Effects of Substances Inhibiting or Uncoupling Respiratory-Chain Phosphorylation of Helicobacter pylori

WERNER BAER¹, HELGA KOOPMANN², and SIEGFRIED WAGNER²

¹ Institut für Mikrobiologie, Carl-Thiem-Klinikum Cottbus, 03048 Cottbus, Germany
² Institute für Medizinische Mikrobiologie, Medizinische Hochschule Hannover, 30625 Hannover, Germany

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Summary

The effects of electron transport inhibitors and uncoupling agents as well as of bismuth compounds on the respiratory activity and oxidative phosphorylation of Helicobacter pylori were investigated. Bismuth gallate and bismuth subsalicylate reduced the respiratory chain-dependent phosphorylation. Inhibition was of the same magnitude as that observed with other known inhibitors. It is concluded that bismuth displays an antibacterial effect by inhibiting the respiratory chain of H. pylori.

Introduction

Bismuth (Bi) compounds are the standard regimen for elimination of Helicobacter (H.) pylori from the infected gastric or duodenal mucosa (9). Although Bi compounds (2) have been used for decades to treat dyspeptic patients, their mode of antibacterial activity on H. pylori has not yet been elucidated. Several possible effects are discussed. One seems to be a protective effect on the gastric mucosa (5). However, a direct antibacterial effect of Bi on H. pylori has also been assumed because it displays an antibacterial effect in vitro (4, 8).

In addition, the effects of Bi on ATP metabolism have been demonstrated previously in other bacteria (12). H. pylori is an obligatory aerobic organism, oxidative phos-
phorylation being its only source of energy (10). Such metabolism, without energy
generation alternatives (e.g. fermentation), makes it highly susceptible to drugs that
interfere with the respiratory chain.

Therefore, we investigated the effects of Bi on the energy metabolism of *H. pylori*. Bi,
being chemically similar to arsenic, appeared to be capable of interfering with the
electron flow of the respiratory chain. To achieve that, we established a system which
allowed the determination of the respiratory activity and oxidative phosphorylation of
ADP and then we tested the effects of several compounds, including Bi, on the respira­
tory activity of *H. pylori*.

### Material and Methods

1. **Bacteria and growth conditions.** Investigations were conducted with *H. pylori* ATCC
   43504. Bacteria were cultured at 37°C in a microaerobic atmosphere (10% CO₂, 5% O₂).
2. **Determination of electron flow.** Respiratory activity was concluded by observing the
   rate of electron transport from the substrate to oxygen. Therefore the respiratory activity was determined by measuring the O₂ consumption in the medium.

   Freshly grown bacteria (final concentration: 1 mg protein/ml) were suspended in PBS
   (0.01 M; pH 7.4) and transferred to the air-tight reaction chamber (volume 2 ml) with
   magnetic stirring. O₂-concentration was registered continuously with an integrated pla­
tinum-electrode (type “Clark”, Backofer, Germany) at 37°C. After equilibration, the reac­
tion was started by injecting the substrate (Table 1), 5 mM final concentration. In the tables,
   the basic rate of metabolism (control without substrate) has been subtracted.
3. **Determination of ATP.** Intracellular ATP concentrations were determined by suspend­
   ing bacteria (2 mg/ml) in PBS at 37°C under normal oxygen pressure.

   The reaction was terminated by chilling in ice-water. Bacteria were centrifuged and the
   pellet was resuspended with a “nucleotide-releasing agent” (13). The ATP concentration
   was determined with the luciferin/luciferase method. The reaction was determined with the
   aid of a commercially available kit (“Lumit-PM”, Lumac, Landgraaf, NL) according to the
   manufacturer’s instructions. The ATP luminescence was measured in a Biolumat LB 9505C
   (Berthold, Germany) and calculated with an ATP calibration curve.

   The inhibition of the electron transport and ATP formation were studied in the presence
   of KCN (Aldrich Chemie, Germany, No. 20, 522-2), antimycine A (Sigma, Germany, No.
   A-8674) or myxothiazole (Boehringer, Germany, No. 775-789).
4. **Compounds used to effect electron flow.** The effects of uncoupling agents on the rate of
   electron transport and the formation of ATP was studied with carbonylcyanide-m-
   chlorophenylhydrazone (Sigma, Germany, No. C-2759) and arsenic acid (Merck, Germany,
   No. 152). The effects of bismuth on the respiratory activity were studied with various
   bismuth compounds (Table 4; from HEK, Lübeck, Germany). For these experiments, satu­
   rated solutions of Bi compounds were used, working at a final concentration of 3 mM. The
duration of these experiments was about five minutes. Results represent mean values of
three experiments, unless indicated otherwise.

