Each well of a flat bottom polystyrene microtitre plate (Dynatech Laboratories, USA; M129A) was coated with 100 μ l antibody solution (10 μ g/ml rabbit- α -CuZn-superoxide dismutase in carbonate buffer, pH 9.6), overnight at 4°C. A second coating followed with a 0.2% gelatin solution for one hour. The plates were washed and each homogenate, diluted 1:100 in PBST/gelatin (0.05% TWEEN 20), was added to a well in duplicate. After two hours of incubation and washing, goat- α -CuZn-superoxide dismutase serum 1:2500 was added to the wells. The plates were incubated for 1.5 hours and washed again. Next, the wells were incubated for one hour with rabbit-goat-peroxidase (Dakopatts P449) 1:7500. Bound antibodies were detected using a solution of 40 mg orthophenylenediamine (OPD) and 40 μ l H_2O_2 in 100 ml citric acid/phosphate buffer, pH 5.0. The incubation time was 30 minutes for each well, the reaction being stopped with 50 μ l 2.5 M sulphuric acid. The optical density was read at 492 nm on a Titertek Multiscan (Flow Laboratories, UK) plate reader. Finally, the CuZn-superoxide dismutase concentration was calculated from a calibration curve based upon 10 standards between 1.25 and 40 ng/ml human recombinant (hr) CuZn-superoxide dismutase and expressed per mg protein of the homogenate.

ELISA for Mn-superoxide dismutase

This procedure closely resembles the ELISA for CuZn-superoxide dismutase. The microtitre plates were coated overnight with 10 μ g/ml rabbit- α -Mn-superoxide dismutase in carbonate buffer. The homogenates were diluted 1:50 in PBST and incubated for two hours. After washing, rabbit- α -Mn-superoxide dismutase-peroxidase 1:250 was added to each well. After one hour bound antibodies were detected as described for CuZn-superoxide dismutase. The standard used in this assay was (hr) Mn-superoxide dismutase.

Human recombinant Mn-superoxide dismutase and CuZn-superoxide dismutase were kindly provided by Dr Z Yavin from the Kyriat Weizmann Institute, Rehovot, Israel. The antibodies against both human superoxide dismutases did not recognise *H. pylori* superoxide dismutase in western blotting nor did pure *H. pylori* extracts provide a signal in the ELISAs. Furthermore, *H. pylori* superoxide dismutase has recently been shown to be an iron containing superoxide dismutase.²⁷

Superoxide dismutase activity measurement

Superoxide dismutase activity was measured in 12 sets of normal tissue and 12 sets of inflamed tissue using the xanthine/xanthine oxidase/cytochrome c method.²⁸ In the presence of xanthine, xanthine-oxidase produces O_2^- , which can be detected by its ability to reduce

Figure 1: Mean concentration of Mn-superoxide dismutase ($\mu\text{g}/\text{mg}$ protein (SEM)) in normal and inflamed antrum and corpus biopsy homogenates. Number of samples between brackets.

cytochrome c. This reduction will be inhibited by superoxide dismutase, through competition with cytochrome c for the flux of O_2^- . In this assay, 80 μl homogenate was added to 890 μl phosphate buffer, pH 7.8. By adding 10 μl xanthine (5 mg in 6.57 ml 0.05 M KOH), xanthine-oxidase (15 μl in 435 μl phosphate buffer), and cytochrome c (25 mg in 2.02 ml phosphate buffer) the reaction started as described above. The reduction of cytochrome c was followed in a spectrophotometer at 550 nm. To provide an estimate of total superoxide dismutase activity in each homogenate a calibration curve based upon five standards between 1.25 and 10 $\mu\text{g}/\text{ml}$ superoxide dismutase was used. Mn-superoxide dismutase activity was determined using the same method, but in the presence of 1 mM potassium cyanide, which inhibits CuZn-superoxide dismutase for $\geq 90\%$. CuZn-superoxide dismutase was thus estimated by subtraction of Mn-superoxide dismutase from total superoxide dismutase activity. The activity is expressed in units/mg protein, where one unit is the superoxide dismutase activity that causes 50% inhibition of the reaction rate in the absence of superoxide dismutase. For CuZn-superoxide dismutase one unit corresponds with 180 ng active (hr) CuZn-superoxide dismutase, while one unit Mn-superoxide dismutase corresponds with 225 ng active (hr) Mn-superoxide dismutase.

