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Pyridoxal isonicotinoyl hydrazone (PIH) prevents copper-mediated *in vitro* free radical formation

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Abstract

Pyridoxal isonicotinoyl hydrazone (PIH) is an iron chelator with antioxidant activity, low toxicity and is useful in the experimental treatment of iron-overload diseases. Previous studies on x-ray diffraction have revealed that PIH also forms a complex with Cu(II). Since the main drug of choice for the treatment of Wilson's disease, d-penicillamine, causes a series of side effects, there is an urgent need for the development of alternative copper chelating agents for clinical use. These chelators must also have antioxidant activity because oxidative stress is associated with brain and liver copper-overload. In this work we tested the ability of PIH to prevent *in vitro* free radical formation mediated by Cu(II), ascorbate and dissolved O_2. Degradation of 2-deoxyribose mediated by 10 μM Cu(II) and 3 mM ascorbate was fully inhibited by 10 μM PIH (I_{50} = 6 μM) or 20 μM d-penicillamine (I_{50} = 10 μM). The antioxidant efficiency of PIH remained unchanged with increasing concentrations (from 1 to 15 mM) of the hydroxyl radical detector molecule, 2-deoxyribose, indicating that PIH does not act as a hydroxyl scavenger. On the other hand, the efficiency of PIH (against copper-mediated 2-deoxyribose degradation and ascorbate oxidation) was inversely proportional to the Cu(II) concentration, suggesting a competition between PIH and ascorbate for complexation with Cu(II). An almost full inhibitory effect by PIH was observed when the ratio PIH:copper was 1:1. A similar result was obtained with the measurement of copper plus ascorbate-mediated O_2 uptake. Moreover, spectral studies of the copper and PIH interaction showed a peak at 455 nm and also indicated the formation of a stable Cu(II) complex with PIH with a 1:1 ratio. These data demonstrated that PIH prevents hydroxyl radical formation and oxidative damage to 2-deoxyribose by forming a complex with Cu(II) that is not reactive with ascorbate (first step of the reactions leading to hydroxyl radical formation from Cu(II), ascorbate and O_2) and does not participate in Haber–Weiss reactions. (Mol Cell Biochem **228**: 73–82, 2001)

Key words: copper overload, free radical, chelator, antioxidant, iron, d-penicillamine, pyridoxal isonicotinoyl hydrazone

Introduction

Previous work carried out by our group has revealed that the iron chelator pyridoxal isonicotinoyl hydrazone (PIH) is a potent antioxidant against $^{\cdot}OH$ formation induced by Fe(III)-EDTA, ascorbate and O_2 or by Fenton reagents Fe(II) and H_2O_2 [1–4]. We demonstrated that iron-mediated lipid peroxidation, 2-deoxyribose oxidative degradation, plasmid DNA strand breaks and 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) hydroxylation is inhibited by PIH. The antioxidant activity of PIH was explained by its ability to form a complex with iron that does not participate in Haber–Weiss reactions [1, 4].

These observations are pharmacologically significant since PIH may be an alternative for iron chelation therapy in iron-overload pathologies such as β-talassemia [5–7]. As opposed to deferrioxamine (DFO), the only drug clinically available for iron-overload therapy, PIH is a low cost chelator that can

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be taken orally and that easily transverses biological membranes [6–8]. A number of *in vivo* studies have demonstrated that PIH has low toxicity, inducing excess iron excretion and negative iron balance [5, 6].

Our interests, however, have recently shifted to Wilson's disease, a pathology characterized by a mutant gene of a copper transporting P-ATPase which causes copper overload. The accumulating copper causes damage primarily to the liver and the brain [9–13]. It has recently been proposed that increased free radical damage and reduction in endogenous antioxidant defenses is connected to copper overload in patients with Wilson's disease or in Long–Evans Cinnamon rats [10, 13–17]. The copper chelator d-penicillamine has been the most widely used drug for Wilson's disease therapy since the late 1950's. Patients require 15–25 mg/kg daily in the early stages of treatment [12]. However, a number of side effects caused by d-penicillamine therapy, including neutropenia, systemic lupus erythematosus, nephrosis and neuronal deterioration [12, 18, 19] has called attention to the need for alternative copper chelating agents for clinical use, such as triethylenetetramine (TRIEN) [9, 12], which is an inhibitor of superoxide dismutase [20] and not totally free of side effects [21, 22].

Rao and Singh [23] have shown, through x-ray studies, that PIH forms a complex with copper in a 1:1 ratio in the solid state. More recently, Richardson [24] observed the formation of a Cu(II) complex with PIH in aqueous solutions. Based on these observations and on the fact that PIH displays antioxidant activity in iron-catalyzed reactions [1–4], we decided to investigate whether PIH could prevent free radical formation mediated by copper ions. The *in vitro* effect of PIH on $^{\cdot}OH$ radical formation, ascorbate oxidation and O_2 uptake from Cu(II), ascorbate and dissolved O_2 was studied and the spectral characteristics of the copper complex with PIH in aqueous solutions was also determined.

