Oxygen scavenging, NADH oxidase and metronidazole resistance in *Helicobacter pylori*

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Failure of triple-therapy regimes to eradicate *Helicobacter pylori* from the stomach is thought to be due to the occurrence of a metronidazole-resistant bacterial population. Exposure of metronidazole-resistant (Mtz\(^R\)) strains of *H. pylori* to an anaerobic environment causes the activation of metronidazole and the loss of resistance. Using metronidazole-sensitive (Mtz\(^S\)) clinical isolates, we selected mutants conferring resistance to metronidazole, which were used to investigate the effect of bacterial cell density upon the activation of metronidazole. The addition of metronidazole, at a final concentration of 10 mg/L, to Mtz\(^R\) cultures of a bacterial cell density >1 x 10\(^6\) cfu/mL, caused a loss in viability. No loss in viability, however, occurred upon addition of metronidazole to Mtz\(^S\) cultures of a cell density of <1 x 10\(^6\) cfu/mL. Mtz\(^S\) cultures lost viability irrespective of the initial cell density, indicating that oxygen scavenging at the site of metronidazole reduction may occur in these cultures. The ability of Mtz\(^S\) wild types, Mtz\(^R\) isogenic mutants and Mtz\(^R\) wild types to scavenge oxygen from the intracellular environment was investigated. *H. pylori* cultures contained NADH and NADPH oxidase activity. NADPH oxidase activity was always more than double the NADH oxidase activity. Mtz\(^R\) mutants possessed approximately one-third the NADH oxidase activity found in their respective Mtz\(^S\) parent wild types. Mtz\(^R\) wild types possessed a low NADH oxidase level similar to that found in the Mtz\(^R\) mutants. We propose that metronidazole resistance may be mediated through an inability of Mtz\(^R\) strains to remove oxygen from the site of metronidazole reduction, thereby preventing metronidazole activation.

Introduction

The occurrence of *Helicobacter pylori* as an aetiological factor in the development of gastroduodenal ulcers\(^1,2\) and gastric cancer\(^3\) is now well established. Approximately half of the world’s population is thought to be infected with *H. pylori*.\(^1\) Current effective triple therapy for the eradication of *H. pylori* includes metronidazole. Failure of treatment regimes involving metronidazole has been attributed to the possession of pre-treatment metronidazole-resistant strains\(^4-6\) or to the development of metronidazole-resistant phenotypes during treatment.\(^4,5,7\) Previous use of metronidazole and the occurrence of metronidazole-resistant *H. pylori* in the community appear to be related. The widespread use of metronidazole in the treatment of anaerobic infections, such as amoebiasis and giardiasis, in hot developing countries is thought to account for the high prevalence of metronidazole-resistant *H. pylori* in these countries.\(^5\) In a similar way, the slightly higher prevalence of metronidazole-resistant strains in women than in men in developed countries may be attributed to the use of metronidazole in the treatment of gynaecological infections.\(^5,8\)

With metronidazole resistance on the increase in the *H. pylori* population, the effectiveness of current triple-therapy regimes containing metronidazole may be compromised. A number of advances to understanding this phenomenon have been achieved in recent years. Resistance to metronidazole is lost on exposure to an anaerobic environment, the degree of sensitivity presented being dependent upon the time of exposure.\(^9,10\) We have shown that the increase in sensitivity upon exposure to anaerobic conditions is due to an increase in the rate of uptake and reduction of the drug.\(^11\) In addition, we have found metronidazole-resistant *H. pylori* to be resistant to other 5-nitroimidazole drugs and that, like with metronidazole, this resistance is lost under anaerobiosis.

In order to understand the mechanism of metronidazole resistance, we have developed an in vitro system that allows exposure of metronidazole-resistant *H. pylori* strains to an anaerobic environment. In this system, the activation of metronidazole is observed as a decrease in viability of the strain. This activation is due to the reduction of metronidazole by the intracellular enzyme, NADH oxidase. We have also shown that the activation of metronidazole is dependent upon the initial cell density of the culture. This is thought to be due to the presence of oxygen scavenging activity in the cells. The ability of metronidazole-resistant *H. pylori* to scavenge oxygen from the intracellular environment was investigated.

