Treatment of *Helicobacter pylori* infection favourably affects gastric mucosal superoxide dismutases

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Abstract

Background and aims—Excessive production of reactive oxygen metabolites (ROMs) by phagocytic cells is thought to contribute to the mucosal pathology of *Helicobacter pylori* infection. Previously, *H. pylori* infection was shown to have a differential effect on some gastric mucosal scavenger enzymes of ROMs – namely, mitochondrial and cytoplasmic superoxide dismutases – reflected by a large increase in the cytokine inducible manganese superoxide dismutase and a marginal decrease in the constitutive copper/zinc superoxide dismutase. The present study was performed to evaluate whether these altered mucosal superoxide dismutase concentrations and activities in *H. pylori* associated gastritis are reversed to normal by successful treatment of the infection.

Patients and methods—In two different treatment groups – namely, omeprazole or ranitidine, in combination with clarithromycin and metronidazole (OME/AB (n=33) and RAN/AB (n=30)) – manganese superoxide dismutase and copper/zinc superoxide dismutase concentrations were evaluated by enzyme linked immunosorbent assays in homogenates of gastric antrum and corpus biopsy specimens obtained before and eight weeks after successful treatment of *H. pylori* infection. Superoxide dismutase activities in these homogenates were determined spectrophotometrically in eight patients of both groups before and after successful treatment. The concentrations of gastric mucosal superoxide dismutases were also determined in 12 patients with a persistent *H. pylori* infection, with (n=4) or without (n=8) eradication therapy. Infection and eradication of *H. pylori* were confirmed by a combination of culture and histology.

Results—Concentrations of manganese superoxide dismutase were significantly lower after than before therapy in antral (p<0.001 in both treatment groups) and corpus (p<0.001 in both treatment groups) mucosa. By contrast, copper/zinc superoxide dismutase concentrations were significantly higher (p<0.001) only in antral mucosa of the OME/AB treated group. Manganese superoxide dismutase activity was significantly lower after than before treatment in antral (OME/AB

p<0.01, RAN/AB p<0.001), but not in corpus mucosa. Copper/zinc superoxide dismutase activity was not significantly altered by therapy. In the 12 patients with a persistent *H. pylori* infection no major changes in the gastric mucosal superoxide dismutase concentrations were found.

Conclusions—The raised manganese superoxide dismutase and reduced copper/zinc superoxide dismutase concentrations and activities in *H. pylori* associated gastritis were reversed towards normal by successful treatment of the infection.

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Keywords: antioxidants, gastric mucosa, gastritis, *Helicobacter pylori*, reactive oxygen species, superoxide dismutase.

Helicobacter pylori infection of the gastric mucosa is strongly associated with gastritis and peptic ulcer disease.¹⁻⁴ Furthermore, long term *H. pylori* associated gastritis is recognised as a causative factor in gastric carcinogenesis.⁵⁻⁶ As well as via locally acting toxic factors such as cytotoxins, urease, and ammonia, mucosal damage by *H. pylori* infection is also caused by attraction and activation of phagocytes producing large quantities of reactive oxygen metabolites (ROMs), primarily to facilitate killing of microorganisms.⁷⁻⁹ These highly toxic ROMs can also cause damage to cellular components in the host, such as structural and regulatory proteins, lipids, carbohydrates, and DNA.¹⁰ Excessive ROM production is thought to contribute to various diseases of the gastrointestinal tract, as shown in animal models and human studies,^{7-9, 11-12} and has been found in association with *H. pylori* infection.^{13, 14}

Organisms possess enzymatic as well as non-enzymatic defence mechanisms against the toxicity of ROMs.^{12, 15, 16} An important scavenging enzyme in the defence against reactive oxygen species, particularly superoxide anion (O₂⁻), is superoxide dismutase.¹⁷ In humans it is present in at least two forms – cytoplasmic copper/zinc superoxide dismutase and mitochondrial manganese superoxide dismutase. Superoxide dismutases are enzymes that catalyse the dismutation of O₂⁻ to hydrogen peroxide, which is decomposed by other enzymes such as catalase and glutathione peroxidase.¹⁰ Our group recently showed that *H. pylori* induced inflammation of the gastric