### Results

1. **Effects of electron donors on the respiratory activity of *H. pylori***

   In preliminary experiments, we investigated a number of organic compounds which
might serve as electron donors for *H. pylori* (Table 1). Numbers represent the mean
values of five experiments. In the presence of D-glucose and formate, essentially no
Table 1. Respiratory activity of *H. pylori* with various electron donors (± standard deviation)

<table>
<thead>
<tr>
<th>Electron donors (final concentration: 5mM)</th>
<th>Respiratory rate (nmol O₂/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Glucose</td>
<td>0.82 (± 0.44)</td>
</tr>
<tr>
<td>Formate</td>
<td>0.56 (± 0.41)</td>
</tr>
<tr>
<td>DL-Lactate</td>
<td>13.73 (± 1.74)</td>
</tr>
<tr>
<td>Succinate</td>
<td>26.46 (± 2.92)</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>18.70 (± 2.59)</td>
</tr>
</tbody>
</table>

electron transport occurred. However, lactate, pyruvate and succinate were readily metabolized by *H. pylori*. The respiratory activity was found to be between 13.73 (lactate) and 26.46 (succinate) nmol O₂/min/mg protein for these compounds (Table 1).

2. Effects of inhibiting agents on the respiratory activity of *H. pylori*

Cyanide, antimycine A and myxothiazole were studied as potential inhibitors of the electron flow of *H. pylori*, using lactate (5 mM) as electron source (Table 2). Experiments represent mean values of three experiments. Cyanide showed maximal inhibition (~87.9%) at about 5 mM. At this concentration intracellular ATP levels also dropped significantly (~86.5%).

Maximum inhibition by antimycine A was observed at about 23 mM. The respiratory rate dropped by 54.1% and ATP levels decreased by 50.7% (Table 2).

Accordingly, maximum activity of mycothiazole was observed at about 0.3 mM. Cellular respiration was reduced by 32.6% and ATP levels dropped by 31.1% (Table 2).

Table 2. Effects of inhibiting agents on the respiratory activity and intracellular ATP levels of *H. pylori* with lactate as electron donor (± standard deviation)

<table>
<thead>
<tr>
<th>Inhibitor (final concentration)</th>
<th>Respiratory rate (nmol O₂/min/mg protein)</th>
<th>ATP concentration (nmol ATP/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>13.73 (± 1.74)</td>
<td>8.91 (± 2.18)</td>
</tr>
<tr>
<td>Cyanide (5 mM)</td>
<td>1.66 (± 0.34)</td>
<td>1.50 (± 0.92)</td>
</tr>
<tr>
<td>Antimycine A (23 mM)</td>
<td>6.30 (± 0.88)</td>
<td>4.39 (± 1.85)</td>
</tr>
<tr>
<td>Myxothiazole (0.3 mM)</td>
<td>9.26 (± 1.31)</td>
<td>6.14 (± 2.13)</td>
</tr>
</tbody>
</table>

3. Effects of uncoupling agents on the respiratory activity of *H. pylori*

In the presence of lactate as electron donor, the uncoupling effects of CCCP and arsenic acid on the flow of electrons were investigated (Table 3). Maximum activity of CCCP was observed at about 0.01 mM; it increased the respiratory rate of *H. pylori* 3.4 times and depressed ATP formation by 63.0%.

Maximum activity of arsenic acid was observed at about 5 mM; at this concentration, a three-fold increase in electron transport was found; intracellular ATP levels dropped by 23.8%.
Table 3. Effects of uncoupling agents on the respiratory activity and intracellular ATP levels of *H. pylori* with lactate as electron donor (± standard deviation)

<table>
<thead>
<tr>
<th>Uncoupler (final concentration)</th>
<th>Respiratory rate (nmol O$_2$/min/mg protein)</th>
<th>ATP concentration (nmol ATP/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>13.73 (± 1.74)</td>
<td>8.91 (± 2.18)</td>
</tr>
<tr>
<td>CCCP (0.01 mM)</td>
<td>46.61 (± 4.84)</td>
<td>3.30 (± 1.42)</td>
</tr>
<tr>
<td>Arsenic acid (5 mM)</td>
<td>40.79 (± 3.92)</td>
<td>6.79 (± 1.44)</td>
</tr>
</tbody>
</table>

4. Effects of bismuth compounds on the respiratory activity of *H. pylori*

The effects of five different bismuth compounds on the respiratory activity of *H. pylori* were investigated, in the presence of lactate as electron donor (Table 4).

Since bismuth salts are poorly soluble at physiological pH, a saturated solution of these salts was used. Neither cellular respiration nor intracellular ATP-levels were significantly influenced by bismuth carbonate, bismuth nitrate and bismuth citrate. Bismuth gallate inhibited the electron flow by 36.6% and lowered ATP levels by 31.3%.

Bismuth subsalicylate reduced electron transport by 52.9% and depressed ATP levels by 44.7%.

In parallel, also Na salicylate was tested; it displayed no significant effects.