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resistance, we investigated the relationship between bacterial cell density and the ability of H. pylori cultures to activate metronidazole. The effective killing of H. pylori cultures that were resistant to metronidazole decreased as the bacterial cell density decreased, to the point where killing no longer occurred. The intracellular NADH oxidase oxygen-scavenging ability in resistant cultures was approximately one-third the activity present in sensitive cultures. Metronidazole resistance in H. pylori may be related to an inability to scavenge oxygen from the site of metronidazole reduction.

Materials and methods

Bacterial strains

H. pylori reference strains NCTC 11637 and NCTC 11638 were obtained from the National Collection of Type Cultures, CPHL, London. Clinical isolates were obtained from the Harold Wood Hospital, Essex; The Royal London Hospital, London; and Brugmann University Hospital, Belgium. The identity of each isolate was confirmed by Gram's stain and a positive reaction in the urease, catalase and oxidase biochemical tests. Strains were classified as resistant (MIC > 32 mg/L) or sensitive (MIC < 2 mg/L) to metronidazole by agar dilution under microaerophilic conditions in an atmosphere of 5% O2, 5% CO2 and 90% N2, at 37°C in 98% humidity (Variable Atmosphere Incubator, Don Whitley Scientific Ltd, Shipley, UK). Strains were stored in liquid nitrogen in tryptone soya broth (TSB) containing 10% (v/v) horse serum and 15% (v/v) glycerol.

Cultures

Frozen cultures were inoculated onto Columbia agar containing 5% oxalated horse blood and DENT H. pylori selective supplement (Oxoid, Unipath, Basingstoke, UK) and incubated for 3 days under microaerophilic conditions. Colonies were suspended in 7 mL of TSB containing 5% horse serum (culture broth) and left static for 3 days under microaerophilic conditions; this was used to inoculate larger fresh culture broth. Cultures were left static under microaerophilic conditions for 4 days before being shaken overnight at 60 rpm.

Selection of mutants conferring resistance to metronidazole

Helicobacter pylori cultures left static under microaerophilic conditions for 3 days were diluted 100-fold in TSB and 100 μL was spread onto TSA plates containing 7% horse blood and a gradient of metronidazole. The gradient plates were incubated under microaerophilic conditions for 3 days. Colonies formed on the highest concentration were resuspended in culture broth. The MIC after each successive passage was determined by the agar dilution method on TSA plates containing 7% horse blood.

Restriction fragment length polymorphism analysis

Five-day-old H. pylori growth was harvested from blood agar plates and resuspended in TE buffer (10 mM Tris, 1 mM EDTA). DNA was extracted using a modified CTAB (hexadeoxytrimethylammonium bromide) method of Doyle & Doyle12; by first incubating the cells for 1 h at 40°C with 0.5 mg/mL Proteinase K (Sigma, Poole, UK) and 10% SDS. Five micrograms of DNA was digested overnight with 10 units of HindIII restriction endonuclease according to the manufacturer's instructions (Promega, Southampton, UK), loaded into a 0.8% agarose gel in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA) and electrophoresed at 7 V/cm.

Effect of bacterial cell density on metronidazole activity

Serial 10-fold dilutions of broth cultures of H. pylori at a bacterial cell density of 10⁷ cfu/mL were prepared in 20 mL TSB in 100 mL conical flasks. The TSB was equilibrated with a microaerophilic atmosphere overnight before use. Metronidazole was added to a final concentration of 10 mg/L and the cultures were left static under microaerophilic conditions. Colony counts were performed by diluting the culture in 10-fold steps in TSB and dropping 20 μL on to TSA plates containing 7% horse blood. The plates were incubated under microaerophilic conditions for 3 days.