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mucosa is accompanied by an increase in manganese superoxide dismutase concentration and activity, both in antral and in corpus mucosa, whereas copper/zinc superoxide dismutase was slightly decreased.¹⁸ We suggested that the alterations in the manganese superoxide dismutase profile in *H. pylori* associated gastritis are a defensive mechanism against possible intracellular damage by free radicals.

The present study was performed to evaluate the effect of eradication of *H. pylori* on the concentration and activity of superoxide dismutases, in particular the manganese type.

Methods

PATIENTS AND BIOPSIES

Biopsy material from 63 dyspeptic patients who were *H. pylori* positive was obtained through gastroscopy. Patients who used or had recently used proton pump inhibitors, corticosteroids, non-steroidal anti-inflammatory drugs, bismuth compounds, sucralfate, or antibiotics were excluded. Use of a low dose H₂ receptor antagonist was not considered to be a reason for exclusion. At endoscopy two biopsy specimens from the antrum, 3–5 cm proximal to the pylorus, and two from the corpus, about 5 cm above the junction between antrum and corpus were obtained for histological examination. These specimens were examined in accordance with the guidelines of the updated Sydney system, by an experienced pathologist.¹⁹ Active (neutrophils) and chronic (mononuclear cells) inflammation were graded on a visual analogue scale and converted to numerical scores (normal=0, mild=1, moderate=2, and severe=3). A single biopsy specimen for *H. pylori* culture was taken from the antrum and was processed as previously described.²⁰ A further two biopsy specimens of antrum and corpus were used for the determination of the superoxide dismutase concentration and activity.

If culture, or histology, or both were positive for *H. pylori* the patients were treated with a combination regimen of acid inhibitory therapy (omeprazole (20 mg twice a day) in 33 patients, 20 men, 13 women, mean age 53 (range 22–75) years, or ranitidine (150 mg twice a day or 300 mg four times a day) in 30 patients, 24 men, six women, mean age 47 (range 22–74) years with clarithromycin (500 mg thrice daily) and metronidazole (500 mg thrice daily) for 14 days, the second only in 50% of the omeprazole treated patients. These combinations are referred to as OME/AB and RAN/AB respectively. Successful *H. pylori* treatment was defined as a negative culture of the antrum biopsy sample and a negative histology, both in the antrum and corpus, eight weeks after the end of therapy. In another four patients (two men, two women, mean age 44 (range 24–60) years) who were treated with OME/AB (double) therapy, *H. pylori* was not eradicated. Furthermore, from eight *H. pylori* positive patients (four men, four women, mean age 51 (range 36–67) years), biopsy specimens

were available at two time points with a mean interval of 9.5 months (range 2–39 months), without an intermediate *H. pylori* eradication treatment and consequently no change in the *H. pylori* status. In these biopsy specimens the gastric mucosal superoxide dismutase concentrations were determined as well.

TISSUE EXTRACTION

Biopsy specimens for superoxide dismutase measurements were combined for each localisation, weighed, and homogenised on ice with a Potter S (B Braun) in 300 µl phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBST). The final concentration of the biopsy specimens was 1 mg biopsy tissue per 100 µl PBST. Protein concentration of the homogenates was determined by the method of Lowry *et al.*²¹