Statistical analysis

The significance of the differences between the

Figure 2: Mean concentration of CuZn-superoxide dismutase ($\mu\text{g}/\text{mg}$ protein (SEM)) in normal and inflamed antrum and corpus biopsy homogenates. Number of samples between brackets.

mean superoxide dismutase concentrations obtained through ELISA were determined with the unpaired Student's *t* test with separate variance estimates if the standard deviations were significantly different according to the *F* test. Where appropriate, the paired Student's *t* test was also applied.

Results

Mn-superoxide dismutase and CuZn-superoxide dismutase concentrations

The mean Mn-superoxide dismutase and CuZn-superoxide dismutase concentrations in normal as well as inflamed antrum and corpus biopsy homogenates are shown in Figs 1 and 2, respectively. The inflamed antrum contained significantly higher concentrations of Mn-superoxide dismutase compared with normal antrum (0.68 (0.02) *v* 0.25 (0.02) ($\mu\text{g}/\text{mg}$ protein (SEM)), $p < 0.001$). The same was true for corpus homogenates (0.62 (0.03) *v* 0.45 (0.02), $p < 0.001$). The CuZn-superoxide dismutase content in the inflamed corpus, however, was decreased in gastritis compared with normal corpus homogenates (0.77 (0.04) *v* 0.88 (0.04), $p = 0.04$). In antrum homogenates a similar, although not significant, trend was found in the CuZn-superoxide dismutase concentration in inflamed and normal mucosa (0.70 (0.03) *v* 0.76 (0.06), NS). The finding of a significant difference ($p < 0.01$) in the Mn-superoxide

Mean Mn-superoxide dismutase and CuZn-superoxide dismutase concentrations ($\mu\text{g}/\text{mg}$ protein) and activity (units/mg protein) in normal stomach and in pangastritis.

	Normal mucosa		Pangastritis	
	Antrum	Corpus	Antrum	Corpus
Concentration	(n=36)	(n=36)	(n=47)	(n=47)
Mn-superoxide dismutase (SEM)	0.25 (0.02)	0.41 (0.03)	0.69 (0.03)	0.61 (0.03)
p Value	<0.001		NS	
Cu-Zn-superoxide dismutase (SEM)	0.80 (0.06)	0.90 (0.05)	0.69 (0.05)	0.77 (0.04)
p Value	NS		NS	
Activity	(n=12)	(n=12)	(n=12)	(n=12)
Mn-superoxide dismutase (SEM)	0.69 (0.46)	0.75 (0.30)	2.18 (0.49)	2.84 (0.58)
p Value	NS		NS	
Cu-Zn-superoxide dismutase (SEM)	9.12 (0.42)	7.31 (0.31)	7.26 (0.70)	5.94 (0.80)
p Value	0.002		NS	

Statistical analysis by the paired Student's *t* test.

Figure 3: Individual activities of Mn-superoxide dismutase (units/mg protein) in normal and inflamed antrum and corpus biopsy homogenates.

dismutase concentration within histologically normal corpus between those patients with a normal antrum and those with antral gastritis (0.41 (0.03) ($n=37$) *v* 0.52 (0.03) ($n=23$), respectively) was of considerable interest.

A significantly higher Mn-superoxide dismutase concentration was found in normal corpus mucosa specimens than in adjacent histologically normal antrum, within the same stomach. This difference was not found in the paired findings of inflamed corpus mucosa and adjacent inflamed antral mucosa, within the same stomach. No difference was found in CuZn-superoxide dismutase concentrations in these groups. The Table shows these paired findings.

Superoxide dismutase activity measurements

Figures 3 and 4 show the mean superoxide dismutase activity values in normal and inflamed stomach. The Table shows the paired findings within normal stomach and inflamed stomach. In accordance with the results of the superoxide dismutase concentrations, the Mn-superoxide dismutase activity was significantly higher in inflamed antral and corpus mucosa than in normal mucosa, while the activity of CuZn-superoxide dismutase seemed to be decreased in the inflamed antrum and corpus, although this difference did not reach statistical significance in the corpus biopsy specimens.

Figure 4: Individual activities of CuZn-superoxide dismutase (units/mg protein) in normal and inflamed antrum and corpus biopsy homogenates.