Crude bacterial extract

Bacterial cells were harvested by centrifuging 75 mL of broth cultures at 4000g for 30 min, discarding the supernatant and washing the pellet once with 50 mM potassium phosphate buffer pH 7.8. The pellet was reconstituted in 5 mL of buffer and sonicated at 20,000 cps for 2 min on ice. Membrane fractions and whole cells were removed by centrifugation at 50,000g for 40 min and the supernatant was assayed for enzyme activity before being stored at –20°C.

NAD(P)H oxidase assay

NAD(P)H oxidase activity in crude bacterial supernatant was assayed in phosphate buffer containing 0.1 mM β-NAD(P)H and 0.2–0.5 mg of protein by measuring the decrease in absorbance of NAD(P)H at 340 nm.13 For the determination of NAD(P)H oxidase activity under anaerobic conditions, oxygen-free nitrogen gas was bubbled through the assay mixture in Thunberg cuvettes for 20 min, the cuvettes were sealed and the absorbance decrease was recorded. Enzyme activity was calculated using the molar extinction coefficient of NAD(P)H at 340 nm of 6.22 x 10³/mol/cm. The concentration of soluble protein was determined by the method of Lowry et al.14, with bovine serum as the standard.

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**Results**

Selection of mutants conferring resistance to metronidazole

Mutants conferring resistance to metronidazole were selected from strains of *H. pylori* metronidazole-sensitive (*Mtz*<sup>S</sup>) wild types. The MIC of the mutants did not change even after subculturing on antibiotic-free media at least three times, thus these mutants were stable for metronidazole resistance. Genomic analysis of *H. pylori* has shown considerable genomic diversity in this species. Each wild type strain of *H. pylori* possessed a unique DNA restriction pattern when digested with *Hin*dIII and each metronidazole-resistant (*Mtz*<sup>R</sup>) mutant possessed the same restriction pattern as its parent strain (Figure 1), confirming that the mutants were derived from the wild types.

Effect of bacterial cell density on metronidazole activity

The effective activation of metronidazole by *H. pylori* cultures, at various bacterial cell densities, was assessed by diluting high density cultures in 10-fold steps and adding metronidazole to a final concentration of 10 mg/L. *Mtz*<sup>S</sup> cultures (NCTC 11638) displayed a continuous loss in viability at all cell densities (Figure 2a). *Mtz*<sup>R</sup> cultures of a high bacterial cell density (10<sup>6</sup>–10<sup>8</sup> cfu/mL) rapidly lost viability when exposed to metronidazole (Figure 2b). Cultures of an initial cell density of 5 × 10<sup>6</sup>–5 × 10<sup>7</sup> cfu/mL lost log 5.5 viability, 1 × 10<sup>8</sup> cultures log 4.5, 5 × 10<sup>7</sup> cultures log 2.0 and 5 × 10<sup>6</sup> cfu/mL cultures log 1.0. Therefore, the amount of cell kill in *Mtz*<sup>R</sup> cultures of a cell density of >10<sup>6</sup> cfu/mL was related to the initial density of the culture. No loss in viability occurred in *Mtz*<sup>S</sup> cultures of an initial cell density of <1 × 10<sup>6</sup> cfu/mL. The wild-type strain NCTC 11637, which was resistant to metronidazole, also displayed this apparent lack of loss in viability in cultures with a bacterial cell density of <1 × 10<sup>6</sup> cfu/mL.

Bacterial cultures of a high cell density possess a lower dissolved oxygen content and a lower redox potential compared with cultures of a lower cell density. Metronidazole has a very low reduction potential (E<sub>1/2</sub> = -486 mV) and would only be activated in conditions in which a low redox status is maintained. It can be reasoned that the loss of viability in *Mtz*<sup>R</sup> cultures of a high cell density is due to the creation of an environment of a suitably low redox status and low oxygen tension, which allows the reduction of metronidazole to occur. As the cultures lose viability so a decrease in the utilization of oxygen occurs and the dissolved oxygen content of the environment increases, creating an environment unfavourable for metronidazole reduction. In cultures diluted to a bacterial cell density of <10<sup>6</sup> cfu/mL the dissolved oxygen content of the environment is such that no significant drug reduction occurs, hence the retention of viability in these cultures. In high-density *Mtz*<sup>R</sup> cultures resistance is temporarily compromised due to the low oxygen content. The ability of low-density *Mtz*<sup>R</sup> cultures to activate metronidazole and lose viability may be due to an ability to maintain a low intracellular redox potential despite the extracellular oxygen concentration. This may be due to a system which is able to remove dissolved oxygen from the intracellular environment.

