HOST RESPONSE TO INFECTION

Susceptibility of *Helicobacter pylori* and its urease activity to the peroxidase-hydrogen peroxide-thiocyanate antimicrobial system

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The susceptibility of *Helicobacter pylori* to the antimicrobial system involving lactoperoxidase, hydrogen peroxide and thiocyanate was investigated. The inhibitory effect of the system on the urease activity of *H. pylori*, which plays a role in its colonisation of the stomach, was also investigated. Twelve *H. pylori* strains examined, including 10 clinical isolates, were all inhibited by the peroxidase system in brain-heart infusion broth supplemented with fetal calf serum, but to different extents. The killing effect was observed within 3 h. Although bacterial viability recovered afterwards, there was still a clear difference between cultures incubated in the presence of the complete system and control cultures incubated in the absence of lactoperoxidase, after incubation for 24 h. The urease activity and viability of *H. pylori* were both inactivated by this system in phosphate buffer. These effects were dependent on the concentrations of both lactoperoxidase and hydrogen peroxide and were abolished by the addition of cysteine. Furthermore, these effects were observed when bovine lactoperoxidase was replaced by recombinant human lactoperoxidase or native or recombinant human myeloperoxidase. The peroxidase system found in saliva and milk may contribute to the host defence against *H. pylori* infection and inhibition of transmission *via* the oral route.

Introduction

Helicobacter pylori, a spiral-shaped gram-negative bacterium with flagella [1], is the cause of human gastritis, peptic gastric ulcers and duodenal ulcers [2]. Recent epidemiological and animal studies have suggested an association between *H. pylori* and gastric cancer, including adenocarcinoma and gastric lymphoma [2]. The urease produced by *H. pylori* is known to be an essential factor for its colonisation of the stomach [1]. Besides neutralising gastric acid, some other roles of this enzyme in *H. pylori* colonisation and pathogenesis have been suggested. The hydrolysis of urea by cytoplasmic urease results in energy generation in the form of a proton-motive force which drives flagellar rotation, allowing bacterial motility in the mucus gel layer of the stomach [1, 3]. The formation of monochloramine from ammonia produced by *H. pylori* urease and neutrophil-derived hypochlorous acid have

been suggested to play an important role in gastric mucosal injury [4, 5]. Although the precise route of transmission and the natural reservoir of *H. pylori* remain unknown, recent clinical studies support the existence of gastro–oral and oral–oral pathways of transmission [6]. *H. pylori* has been isolated successfully from the oral cavity by culture [6]. In a study employing the PCR method in which 42 patients were examined, *H. pylori* DNA was identified in dental plaque of 97% of the patients and in the saliva of 55% of the patients, showing a distribution relatively independent of the infection status of the stomach [7].

Human saliva, which continuously bathes the oral mucosa and teeth, contains various antimicrobial factors such as lysozyme, lactoferrin, peroxidases and secretory IgA [8]. Two different haem-containing peroxidases, salivary peroxidase secreted from the salivary gland and myeloperoxidase (MPO) derived from neutrophil polymorphonuclear leucocytes (neutrophils), have been identified in whole saliva [9, 10]. Recent genetic studies have revealed that salivary peroxidase is another product of the gene encoding lactoperoxidase (LPO) [11–14], a secretory peroxidase

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found in breast milk [15–17]. LPO is a monomeric glycoprotein that varies in molecular mass from *c.* 80 to 100 kDa [9, 13, 15–17]. MPO is a tetrameric glycoprotein consisting of two 59-kDa heavy chains and two 13.5-kDa light chains in its mature form and found in the azurophilic granules of neutrophils [18]. These enzymes catalyse the hydrogen peroxide (H_2O_2)-dependent oxidation of thiocyanate (SCN^-) to yield the hypothiocyanite anion (OSCN^-) with inhibitory activity against a wide variety of micro-organisms including gram-positive and gram-negative bacteria, fungi and viruses [19–23]. The product, OSCN^- , reacts with microbial sulph-hydryls [24] and inhibits various cellular processes such as glycolysis [25, 26], membrane transport of sugars and amino acids [27, 28] and respiration [29, 30]. It has been estimated that human saliva samples under resting and stimulated conditions contain OSCN^- at average concentrations of 61 and 34 μM , respectively [31]. Therefore, it is of interest to study the effects of the peroxidase antimicrobial system, a natural component of secretions such as saliva and milk, against *H. pylori*. This study reports for the first time the influence of the peroxidase- H_2O_2 - SCN^- antimicrobial system on the viability and urease activity of *H. pylori*.