Table 4. Effects of bismuth compounds on the respiratory activity and intracellular ATP concentrations of *H. pylori* with lactate as electron donor (± standard deviation)

<table>
<thead>
<tr>
<th>Bismuth compounds</th>
<th>Respiratory rate (nmol O$_2$/min/mg protein)</th>
<th>ATP concentration (nmol ATP/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>13.73 (± 1.74)</td>
<td>8.91 (± 2.18)</td>
</tr>
<tr>
<td>Bi carbonate</td>
<td>13.61 (± 2.09)</td>
<td>8.52 (± 3.20)</td>
</tr>
<tr>
<td>Bi nitrate</td>
<td>12.44 (± 2.32)</td>
<td>7.94 (± 2.88)</td>
</tr>
<tr>
<td>Bi citrate</td>
<td>11.94 (± 1.86)</td>
<td>7.46 (± 2.51)</td>
</tr>
<tr>
<td>Bi gallate</td>
<td>8.70 (± 1.13)</td>
<td>6.12 (± 2.02)</td>
</tr>
<tr>
<td>Bi subsalicylate</td>
<td>6.46 (± 0.90)</td>
<td>4.93 (± 1.78)</td>
</tr>
</tbody>
</table>

Discussion

We investigated the endogenous respiratory activity and oxidative phosphorylation of *H. pylori* by determining the O$_2$ consumption and intracellular ATP levels under defined conditions. Under conditions of equilibrium, without external electron source, cells of *H. pylori* consumed about 3–4 nmol O$_2$/min/mg protein. This basic rate of metabolism probably reflects the minimum requirement of respiration which is needed to maintain viability of *H. pylori* under these conditions. For additional metabolic activity (protein synthesis etc.), more energy and thus more oxidizable substrate is needed.
Glucose as well as other sugars (data not shown) did not significantly influence the turnover rate of electrons. This confirms previous publications (10), who also stated that *H. pylori* could not utilize sugars.

Likewise, formate was not utilized, indicating that *H. pylori* does not possess the enzyme formate dehydrogenase. This observation is in contrast to previous reports who showed the presence of this enzyme in related bacteria such as *Campylobacter jejuni* (6).

The effects of inhibitors on respiratory electron flow were studied, because they interact specifically with certain components of the respiratory chain. Cyanide showed the most prominent effects, because it blocked the terminal cytochrome oxidase. With antimycine A and myxothiazole, some electron flow has been observed, since these substances inhibit the bc 1 cytochrome-complex, thus allowing the entrance of electrons into the respiratory cascade on a level below the redox potential of this cytochrome (1).

In parallel to the reduced electron flow, a decreased energy yield of the respiratory chain could also be demonstrated: Cyanide, being the most potent inhibitor of the electron cascade, also induced the most severe depression of intracellular ATP levels. Corresponding to the milder inhibition of electron transport by antimycine A and myxothiazole, ATP levels were also reduced to a lesser degree (7), (Table 2).

We also investigated the effects of uncoupling agents such as CCCP and arsenic acid. These substances interfere with the proton flow across the membrane, which is the driving force for the generation of ATP (3, 11).

As a consequence, the energy derived from the electron transport is not conserved as ATP, but is released as thermal energy, thus allowing the electrons to pass the respiratory chain faster (1).

Finally, we investigated the effects of bismuth salts on the energy metabolism of *H. pylori*, because it had been shown that these substances exert antibacterial effects *in vitro* (14). One problem with bismuth compounds is their poor solubility at physiological pH. For our experiments, saturated solutions of bismuth salts were used. However, the amount of bismuth becoming dissolved has not been determined.

From the substances investigated, only in the presence of bismuth gallate and bismuth subsalicylate a significant reduction of the respiratory rate and intracellular ATP concentration has been found. This finding parallels the observation that the former bismuth compounds are less soluble than bismuth gallate or bismuth subsalicylate and therefore less effectively inhibit electron flow. *In vivo*, however, all these bismuth compounds have been used successfully for the treatment of *H. pylori* infection (2).

One should keep in mind, however, that the *in vivo* situation is quite different from our experimental design: The period of exposure is much longer in the stomach than under our experimental conditions (about five minutes). The solubility is pH-dependent while we used consistently a neutral pH for our investigations, whereas in the stomach a pH gradient is found. Finally, other effects of bismuth, such as protection of the gastric mucosa, have also to be assumed (15).

The nature of the mechanism of action of bismuth on the bacterial respiration appears to be an inhibition of the respiratory chain. Both respiratory rate and ATP levels are similar in the presence of bismuth subsalicylate and antimycine A. Effects like these have been described for other bacteria (12). We could rule out that salicylate alone did induce these effects under physiological conditions, as already described previously for other bacteria (12).
References


PD Dr. med. Werner Baer, Institut für Mikrobiologie, Carl-Thiem-Klinikum, Thiemstr. 111, 03048 Cottbus, Germany