NAD(P)H oxidase activity in crude bacterial extracts

Some aerobic and anaerobic bacteria are known to possess soluble NAD(P)H oxidases which are able to remove oxygen from the environment. Crude bacterial supernatants of *H. pylori* were found to possess measurable NADH and NADPH oxidase activity. Under anaerobic conditions almost no loss of NADH and NADPH occurred, indicating that oxygen was required for the loss of NADH and NADPH from the assay system. *Mtz*<sup>S</sup> mutants contained approximately a third of the NADH oxidase activity found in the *Mtz*<sup>R</sup> wild types (Table). This
The difference was found to be significant ($\alpha = 0.01$). M tz$^5$ wild types (NCTC 11637 and T 5410) contained a low level of NADH oxidase activity similar to that displayed in the M tz$^R$ mutants. The level of NADPH oxidase activity was nearly always over twice as high as the level of NADH oxidase. Overall, NADPH was slightly higher in M tz$^5$ wild types than in their respective M tz$^R$ mutants, though statistically no significant difference between the levels in sensitive and resistant strains was apparent ($\alpha = 0.01$). In addition, M tz$^R$ mutants often contained the same level of NADPH oxidase as their M tz$^5$ wild-type parent strains and the M tz$^5$ wild types contained a NADPH oxidase level on par with that found in the M tz$^5$ wild types. This was not the case with the NADH oxidase activity which was always considerably greater in the M tz$^5$ wild types than in their respective M tz$^R$ mutants.

**Discussion**

Metronidazole is regarded as the drug of choice for anaerobes and as such would not be expected to be effective in the presence of oxygen. The fact that it is an essential component of current triple-therapy regimes suggests either that H. pylori exists in an anaerobic or hypoxic environment in gastric pits, or that the organism is capable of scavenging oxygen, creating an environment suitable for the reduction of the drug. This report shows that a relationship exists between the intracellular oxygen-scavenging ability of H. pylori and the sensitivity of the bacterium to metronidazole. M tz$^R$ strains of H. pylori possessed considerably lower soluble cytosolic NADH oxidase activity than M tz$^5$ strains. Soluble cytosolic NAD(P)H oxidase enzymes have been purified from anaerobic and aerobic bacteria. These enzymes are able to reduce oxygen directly to hydrogen peroxide or water. The function of these enzymes in vivo is not known though it is thought that they may be involved in scavenging oxygen from the intracellular environment or in the regeneration of NAD$^+$. Nitroimidazoles are activated by reduction of the nitro group attached to the imidazole ring. Metronidazole and other 5-nitroimidazole drugs possess low reduction potentials and are unable to be activated to a clinically significant extent by aerobic organisms. This makes the 5-nitroimidazoles selectively toxic towards anaerobic organisms which are able to attain the low intracellular redox environment required for drug activation. H. pylori and other microaerophilic bacteria contain elements of both aerobic and anaerobic metabolism and are sensitive to metronidazole and other 5-nitroheterocyclic drugs. In anaerobes metronidazole reduction is mediated via the electron carrier protein ferredoxin, which is a component of the oxygen-sensitive pyruvate:ferredoxin oxidoreductase (PFOR) complex. The PFOR is part of a unique pathway in which pyruvate is decarboxylated to acetate or acetyl-coenzyme A and carbon dioxide. A pyruvate: flavodoxin oxidoreductase (PFIOR) has recently been discovered in H. pylori and shown to cause the reduction of metronidazole.

