Iron enhances the antituberculous activity of pyrazinamide

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Background: Pyrazinamide is a paradoxical frontline tuberculosis drug characterized by high in vivo sterilizing activity but poor in vitro activity. This separation in pyrazinamide activity reflects differences between the in vivo tissue environment and in vitro culture conditions. The well-known acid pH requirement for pyrazinamide activity was discovered previously based on such reasoning but does not completely explain the discrepancy between in vivo and in vitro activity of pyrazinamide. This study examined the effect of iron, which could potentially be elevated in local inflammatory lesions, on pyrazinamide activity in vitro.

Materials and methods: The effect of iron on the activity of pyrazinamide or its active derivative pyrazinoic acid against Mycobacterium tuberculosis was assessed in liquid medium in a drug exposure assay or in solid medium with pyrazinamide plus iron or pyrazinamide alone. The effect of iron on pyrazinamide or pyrazinoic acid was expressed as percentage of growth inhibition.

Results: We have shown that iron enhances the activity of pyrazinamide and pyrazinoic acid against M. tuberculosis in both liquid and solid media at acid pH 5.6. Iron enhanced the activity of pyrazinoic acid but not pyrazinamide against the naturally pyrazinamide-resistant Mycobacterium bovis BCG. Other metal ions such as magnesium, calcium and zinc did not enhance the activity of pyrazinamide or pyrazinoic acid.

Conclusions: Iron increased the activity of pyrazinamide or pyrazinoic acid against M. tuberculosis in vitro. These findings may have implications for the study of mechanism of action of pyrazinamide and possible iron supplement for improving the activity of pyrazinamide.

Keywords: pyrazinamide, iron, drug susceptibility, Mycobacterium tuberculosis

Introduction

Pyrazinamide is an important front-line tuberculosis (TB) drug that forms the most effective TB chemotherapy along with isoniazid, rifampicin and ethambutol. Pyrazinamide plays a key role in shortening the TB therapy from previously 9–12 months to 6 months. The ability of pyrazinamide to shorten the therapy seems to correlate with its ability to kill a special bacterial population with low metabolic activity residing in acidic pH environments that are not killed by other TB drugs and also its ability to kill old non-growing bacilli more effectively than young bacilli. Pyrazinamide is a paradoxical TB drug. Despite its high sterilizing activity in vivo, surprisingly, pyrazinamide has no activity under normal culture conditions at close to neutral pH. This discrepancy between the in vitro and in vivo activity of pyrazinamide reflects possible differences between in vivo and in vitro conditions that affect the drug activity. McDermott and colleagues reasoned that one of the differences was the acid pH, which is present in vivo at local lesions owing to production of lactic acid by infiltrating inflammatory cells. This reasoning led to the discovery that acid pH increases the activity of pyrazinamide. Even at acid pH, pyrazinamide kills tubercle bacilli ineffectively, killing no more than 76% of bacilli with a high pyrazinamide concentration of 1000 mg/L at pH 4.8 over a period of 2 weeks. The MIC of pyrazinamide is 50–100 mg/L at acid pH of 5.5–6.0. This MIC is somewhat higher than the serum pyrazinamide concentration of 30–60 mg/L. Thus, it appears that acid pH cannot completely explain the discrepancy between high in vivo activity and poor in vitro activity of pyrazinamide. Various factors besides acid pH, such as inoculum size, serum albumin, age of cultures and efflux inhibitors, could also influence the activity of pyrazinamide. During our ongoing study of factors that potentially influence the activity of pyrazinamide, we tested the effect of iron on the activity of pyrazinamide and its active derivative pyrazinoic acid. We reasoned that another possible difference besides acid pH between in vivo and in vitro conditions is the local iron concentration in the lesions, which could be increased during inflammation. We show in this study that iron can indeed enhance the activity of pyrazinamide and pyrazinoic acid in various in vitro tests.

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Materials and methods

Effects of iron on the activity of pyrazinamide and pyrazinoic acid as assessed in liquid medium

To assess the effect of iron on pyrazinamide or pyrazinoic acid activity, a 3-week-old Mycobacterium tuberculosis H37Ra culture grown in 7H9 medium with albumin-dextrose-catalase (ADC) supplement (Difco) was diluted at 1:200 in Sauton’s medium (pH 5.6) containing ferrous sulphate or ferric chloride at 1.2, 0.6, 0.3, 0.15 or 0 mM and pyrazinoic acid at 60, 30, 15 or 7.5 mg/L in a 96-well microtiter plate. The microtiter plate was incubated at 37°C for 7 days before the viability of the bacteria was assessed by MTT redox dye using the method described previously. The degree of growth inhibition was calculated by the following equation: ([OD value of control–OD value of sample]/OD value of control) x 100.