Materials and methods

Bacteria and growth conditions

H. pylori ATCC 43504 and 43629 were obtained from the American Type Culture Collection (Rockville, MD, USA). Ten different human clinical isolates of *H. pylori* isolated from gastric tissue of *H. pylori*-positive patients treated at Mie University Hospital (Mie, Japan), of which four isolates had been used in a previous study [32], were used. The bacteria were grown either on Trypticase Soy Agar (Becton Dickinson) supplemented with defibrinated horse blood (Nihon Biosupp. Center, Tokyo, Japan) 5% (TSB) or Brain-Heart Infusion Broth (Difco) supplemented with fetal calf serum (Nihon Biosupp. Center) 5% (BHI-serum). The cultures were incubated at 37°C under micro-aerobic conditions (CO_2 10%).

Peroxidases

LPO purified from bovine milk ($A_{412}/A_{280} = 0.90$) was purchased from Sigma. Native human MPO purified from leucocytes ($A_{430}/A_{280} = 0.73$) was obtained from Athens Research and Technology (Athens, GA, USA). Recombinant human LPO and MPO were expressed in a baculovirus-insect cell system and purified according to the procedure described by Shin *et al.* [13].

Peroxidase system

The LPO system composed of bovine LPO 8 $\mu\text{g}/\text{ml}$, 0.6 mM H_2O_2 and 0.66 mM SCN^- was tested to assess its influence on the growth of *H. pylori* in 12-well

plates containing BHI-serum broth, with a total volume of 2 ml/well. The wells were inoculated with *H. pylori* cells, precultured in BHI-serum broth, at a cell density of *c.* 10^6 or 10^7 cfu/ml. The LPO system was initiated by the addition of H_2O_2 to the wells containing all other components pre-warmed to 37°C. The plates were incubated under micro-aerobic conditions at 37°C and the number of viable bacterial cells was determined at indicated times by plating on TSB agar after appropriate dilution in phosphate-buffered saline (PBS; 138 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer, pH 7.4). The colonies formed on the plates were counted after incubation under micro-aerobic conditions at 37°C for 4 days. The detection limit in determination of the number of *H. pylori* cells was $\log_{10}$ 1.70 cfu/ml.

Urease activity

The LPO system with the same composition was also tested to assess its influence on the urease activity of *H. pylori* in 50 mM sodium phosphate buffer, pH 6.6. *H. pylori* cells were treated at a cell density of *c.* 10^7 cfu/ml under micro-aerobic conditions at 37°C for 1 h. The reaction of the LPO system was terminated by mixing with an equal volume of 10 mM sodium azide. Sodium azide added at this concentration did not show any inhibitory effect on the urease activity. As a reference, urease purified from Jack bean (Wako Pure Chemical, Osaka, Japan) was also treated with the system at a concentration of 1 mg/ml. After the treatment, the urease activity in the bacterial suspension or the enzyme solution was determined without any further treatment. Measurement of urease activity was based on the phenol-hypochlorite reaction as described by Takebe and Kobayashi [33]. The activity of urease is expressed as international units (IU), where one unit is defined as the amount of enzyme hydrolysing 1 μmol of urea/min at 37°C. The influence of the concentrations of LPO and H_2O_2 , the influence of cysteine added as L-cysteine hydrochloride monohydrate (Kanto Chemical, Tokyo, Japan) and the effect of replacement of bovine LPO with recombinant human LPO, or native or recombinant human MPO were also examined. The number of viable bacterial cells in the reaction mixture of the peroxidase system was also determined without addition of sodium azide by serially diluting the mixture in PBS and plating the diluted samples on TSB agar as described above.

Results

Effects of the peroxidase system on the growth of H. pylori

The 12 *H. pylori* strains examined were all inhibited by the LPO system in BHI-serum broth, but to different extents (Table 1). Among them, clinical isolate no. 32 was most susceptible to the LPO system, as the number of viable bacterial cells was reduced by more than

Table 1. Effect of the LPO system on the number of *H. pylori* in BHI-serum broth

Strain no.	Mean (SD) bacterial number (log ₁₀ cfu/ml)*	
	Control	LPO system
ATCC 43504	7.73 (0.02)	6.23 (0.33)
ATCC 43629	8.34 (0.06)	7.79 (0.12)
Clinical isolates		
2	7.90 (0.06)	4.86 (0.46)
3	8.08 (0.07)	7.17 (0.15)
19	7.97 (0.04)	5.83 (0.25)
32	7.98 (0.08)	3.18 (0.39)
33	8.11 (0.03)	5.80 (0.16)
38	7.98 (0.07)	5.81 (0.60)
A	7.85 (0.22)	6.10 (0.14)
B	8.10 (0.04)	6.54 (0.26)
C	7.17 (0.08)	6.16 (0.12)
D	7.49 (0.03)	4.80 (0.78)