A anaerobic resistance to metronidazole in the protozoan Trichomonas vaginalis and the bacterium Bacteroides fragilis has been reported to be due to a decrease in PFOR activity. In B. fragilis this is due to a decreased transcription of the ferrodoxin gene. If metronidazole resistance in H. pylori is due to a decrease in PFIO R activity, it is feasible that PFIO R activity may be restored upon exposure to anaerobic conditions, thereby reducing the sensitivity to drug when exposed to anaerobic conditions. Likewise, the killing of M tz$^5$ cultures that occurs at a high bacterial cell density could be accounted for by a conver-
sion in the metabolism to primarily anaerobic metabolic pathways in cultures as they grow and outstrip the supply of oxygen. In such a scenario, as the culture loses viability upon exposure to metronidazole so the oxygen tension of the environment may be increased and anaerobic metabolism involving PFOR activity is switched off; metronidazole would no longer be activated and no more killing would occur. All this is a reasonable explanation but does not explain the phenomenon that when a MtzR culture of high bacterial cell density is diluted over a thousand-fold no kill occurs. Logically, the diluted cultures would be expected to possess the same level of PFOR activity, relative to the number of bacteria cells present, as the original high density culture and therefore the same proportional kill of the population would be expected. This did not occur when the population was diluted below a cell density of 10^6 cfu/mL. It is probable that MtzS and MtzR strains do contain PFOR activity though it is hard to see how a mechanism in the metabolism to primarily anaerobic metabolic pathways in cultures as they grow and outstrip the supply of oxygen. In such a scenario, as the culture loses viability upon exposure to metronidazole so the oxygen tension of the environment may be increased and anaerobic metabolism involving PFOR activity is switched off; metronidazole would no longer be activated and no more killing would occur. All this is a reasonable explanation but does not explain the phenomenon that when a MtzR culture of high bacterial cell density is diluted over a thousand-fold no kill occurs. Logically, the diluted cultures would be expected to possess the same level of PFOR activity, relative to the number of bacteria cells present, as the original high density culture and therefore the same proportional kill of the population would be expected. This did not occur when the population was diluted below a cell density of 10^6 cfu/mL. It is probable that MtzS and MtzR strains do contain PFOR activity though it is hard to see how a mechanism based on the loss of PFOR could explain fully these results. In addition, Daucher & Krieg could find no relationship between the level of PFOR activity in microaerophilic Campylobacter species, to which H. pylori is closely related, and resistance to metronidazole.

Oxygen has a very high affinity for electrons (E_f = -150 mV) and would be able to outcompete 5-nitroimidazole drugs for abstraction of electrons from reduced flavodoxin or be able to abstract the electron from the nitro group of the reduced drug, thereby reforming the inactive parent compound (a futile cycling mechanism). In metronidazole-sensitive strains NADH oxidase activity may be able to maintain a sufficiently low oxygen concentration at the site of metronidazole activation to allow reduction to occur. In resistant strains the presence of a higher intracellular oxygen concentration may prevent the proper functioning of the PFOR complex or the oxygen may be reduced to superoxide by the flavoprotein component of the PFOR, thereby preventing electron flow to metronidazole (Figure 3). This mechanism displays characteristics of the aerobic type of metronidazole resistance found in T. vaginalis. Here resistance to metronidazole occurs as the oxygen concentration of the environment is increased and is thought to be due to the hydrogenosomal NAD(P)H oxidases having an apparent lower affinity for oxygen than those in sensitive strains. We speculate that NADH oxidase activity in H. pylori may be somehow closely linked to the PFOR complex so as to allow proper functioning of the complex in a low oxygen environment.

NADH oxidases purified from aerobic bacteria are also able to use NADPH as an electron donor and the enzyme or enzymes possessing the NADH oxidase activity in H. pylori may have an affinity for NADPH as a substrate also. Since statistically the level of NADPH oxidase activity was no different in sensitive and resistant strains, the NADPH oxidase activity in H. pylori may not play a role in maintaining an environment suitable for the activation of metronidazole.