The effect of iron or other divalent cations on pyrazinamide activity was also assessed in a drug exposure experiment followed by cfu assay. A 10-day-old H37Ra culture was centrifuged at 7000 rpm for 10 min at 4°C and the cells were resuspended in 7H9 medium at pH 5.5 to a cell density of about 10⁶–10⁷ bacilli/mL. The cell suspensions were treated with nothing (control) or pyrazinamide (100 mg/L), ferrous sulphate (1 mM), or pyrazinamide plus ferrous iron, followed by incubation at 37°C for 5 days. A control that received no drug was also included. The cells were washed with 7H9 medium and plated on 7H11 agar plates, which were then incubated at 37°C for 3–4 weeks when the cfu was determined. The effect of other divalent cations such as zinc, magnesium and calcium was also assessed in a similar manner as above.

Effect of iron on the activity of pyrazinamide and pyrazinoic acid as assessed on 7H11 agar

M. tuberculosis H37Ra, H37Rv or BCG cultures were grown in 7H12B (Becton Dickinson, Sparks, MD, USA) in the BACTEC 460 system. When a growth index of 999 was achieved (which indicates saturation of growth or stationary phase), 200 µL aliquots from the BACTEC 12B (Becton Dickinson) cultures were used to prepare 10-fold serial dilutions (10⁻¹, 10⁻², 10⁻³ and 10⁻⁴) in sterile distilled water for inoculation. Middlebrook 7H11 plates at pH 5.6 with ADC supplement were prepared with the addition of pyrazinamide (with concentrations of 50, 25, 12.5 and 6.25 mg/L) plus ferrous ammonium sulphate or ferric ammonium citrate (with concentrations of 1.0 and 0.5 mM), or pyrazinamide (with concentrations of 50, 25, 12.5 and 6.25 mg/L) plus ferrous ammonium sulphate or ferric ammonium citrate (with concentrations of 1.0 and 0.5 mM). Control plates (pH 5.6) with the indicated concentrations of pyrazinamide, pyrazinoic acid, ferrous ammonium sulphate, and ferric ammonium citrate alone and plates lacking these compounds were also included. Finally, 30 µL aliquots of the four dilutions (10⁻¹, 10⁻², 10⁻³ and 10⁻⁴) of the H37Ra or H37Rv were used to inoculate each quadrant of the 7H11 plates. All plates were incubated at 37°C in an incubator with an atmosphere of 5% CO₂ and 95% air for 3 weeks before cfu determination. The enhancement effect of ferrous or ferric iron on pyrazinamide or pyrazinoic acid was expressed as the percentage of growth inhibition by the equation: [cfu of control – cfu of sample]/cfu of control x 100.

Results and discussion

We initially tested the effect of iron on pyrazinamide or pyrazinoic acid activity against M. tuberculosis H37Ra in liquid Sauton’s medium at pH 5.6. The results for pyrazinoic acid are shown in Figure 1. The antituberculous activity of pyrazinoic acid was enhanced at ferrous sulphate concentrations of 0.15–0.6 mM. This was shown by a greater degree of inhibition by pyrazinoic acid plus ferrous iron than by pyrazinoic acid alone. Although the concentration of ferrous iron at 1.2 mM was high, showing considerable growth inhibition by itself, the iron enhancement effect could still be seen at this concentration (Figure 1). Using similar experimental conditions, we assessed the effect of ferrous iron on the activity of pyrazinamide with lower iron concentrations. Indeed, ferrous iron at 0.15–0.6 mM also enhanced the activity of pyrazinamide (Figure 2). However, one difference was that ferrous iron enhancement of pyrazinamide activity was seen at somewhat higher pyrazinamide concentrations of 25 and 50 mg/L, but not at 12.5 and 6.25 mg/L (Figure 2). This presumably results from the fact that only a portion of the prodrug pyrazinamide was converted into the active component pyrazinoic acid that reacted with ferrous iron to show activity. We also tested the effect of ferrous iron on pyrazinamide and pyrazinoic acid activity using naturally pyrazinamide-resistant BCG in a similar experiment as described above. Mycobacterium bovis BCG is known to have a characteristic single point mutation in its pncA gene which is responsible for its natural pyrazinamide resistance, but remains susceptible to pyrazinoic acid. Ferric iron at 0.15–0.6 mM increased the activity of pyrazinoic acid, but not pyrazinamide against BCG as expected (Table 1). In a similar type of experiment, ferric iron (ferric chloride) was also found to enhance the activity of pyrazinoic acid and pyrazinamide (Table 2). It is worth noting that the enhancement effect of ferric iron on pyrazinamide activity was more obvious when the pyrazinoic acid concentrations were below its MIC (50 mg/L).
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(Table 2). This is because at the MIC of 50 mg/L, pyrazinoic acid showed a significant growth inhibitory effect by itself and the effect of iron was not as evident as when pyrazinoic acid concentrations were at 25, 12.5 and 6.25 mg/L, where there was a much higher iron enhancement effect (Table 2).