ELISA FOR COPPER/ZINC SUPEROXIDE DISMUTASE

The copper/zinc superoxide dismutase concentration in the tissue homogenates was determined by an adapted enzyme linked immunosorbent assay (ELISA).^{18, 22} Briefly, each well of a flat bottomed polystyrene microtitre plate (Dynatech Laboratories, USA; M129A) was coated with 100 µl antibody solution (10 µg/ml goat α-copper/zinc 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, 100 µl diluted 1:100 in PBST/gelatin (0.05% Tween 20), was added to each well in duplicate. After two hours of incubation and washing, 100 µl rabbit α-copper/zinc superoxide dismutase serum diluted 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 100 µl preabsorbed goat α-rabbit peroxidase (Dakopatts P448) diluted 1:5000. Bound antibodies were detected using 100 µl of a solution of 40 mg orthophenylenediamine and 40 µl H₂O₂ in 100 ml citric acid/phosphate buffer, pH 5.0. The incubation time was 20 minutes for each well, the reaction being stopped with 50 µl 2.5 M sulphuric acid. The optical density was read at 492 nm on a Titertek Multiscan (Flow Laboratories, UK) plate reader. The copper/zinc superoxide dismutase concentration was calculated from a calibration curve based on 10 standards between 1.25 and 40 ng/ml human recombinant copper/zinc superoxide dismutase and expressed per mg protein of the homogenate.

ELISA FOR MANGANESE SUPEROXIDE DISMUTASE

This procedure closely resembles the ELISA for copper/zinc superoxide dismutase. The microtitre plates were coated overnight with 10 µg/ml rabbit α-manganese superoxide dismutase in carbonate buffer. The homogenates were diluted 1:50 in PBST and

incubated for two hours. After washing, 1:250 rabbit α -manganese superoxide dismutase peroxidase was added to each well. After one hour bound antibodies were detected as described for copper/zinc superoxide dismutase. The standard used in this assay was human recombinant manganese superoxide dismutase.

Human recombinant manganese superoxide dismutase and copper/zinc superoxide dismutase were kindly provided by Dr Z Yavin from the Kyriat Weizmann Institute, Rehovot, Israel.

SUPEROXIDE DISMUTASE ACTIVITY MEASUREMENT

Superoxide dismutase activity was measured in tissue homogenates of biopsy specimens

obtained before and after treatment from eight patients of both treatment groups, using the xanthine/xanthine oxidase/cytochrome c method.^{18,23} In the presence of xanthine, xanthine oxidase produces $O_2^{\cdot-}$, which is detected by its ability to reduce cytochrome c. This reduction will be inhibited by superoxide dismutase, through competition with cytochrome c for the dismutation of $O_2^{\cdot-}$. In this assay, 80 μ l homogenate was added to 890 μ l phosphate buffer, pH 7.8. By adding 10 μ l xanthine (5 mg in 6.57 ml 0.05 M KOH), xanthine oxidase (15 μ l in 435 μ l 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 on six standards between 25 and 250 ng superoxide dismutase was used. Manganese superoxide dismutase activity was determined using the same method, but in the presence of 1 mM potassium cyanide which inhibits copper/zinc superoxide dismutase by $\geq 90\%$. Copper/zinc superoxide dismutase was thus estimated by subtraction of manganese superoxide dismutase from total superoxide dismutase activity. The activity is expressed in units/mg protein, in which one unit is the superoxide dismutase activity which causes 50% inhibition of the reaction rate in the absence of superoxide dismutase. For copper/zinc superoxide dismutase one unit corresponds to 180 ng active human recombinant copper/zinc superoxide dismutase, whereas one unit manganese superoxide dismutase corresponds to 225 ng active human recombinant manganese superoxide dismutase.

Figure 1: Effect of eradication of *H pylori* by omeprazole based therapy (OME/AB, $n=33$) and by ranitidine based therapy (RAN/AB, $n=30$) on manganese superoxide dismutase (Mn-SOD) concentrations in antral and corpus biopsy specimens. Concentrations in μ g/mg protein (SEM) (paired analysis). Mean (—) and median (···) concentrations are indicated. (a) After treatment, (b) before treatment.