BHI-serum broth was inoculated with *H. pylori* cells at *c.* 10⁷ cfu/ml in the case of each strain examined and the cultures were incubated with the LPO system composed of bovine LPO 8 mg/L, 0.6 mM H₂O₂ and 0.66 mM SCN⁻. After incubation at 37°C for 24 h under micro-aerobic conditions, the number of viable bacteria was compared with that in control cultures incubated under the same conditions in the absence of LPO.

*Results represent the mean (SD) of three experiments.

log₁₀ 4 compared with control cells incubated in the absence of LPO. Strain ATCC 43629 was the most resistant, as the number of viable cells was reduced by log₁₀ <1. Most *H. pylori* strains other than ATCC 43629 and clinical isolate no. 3 showed lower viability after exposure to the LPO system for 24 h than that at the time of inoculation, indicating that the LPO system is bactericidal against *H. pylori*.

The type strain ATCC 43504 and clinical isolate no. 19 were used to study the mode of action of the LPO system. The killing effect of the LPO system was observed against both strains within 3 h and the viability recovered afterwards (Fig. 1). However, the number of viable bacteria in the cultures incubated with the complete system was clearly different from that in control cultures incubated in the presence of SCN⁻ only or in the presence of LPO and SCN⁻ only for up to 24 h in the case of strain ATCC 43504 and for up to 48 h in the case of clinical isolate no. 19. The influence of bacterial inoculum size was investigated with two different inocula, with the same concentration (0.6 mM) of H₂O₂ in each instance. The viability of

clinical isolate no. 19 was significantly reduced in the presence of SCN⁻ and H₂O₂ when an inoculum of 10⁶ cfu/ml was used (Fig. 1d). However, no such decrease in the viability of this strain in the presence of SCN⁻ and H₂O₂ was observed when an inoculum of 10⁷ cfu/ml was used (Fig. 1c). No inhibition of strain ATCC 43504 was observed at the same concentration of SCN⁻ and H₂O₂ with an inoculum of 10⁶ or 10⁷ cfu/ml (Fig. 1a and b).

Effects of the peroxidase system on the urease activity and viability of H. pylori in phosphate buffer

As ammonia was detected in BHI-serum broth at a level that significantly interfered with the urease assay, phosphate buffer was used to assess the effect of the LPO system on the urease activity of *H. pylori*. The urease of *H. pylori* and the urease purified from Jack bean were both almost completely inactivated by the LPO system (Table 2). The inactivation of urease was partially abolished by the addition of cysteine hydrochloride, suggesting that such inactivation is due to the oxidation of functionally important sulph-hydryls of the enzyme. The inactivation of urease by this system was found to be dependent on the concentration of LPO and complete inactivation was achieved at an LPO concentration of 1 mg/L (Fig. 2a). The inactivation of urease was also found to be dependent on the concentration of H₂O₂ and no activity of the *H. pylori* urease or the Jack bean enzyme remained when treated at an H₂O₂ concentration of 0.3 and 0.01 mM, respectively (Fig. 2b).

H. pylori lost viability in phosphate buffer containing the complete LPO system; the number of viable cells decreased to a level below the detection limit (Table 2). Cysteine hydrochloride protected *H. pylori* from the bactericidal effect of the LPO system, suggesting that the oxidation of bacterial sulph-hydryls by OSCN⁻ may occur. The killing effect of this system was found to be dependent on the concentration of LPO and the number of viable cells decreased to a level below the detection limit at an LPO concentration of 1 mg/L (Fig. 2c). The killing effect was also found to be dependent on the concentration of H₂O₂ and no viable

Table 2. Effects of the LPO system on the urease activity and viability of *H. pylori* in phosphate buffer

Condition	Jack bean	<i>H. pylori</i> ATCC 43504		<i>H. pylori</i> no. 19	
	Urease (mIU/μg protein)*	Urease (mIU/10 ⁷ cfu of inoculum)*	Mean (SD) bacterial number (log ₁₀ cfu/ml)†	Urease (mIU/10 ⁷ cfu of inoculum)*	Mean (SD) bacterial number (log ₁₀ cfu/ml)†
Control	95.2	5.45	7.06 (0.05)	2.71	7.21 (0.06)
LPO system	0.00	0.03	<1.70 ...	0.01	<1.70 ...
LPO system with cysteine	100	4.33	6.97 (0.07)	1.42	7.09 (0.17)