NADH oxidase and metronidazole resistance

<table>
<thead>
<tr>
<th>Strain</th>
<th>MtzS NADH oxidase M ± S</th>
<th>MtzR NADH oxidase M ± S</th>
<th>MtzS NADPH oxidase M ± S</th>
<th>MtzR NADPH oxidase M ± S</th>
</tr>
</thead>
<tbody>
<tr>
<td>L M C H 37</td>
<td>8.2 ± 1.4</td>
<td>1.8 ± 0.8* , 3.0 ± 2.0</td>
<td>13.2 ± 0.9</td>
<td>7.6 ± 2.1*, 10.5 ± 3.7</td>
</tr>
<tr>
<td>N C T C 11638</td>
<td>8.1 ± 1.6</td>
<td>2.5 ± 0.8</td>
<td>18.3 ± 4.2</td>
<td>12.8 ± 2.2</td>
</tr>
<tr>
<td>T 5431</td>
<td>7.4 ± 2.7</td>
<td>3.2 ± 2.4</td>
<td>10.2 ± 1.7</td>
<td>12.5 ± 4.7</td>
</tr>
<tr>
<td>H W 13</td>
<td>6.5 ± 1.1</td>
<td>3.2 ± 2.2</td>
<td>14.5 ± 2.5</td>
<td>8.5 ± 4.3</td>
</tr>
<tr>
<td>N C T C 11637</td>
<td>2.6 ± 1.5</td>
<td>1.7 ± 0.6</td>
<td>14.0 ± 1.9</td>
<td>11.2 ± 4.7</td>
</tr>
</tbody>
</table>

*MtzR isogenic mutant had an MIC of 16 mg/L.

Table. Soluble cytosolic NAD(P)H oxidase specific activity (nmol/min/mg soluble protein) in MtzS wild type, MtzR isogenic mutants and MtzR wild-type strains of H. pylori. NCTC 11637 and T 5410 are MtzR wild-type strains. Determinations performed at least in triplicate.
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SENSITIVITY
(high NADH oxidase activity)

Pyruvate + CoA

PFIO R 1

Acetyl-CoA + CO₂

Flod ox

Mtz-NO₂⁻ S3 DNA damage S₄ Cell death

RESISTANCE
(low NADH oxidase activity)

Pyruvate + CoA

PFIO R 1

Acetyl-CoA + CO₂

Flod red

NADH oxidase

2 NADH S₁ 2 NAD⁺

O₂ + 2H⁺ 2 H₂O

O₂⁻ or Mtz-NO₂⁻ R₂ Mtz-NO₂⁻ R₁

O₂⁻ or Mtz-NO₂⁻ R₃

Figure 3. Proposed involvement of NADH oxidase in metronidazole resistance. Pyruvate is decarboxylated to acetyl-CoA by the PFIO R complex and the flavodoxin (Fld) component of the PFIO R is reduced (1). In the following reaction, sensitivity or resistance to metronidazole depends upon the absence or presence of oxygen at the site of metronidazole reduction. S denotes a pathway involved in sensitivity and R a resistance pathway. In sensitive strains, NADH oxidase removes molecular oxygen from the site of metronidazole reduction (S₁), hence metronidazole is reduced by flavodoxin (S₂). The reduced drug causes DNA damage (S₃) which leads to cell death (S₄). In resistant strains, oxygen outcompetes metronidazole for the electrons from the flavodoxin and consequently no metronidazole activation occurs (R₁) or metronidazole is reduced by the flavodoxin (R₂) but is subsequently oxidized back to the inactive parent compound by a futile cycling mechanism (R₃).

to the development of resistance. Further studies into the nature of this phenomenon will increase our understanding of metronidazole resistance in H. pylori and may be applicable to other microaerophilic bacteria.

Acknowledgements

We thank Dr Y. Glupczynski, Brugmann Hospital, Brussels, Belgium, Dr J. Hardie, Royal London Hospital Trust, London and Mr G. Whittaker, Harold Wood Hospital, Essex for providing strains of H. pylori.

References

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Received 30 April 1996; returned 29 May 1996; revised 6 August 1996; accepted 27 September 1996