Figure 2: Effect of eradication of *H pylori* by omeprazole based therapy (OME/AB, $n=33$) and by ranitidine based therapy (RAN/AB, $n=30$) on copper/zinc superoxide dismutase (CuZn-SOD) concentrations in antral and corpus biopsy specimens. Concentrations in μ g/mg protein (SEM) (paired analysis). Mean (—) and median (···) concentrations are indicated. (a) After treatment, (b) before treatment.

STATISTICAL ANALYSIS

The significance of the differences between the pretreatment and post-treatment superoxide dismutase concentrations and activities in the biopsy homogenates was assessed by paired Student's *t* test.

Results

The two treatment groups were analysed separately to evaluate whether *H pylori* therapy based on the proton pump inhibitor omeprazole or the H_2 receptor antagonist ranitidine had similar effects on the superoxide dismutase concentrations in the gastric mucosa and to exclude concealment of different findings. In addition, patients in the RAN/AB group were found to have significantly higher ($p < 0.02$ – $p < 0.001$) concentrations of manganese superoxide dismutase in the antral mucosa and copper/zinc superoxide dismutase in the corpus mucosa compared with the OME/AB group before treatment, which remained significant even after treatment (see data below and Figs 1 and 2).

After therapy manganese superoxide dismutase concentrations were significantly lower (all $p < 0.001$) compared with before therapy in

both treatment groups in antral (OME/AB 0.14 (0.04) *v* 0.65 (0.05); RAN/AB 0.29 (0.05) *v* 1.10 (0.07)) as well as corpus mucosa (OME/AB 0.34 (0.05) *v* 0.57 (0.05); RAN/AB 0.45 (0.05) *v* 0.71 (0.05)), as illustrated in Fig 1. By contrast, copper/zinc superoxide dismutase concentrations were higher after than before treatment, although only significantly in antral mucosa of the OME/AB group (0.73 (0.03) *v* 0.60 (0.02), *p*<0.001; RAN/AB 0.72 (0.04) *v* 0.66 (0.03), NS). In corpus mucosa no significant change in the copper/zinc superoxide dismutase antigen concentration was found compared with before treatment (OME/AB 0.58 (0.04) *v* 0.53 (0.04); RAN/AB 0.78 (0.04) *v* 0.75 (0.03) respectively; Fig 2).

TABLE I Manganese superoxide dismutase (Mn-SOD) and copper/zinc superoxide dismutase (CuZn-SOD) activity in gastric mucosal biopsy specimens of *H. pylori* positive patients before and after successful omeprazole based (OME/AB) and ranitidine based eradication therapy (RAN/AB)

	Mn-SOD (n=8)			CuZn-SOD (n=8)		
	Before	After	<i>p</i> Value	Before	After	<i>p</i> Value
OME/AB:						
Antrum	4.76 (0.91)	0.94 (0.58)	<0.01	4.65 (0.50)	6.59 (0.85)	NS
Corpus	2.87 (0.74)	2.33 (0.64)	NS	5.14 (0.76)	5.27 (0.65)	NS
RAN/AB:						
Antrum	3.17 (0.55)	0.96 (0.35)	≤0.001	5.41 (0.61)	5.91 (0.68)	NS
Corpus	2.34 (0.30)	1.44 (0.37)	NS	5.23 (0.70)	5.72 (0.55)	NS

Activity is expressed in units/mg protein (SEM) (paired analysis).

TABLE II Active (neutrophils) and chronic (mononuclear cells) inflammation scores based on the updated Sydney system for the histological evaluation of gastric mucosal biopsy specimens of *H. pylori* positive patients before and after successful omeprazole based eradication treatment (OME/AB, n=33) or ranitidine based eradication therapy (RAN/AB, n=30)

	Active inflammation			Chronic inflammation		
	Before	After	<i>p</i> Value	Before	After	<i>p</i> Value
OME/AB:						
Antrum	1.2 (0.1)	0.1 (0.1)	<0.001	1.9 (0.1)	1.2 (0.1)	<0.001
Corpus	0.5 (0.1)	0.1 (0.1)	0.002	1.5 (0.1)	1.0 (0.1)	<0.001
RAN/AB:						
Antrum	1.0 (0.1)	0 (0)	<0.001	1.7 (0.1)	1.0 (0)	<0.001
Corpus	0.4 (0.1)	0 (0)	<0.001	1.2 (0.1)	1.0 (0)	<0.02

Scores are means (SEM).