Jack bean urease at 1 mg/ml or cells of *H. pylori* ATCC 43504 or clinical isolate no. 19 at *c.* 10⁷ cfu/ml were treated with the LPO system with or without 10 mM cysteine in 50 mM phosphate buffer, pH 6.6, at 37°C for 1 h under micro-aerobic conditions; controls did not contain H₂O₂. Urease activity and the number of viable bacteria in the mixture were assayed as described in the text.

*Results represent the mean of three experiments.

†Results represent the mean (SD) of three experiments.

Fig. 1. Antibacterial effect of the LPO system against *H. pylori* in BHI-serum broth. *H. pylori* ATCC 43504 (**a, b**) and clinical isolate no. 19 (**c, d**) in BHI-serum broth at initial cell density of $c. 10^7$ cfu/ml (**a, c**) or 10^6 cfu/ml (**b, d**) were incubated with the LPO system composed of bovine LPO 8 mg/L, 0.6 mM H₂O₂ and 0.66 mM SCN⁻ at 37°C under micro-aerobic conditions. The number of viable bacteria was determined at the indicated times. Experiments were carried out with the complete LPO system (□) and with the following components: SCN⁻ (○), LPO and SCN⁻ (▲), SCN⁻ and H₂O₂ (△). Results represent the mean of three experiments (bar, SD).

bacteria were found when treated at an H₂O₂ concentration of 0.6 mM (Fig. 2d).

Effects of peroxidases from different sources

Inhibitory effects of the peroxidase system on the urease activity and viability of *H. pylori* were observed in phosphate buffer when bovine LPO was replaced by recombinant human LPO, or native or recombinant human MPO (Table 3).

Discussion

The results of the present study show that the system composed of peroxidase, H₂O₂ and SCN⁻, which is a natural component of secretions such as saliva and milk, exerts antimicrobial effects against *H. pylori*. The

short-lived bactericidal effect of the system on *H. pylori* is in agreement with previous reports of studies with other species of bacteria, in which H₂O₂ was introduced only at the initiation of the reaction [34, 35]. SCN⁻ was used at a concentration of 0.66 mM throughout this study. This concentration of SCN⁻ is within the range reported in human saliva samples (1.21 ± 0.66 mM for resting condition and 0.84 ± 0.48 mM for stimulated condition) [31]. In a previous study, it was observed that the LPO system with a composition similar to that used in this study (0.66 mM SCN⁻, 0.6 mM H₂O₂ and bovine LPO 8 mg/L in 50 mM phosphate buffer, pH 6.6) yielded OSCN⁻ at concentrations of 171 and 48 μ M at 5 and 60 min after the start of the reaction, respectively [30]. These concentrations of OSCN⁻ are also within the range of that reported in human parotid saliva samples collected directly from Stensen's duct (range 10–210 μ M, av-

Fig. 2. Influence of the concentrations of LPO and H₂O₂ on inactivation of urease and their bactericidal effect against *H. pylori*. Jack bean urease at 1 mg/ml (○) or cells of *H. pylori* ATCC 43504 (△) or clinical isolate no. 19 (▲) at $c. 10^7$ cfu/ml were treated with the LPO system composed of various concentrations of bovine LPO, 0.6 mM H₂O₂ and 0.66 mM SCN⁻ in 50 mM phosphate buffer, pH 6.6, at 37°C for 1 h under micro-aerobic conditions (a). In other experiments, bovine LPO was added at 8 mg/L and H₂O₂ was added at various concentrations (b). Urease activity was assayed as described in the text. Bacterial viability was examined under the same conditions as indicated for panels a and b and the results are shown in panels c and d, respectively. Results represent the mean of two experiments.

Table 3. Effects of LPO and MPO from different sources on the urease activity and viability of *H. pylori* in phosphate buffer

Peroxidase	<i>H. pylori</i> ATCC 43504		<i>H. pylori</i> no. 19	
	Urease (mIU/10 ⁷ cfu of inoculum)*	Mean (SD) bacterial number (log ₁₀ cfu/ml) [†]	Urease (mIU/10 ⁷ cfu of inoculum)*	Mean (SD) bacterial number (log ₁₀ cfu/ml) [†]
Bovine LPO	0.01	<1.70 ...	0.02	<1.70 ...
Recombinant human LPO	0.00	<1.70 ...	0.01	<1.70 ...
Human MPO	0.00	<1.70 ...	0.01	<1.70 ...
Recombinant human MPO	0.00	2.22 (0.20)	0.00	<1.70 ...
Control (without peroxidase)	4.09	7.06 (0.05)	1.88	7.33 (0.04)

Experiments were done under the same conditions as described in Table 2 except that bovine LPO was replaced with recombinant human LPO, or native or recombinant human MPO at the same concentration; controls did not contain peroxidase.