Discussion

In *H. pylori* infected antrum and corpus the amount and activity of Mn-superoxide dismutase were found to be significantly increased, thus contributing to protection against ROM damage. The concentration and activity of CuZn-superoxide dismutase, however, were slightly decreased in the inflamed gastric mucosa.

These results are comparable to those reported by Verspaget *et al.*²³ who investigated the superoxide dismutase concentrations and activity values in the intestine of patients with inflammatory bowel disease. As in this study, they also found a large increase in the amount of Mn-superoxide dismutase in the inflamed tissue, although the Mn-superoxide dismutase activity values in inflammatory bowel disease patients were only marginally increased. Furthermore, they also found CuZn-superoxide dismutase values, concentration as well as activity, to be decreased in the inflamed tissue.

From this study it is not conclusive whether the effects on superoxide dismutase values are caused specifically by *H. pylori* or by gastric mucosal inflammation, as most patients with chronic active gastritis have an *H. pylori* infection. A direct interference of *H. pylori*, containing a distinct iron-superoxide dismutase,²⁷ on the Mn- and CuZn-superoxide dismutase determinations, however, was excluded by the absence of cross reactivity in the assays.

There are several possible explanations for the results obtained in this study. As for the increase in Mn-superoxide dismutase values, it is known that Mn-superoxide dismutase, and not CuZn-superoxide dismutase, can be induced by several cytokines including tumour necrosis factor α (TNF α), interferon γ (IFN γ), and interleukin 1 (IL1).²⁹⁻³¹ This is particularly relevant as mucosal cytokines, like TNF α and IL8, have been found to be increased because of infection with *H. pylori*.³²⁻³³ The fact that inflammation of the antrum causes an increase of the Mn-superoxide dismutase concentration in the corpus, even when the corpus shows no histological signs of *H. pylori* infection, might be caused by paracrine effects of gastric mucosal release of cytokines. The induction by cytokines seems paradoxical as TNF α and IFN γ also induce production of O $_2^-$ during inflammation. The competition, however, may provide a mechanism for selective killing of infected or damaged cells during an immune response. As many viruses and some bacteria shut off synthesis of host proteins in infected cells, the production of superoxide dismutase might also be blocked. As a result, infected cells may be more vulnerable to damage by O $_2^-$.³⁴

The impairment of CuZn-superoxide dismutase in the inflamed tissue might also be caused by inactivation through high local concentrations of free radicals or by a reduced local concentration of the enzyme, or both.³⁴ Salo *et al.*³⁵ found that CuZn-superoxide dismutase is irreversibly inactivated by its own product H $_2$ O $_2$. Exposure of red blood cells to H $_2$ O $_2$ resulted in an inactivation of CuZn-superoxide dismutase up to 50%, while exposure to O $_2^-$ and H $_2$ O $_2$ together caused an

inactivation of up to 75%. Accompanying this loss of activity, the copper binding ability of the enzyme also diminishes with H_2O_2 exposure. As a result of these processes, CuZn-superoxide dismutase may become more susceptible to proteolysis and fragmentation.

Tissue injury by reactive oxygen metabolites has been implicated in the pathogenesis of peptic ulcer disease and gastric carcinoma, both *H. pylori* associated diseases.¹⁵

Although increased tissue concentrations of ROMs are not direct evidence for a pathogenic role,³⁶ we showed for the first time that in *H. pylori* related gastritis a free radical scavenging system (Mn-superoxide dismutase) is changed, probably to compensate for the rate of radical generation.

Individual differences in this mechanism, either because of characteristics of *H. pylori*, such as cytotoxin or urease production, or because of host factors, such as diet, smoking, or primary dysfunction of the antioxidant system, might play a part in susceptibility to diseases like peptic ulcer disease or gastric carcinoma.

In conclusion, gastritis in the presence of *H. pylori* is characterised by a pronounced increase in the amount and activity of Mn-superoxide dismutase, but a slight decrease in CuZn-superoxide dismutase values. Further studies are needed to determine the exact contribution of changes in the superoxide dismutase values and *H. pylori* induced ROMs in patients with *H. pylori* associated gastritis, peptic ulcer disease, and gastric carcinoma, and the effect of treatment on superoxide dismutase values.

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