Updated Sydney system¹⁹ score grading: normal=0; mild=1; moderate=2; and severe=3.

TABLE III Manganese superoxide dismutase (Mn-SOD) and copper/zinc superoxide dismutase (CuZn-SOD) concentrations in gastric mucosal biopsy specimens of *H. pylori* positive patients before and after unsuccessful treatment (no eradication of *H. pylori*)

	Mn-SOD (n=4)			CuZn-SOD (n=4)		
	Before	After	<i>p</i> Value	Before	After	<i>p</i> Value
Antrum	0.76 (0.05)	0.82 (0.08)	NS	0.61 (0.07)	0.72 (0.09)	NS
Corpus	0.58 (0.19)	0.69 (0.09)	NS	0.50 (0.16)	0.54 (0.11)	NS

Results expressed as µg/mg protein (SEM) (paired analysis).

TABLE IV Manganese superoxide dismutase (Mn-SOD) and copper/zinc superoxide dismutase (CuZn-SOD) concentrations in gastric mucosal biopsy specimens of consistently *H. pylori* positive patients obtained on two separate occasions with no intermediate *H. pylori* eradication therapy

	Mn-SOD (n=8)			CuZn-SOD (n=8)		
	First	Second	<i>p</i> Value	First	Second	<i>p</i> Value
Antrum	0.89 (0.06)	0.87 (0.10)	NS	0.67 (0.09)	0.61 (0.05)	NS
Corpus	0.65 (0.09)	0.80 (0.07)	NS	0.58 (0.06)	0.67 (0.06)	<0.01

Results expressed as µg/mg protein (SEM) (paired analysis).

Mean (range) interval between measurements 9.5 (2–39) months.

Manganese superoxide dismutase activity in both treatment groups was also significantly lower (*p*<0.01) after than before treatment in antral but not in corpus mucosa, whereas copper/zinc superoxide dismutase activity was not significantly altered by therapy, neither in antral nor in corpus mucosa (Table I).

Histological evaluation of the gastric mucosal biopsy specimens disclosed that the active inflammation was almost completely resolved after treatment in both treatment groups, whereas the chronic inflammation persisted although significantly reduced in both antrum and corpus (Table II).

In the four patients in whom *H. pylori* was not eradicated after therapy no major alterations in the gastric mucosal superoxide dismutase concentrations were found (Table III). Similarly, in the eight consistently *H. pylori* positive patients on two separate occasions, without intermediate eradication treatment, no significant changes in superoxide dismutase concentrations were found, except for copper/zinc superoxide dismutase in corpus mucosa (Table IV). Particularly, manganese superoxide dismutase concentrations remained high or showed a tendency to increase further in both groups.

Discussion

Oxygen radicals are molecules with one or two unpaired electrons in their outer orbital. Some examples are the superoxide anion (O_2^-) and the hydroxyl radical ($OH^\cdot$). The superoxide radical is the primary product of an activated NADPH oxidase system in the membrane of inflammatory cells, such as neutrophils and macrophages. The free radicals attack lipid components of cell membranes, proteins, and nucleic acids of microorganisms, but possibly also of the host.^{7-10 12 15 16 23} The most important enzymatic antioxidants are superoxide dismutase, catalase, and glutathione reductase.^{8 10 24} In humans, at least two forms of superoxide dismutase are known, a constitutive cytoplasmic form (copper/zinc superoxide dismutase), and an inducible mitochondrial form (manganese superoxide dismutase).²³ *H. pylori* has been shown to possess an iron containing superoxide dismutase,²⁵ which does not cross react with the antibodies against the human superoxide dismutases in our ELISAs.¹⁸