We also tested the effect of iron on pyrazinamide activity in a drug exposure assay after incubation for 5 days followed by cfu count on 7H11 plates. As can be seen in Figure 3, ferrous iron had a significant enhancement effect on pyrazinamide activity with a more than 10-fold reduction in cfu compared with pyrazinamide alone. The difference was statistically significant ($P < 0.05$) when comparing pyrazinamide plus iron with either pyrazinamide alone or with iron alone. Unlike iron, zinc chloride as a control divalent cation did not have any enhancement effect on pyrazinamide activity (Figure 3). Other divalent cations such as magnesium or calcium also did not enhance the activity of pyrazinamide (data not shown).

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Table 2. Effect of ferric (Fe$^{3+}$) iron on pyrazinoic acid and pyrazinamide activity against H37Ra expressed as inhibition rate (%)

<table>
<thead>
<tr>
<th>POA (mg/L)</th>
<th>PZA (mg/L)</th>
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<tbody>
<tr>
<td>Fe$^{3+}$ (mM)</td>
<td>50</td>
</tr>
<tr>
<td>0.6</td>
<td>81</td>
</tr>
<tr>
<td>0.3</td>
<td>61</td>
</tr>
<tr>
<td>0.15</td>
<td>67</td>
</tr>
<tr>
<td>0</td>
<td>60</td>
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Figure 3. Effect of ferrous iron and zinc on pyrazinamide activity against M. tuberculosis H37Ra. The H37Ra cells were treated with pyrazinamide (100 mg/L) in the presence or absence of ferrous sulphate (1 mM) or zinc chloride (1 mM) for 5 days when the cfu was determined on 7H11 agar plates. For details of the experiment, see Materials and methods.
Iron enhancement of pyrazinamide activity

Iron enhancement of pyrazinamide activity in vitro versus in vivo as the MIC of pyrazinamide (50–100 mg/L at pH 5.5–6.0) is higher than the serum pyrazinamide concentration (30–60 mg/L). It is worth noting that iron can be released from iron binding proteins (e.g., transferrin, lactoferrin, ferritin, haem-containing enzymes) at acid pH during active inflammation, which could potentially cause increased local iron concentration in tuberculous lesions. It is significant that we have shown in this study that iron could enhance the activity of pyrazinamide and pyrazinoic acid against M. tuberculosis. The elevated iron concentration in the lesions could be another factor besides acid pH that can potentiate pyrazinamide activity. However, there is no information available on iron concentrations in local tuberculous lesions, and future studies are needed to assess this in the context of iron enhancement of pyrazinamide activity.

We have shown in this study that various iron salts with different anion groups complexed with iron such as ferrous sulphate, ferrous ammonium sulphate, ferric chloride and ferric ammonium citrate could all enhance the activity of pyrazinamide or pyrazinoic acid. This indicates that it is the iron that is important for the activity, not the anion group in complex with the iron.

The mechanism of iron enhancement of pyrazinoic acid and pyrazinamide activity is not known. It is possible that iron causes lipid peroxidation of the M. tuberculosis membrane and membrane damage, leading to disruption of membrane potential through increased permeability to protons or inhibition of membrane-embedded respiratory chain enzymes such as H+-ATPase, NADH dehydrogenase and cytochrome c oxidase involved in membrane potential and energy production. The iron-mediated disruption of membrane potential could then serve as a ‘weak point’ for attack by pyrazinoic acid, which we have shown to decrease membrane potential at acid pH. Further studies are needed to address these possibilities and to determine whether iron can be used as a supplement for enhancement of pyrazinamide activity in animal models of TB infection. However, one must be cautious regarding the iron supplement used for enhancement of pyrazinamide activity, as iron could also serve as a bacterial growth factor.

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References