*Results represent the mean of three experiments.

[†]Results represent the mean (SD) of three experiments.

erage 52 μM) [31]. Therefore, it seems reasonable to extrapolate the results obtained in this study to the conditions *in vivo*.

It is well known that H_2O_2 in itself has an inhibitory effect on various micro-organisms including *H. pylori* [36, 37]. Indeed, the viability of clinical isolate no. 19 was significantly reduced in the presence of 0.66 mM SCN^- and 0.6 mM H_2O_2 when the strain was inoculated at a lower density (10^6 cfu/ml). As *H. pylori* is catalase positive [38] and not susceptible to SCN^- at the concentration used in this study, a larger amount of endogenous catalase from the higher density of cells (10^7 cfu/ml) may be needed to diminish the toxicity of H_2O_2 in the case of a strain with high H_2O_2 sensitivity such as clinical isolate no. 19. However, most of the results in this study showed that the antimicrobial activity of the product of the reactions catalysed by peroxidases was greater than that of SCN^- and H_2O_2 .

The results of the present study show that the peroxidase system inactivates ureases from *H. pylori* and Jack bean. It has been shown that the chemical modification of a cysteine residue near the active site of the urease from Jack bean, or that of *Klebsiella aerogenes*, can eliminate enzyme activity [39, 40]. A high degree of similarity in the primary structure has been found by comparing the ureases from various bacteria, including *H. pylori* and *K. aerogenes*, and the Jack bean urease [41]. Therefore, it seems reasonable to assume that OSCN $^-$, a reactive intermediate produced by the peroxidase system, inactivates the urease of *H. pylori* and that of Jack bean by reacting with an important cysteine residue near the active site. The influence of the LPO system on urease activity of strains ATCC 43629 and clinical isolate no. 3, which were the most resistant to the system in BHI-serum broth, was also examined. The sensitivity of ureases of these strains was quite similar to that observed for ATCC 43504 and clinical isolate no. 19 (details not shown). Therefore, urease inactivation by the LPO system is not likely to correlate directly to the bactericidal effect of the system. Although the urease of *H. pylori* is principally involved in neutralising gastric acid, recent studies have suggested some other roles of this enzyme in *H. pylori* colonisation and pathogenesis, such as a role in flagellar rotation and a role in triggering gastric mucosal injury through the inflammatory response [1, 3–5]. The peroxidase system in secretions may reduce the degree of pathogenesis of *H. pylori* by inhibiting the urease.

Recent genetic studies have demonstrated that salivary peroxidase is expressed from the chromosomal gene encoding LPO [11–14]. The peroxidase system may exert its antimicrobial effect on *H. pylori* in the oral cavity, as whole human saliva contains large amounts of LPO and MPO at average concentrations of 1.9 and 3.6 mg/L [10], respectively. The concentration of peroxidases, 8 mg/L, used in most of the experiments

of this study is slightly above the sum of LPO and MPO concentrations reported in saliva [10] and it is within the range of total peroxidase estimated in saliva with bovine LPO as a standard [42]. The influence of the LPO concentration was tested and it was found that LPO in amounts as small as 1 mg/L completely abolished both urease activity and the viability of *H. pylori* in phosphate buffer. Furthermore, saliva contains OSCN $^-$, the antimicrobial intermediate produced by the system, at an average concentration ranging from 34 to 61 μM [8, 31]. Although the formation of hypochlorous acid by neutrophil-derived MPO has been suggested to serve as a pathogenic factor of *H. pylori* causing gastric mucosal injury through an inflammatory response [4, 5], the system composed of MPO, H_2O_2 and SCN^- kills the bacteria and can at least contribute to host protection against *H. pylori* in the early stage of infection. The peroxidase system derived from saliva or milk may be active in the oesophagus and the stomach, as it has been shown that peroxidase activity is not rapidly inactivated in gastric juice [43]. Further clinical studies will be needed to determine the influence of the peroxidase system on *H. pylori* colonisation.

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