Previously, we have shown that in *H. pylori* associated gastritis an increase in concentration and activity of manganese superoxide dismutase exists, as well as a minor decrease in copper/zinc superoxide dismutase, which might be regarded as a response by the host to minimise gastric mucosal damage due to free radicals produced in response to *H. pylori* infection.¹⁸ In the present study we assessed the effect of eradication of *H. pylori* on the gastric concentrations and activities of mucosal superoxide dismutase in biopsy specimens from the gastric antrum and corpus, in patients who were treated with either an omeprazole based (OME/AB) or a ranitidine based (RAN/AB) *H. pylori* eradication treatment. Although

there were pretreatment and post-treatment differences in the gastric mucosal superoxide dismutase concentrations between the two treatment groups, which might be related to differences in the age and sex distribution, we found with both therapies that the increased concentration and activity of manganese superoxide dismutase was reversed by successful treatment of the *H pylori* infection. These concentrations of gastric mucosal manganese superoxide dismutase became comparable with those of patients without an *H pylori* infection and with a histologically normal gastric mucosa, as described previously.¹⁸ The finding of a persistently high manganese superoxide dismutase concentration in the gastric mucosa of patients when *H pylori* eradication either failed or was not attempted, indicates that normalisation of this variable is directly related to the elimination of the organism. Conceivably, this is a result of the disappearance of the antigenic stimulus, such as *H pylori* derived neutrophil activating and cytotoxic factors, thus leading to a disappearance of active inflammation and subsequently to decreasing concentrations of tumour necrosis factor- α and interleukins,²⁶ particularly as these compounds have been shown to increase manganese superoxide dismutase expression.²⁷ The manganese superoxide dismutase activity in the antrum was lower after than before therapy in both treatment groups, but did not change in corpus mucosa. We ascribe this finding to the fact that *H pylori* associated gastritis is antrum predominant, with less active inflammation in the corpus.

Furthermore, no major changes occurred in the copper/zinc superoxide dismutase antigen concentration and activity, neither in antral nor in corpus mucosa, after the eradication of *H pylori*. This finding is not surprising as the constitutive copper/zinc superoxide dismutase is not inducible by cytokines, and no impressive alterations in this enzyme were previously found in *H pylori* associated gastritis.¹⁸ Moreover, although eradication of *H pylori* was achieved, complete histological normalisation during this relatively short follow up was not obtained, as illustrated by the decreased but not fully resolved chronic inflammation. Perhaps only then the copper/zinc superoxide dismutase concentrations in the gastric mucosa might return to the previously described higher values in patients without an *H pylori* infection.¹⁸

H pylori has been recognised as a causal agent in gastric carcinogenesis, and damage to DNA by free radicals in chronic active gastritis might be an important link in this connection.¹⁰⁻²³ Furthermore, it has been established that concentrations of manganese superoxide dismutase are raised in several malignant diseases of the digestive tract, in both serum and tissue homogenates.²⁸⁻²⁹ These raised concentrations in colorectal carcinoma were even found to be related to the clinical outcome of the patients.²⁹ We showed in the present study that the upregulation of the free radical scavenging system related to man-

ganese superoxide dismutase is reversed by successful treatment of *H pylori*, possibly reflecting a diminishing free radical contribution in the pathological process. Similarly, we have previously shown a (pre)malignant profile in gastric mucosal plasminogen activators in *H pylori* infection that approached normal values after successful treatment of the infection.³⁰⁻³¹ Others have shown that the increased gastric epithelial cell proliferation in *H pylori* associated gastritis is reduced after successful treatment.³²⁻³³ Furthermore, in accord with our findings *H pylori* eradication restores decreased vitamin C concentrations in gastric juice.³⁴⁻³⁵ Whether all these effects will ultimately result in a decreased risk for gastric cancer, however, remains to be established.

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