Materials and methods

Reagents and solutions

Ascorbic acid, catalase, 2-deoxyribose, deferoxamine mesylate (DFO), EDTA, neocuproine, d-penicillamine and thiobarbituric acid (TBA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The synthesis of 3-hydroxy-5-(hydroxymethyl)-2-methyl-4-pyridine-carboxaldehyde-4-pyridinecarbonylhydrazone (pyridoxal isonicotinoyl hydrazone, PIH) and its analogs (SIH and PBH) was performed by a Schiff base condensation of either pyridoxal or salicylaldehyde with acid hydrazides. Details of this procedure and of the chemical identification of the products have been previously published [4, 25, 26]. The hydrazides used to react with pyridoxal were isonicotinic acid hydrazide (forming PIH) and benzoic acid hydrazide (forming PBH). The hydrazide used to react with salicylaldehyde was isonicotinic acid hydrazide (forming SIH). PIH and its analogues were prepared by Dr. Prem Ponka (Lady Davis Institute for Medical Research, Jewish General Hospital, Montreal, Canada) and donated to our research group. All the other reagents were of analytical purity.

Stock solutions (1 mM) of the chelators were prepared daily in phosphate buffer pH 7.2 (100 mM NaOH was used to dissolve PIH, SIH and PBH before neutralization with HCl). Copper sulfate solutions (1 mM) were prepared daily in 10 mM HCl. Stock solutions of 1% TBA were prepared in 50 mM NaOH and used within a week. All the solutions were prepared with milli-Q deionized water.

The assay of 2-deoxyribose degradation

The formation of $^{\cdot}OH$ radicals was measured using 2-deoxyribose oxidative degradation as previously described [27]. The principle of the assay is the quantification of the 2-deoxyribose degradation product, malonaldehyde, after its condensation with TBA [28]. Typical reactions were started by the addition of ascorbate (3 mM final concentration) to 0.5 mL solutions containing 20 mM phosphate buffer (pH 7.2), 5 mM 2-deoxyribose, 10 μM Cu(II) and 7 μM PIH (or no PIH). Ascorbate was added approximately 15 min after the addition of PIH. Reactions were terminated by the addition of 0.5 mL 4% phosphoric acid (v/v) followed by 0.5 mL 1% TBA solution. After boiling for 15 min, the absorbance of solutions was measured at 532 nm.

The assays of ascorbate oxidation and O_2 uptake

The oxidation of ascorbate was performed as previously described [1] in 1 mL solutions containing 100 μM ascorbate, 20 mM phosphate buffer (pH 7.2), Cu(II) (0–10 μM) and PIH (0–20 μM). The reactions were started at room temperature by the addition of ascorbate and followed at 265 nm for 5 min. Slopes were calculated using the linear part of the curve (the first 50–60 sec of reaction), with a minimal r^2 value of 0.995.

Oxygen uptake was measured using a Clark-type electrode (Yellow Springs Instruments Co.) in a 3 mL glass chamber equipped with magnetic stirring. Measurements were started at room temperature after the addition of ascorbate (3 mM of final concentration) to solutions containing 20 mM phosphate buffer (pH 7.2), 10 μM Cu(II) and PIH (0–15 μM). The initial concentration of dissolved O_2 was 220 μM at 1000 meters above sea level.

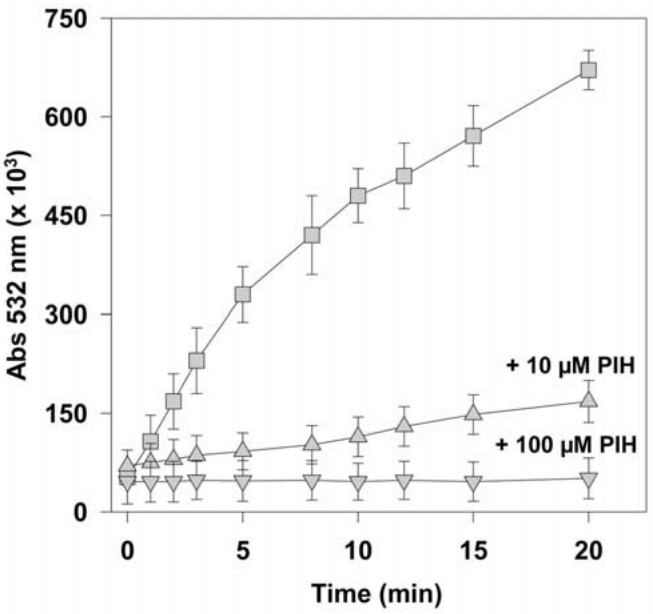

Fig. 1. Time course of the oxidative degradation of 2-deoxyribose caused by Cu(II) plus ascorbate in the absence or presence of PIH. Solutions were incubated at different time intervals (as indicated on the *x*-axis) at room temperature and contained 20 mM phosphate buffer (pH 7.2), 5 mM 2-deoxyribose, 10 µM Cu(II), PIH (0, 10 or 100 µM) and 3 mM ascorbate. Data are means ± S.D. (n = 4).

Absorption spectra

Spectra of the complexes of PIH with copper were obtained with a Hitachi U-2001 spectrophotometer. The complexes were pre-incubated for 10–15 min at room temperature in phosphate-buffered solutions before measurement against a blank containing PIH plus buffer, or against buffer only.

Results and discussion

PIH inhibits copper-mediated 2-deoxyribose degradation

Figure 1 shows the time course of 5 mM 2-deoxyribose degradation induced by 10 µM Cu(II), 3 mM ascorbate and dissolved O_2. The degradation of 2-deoxyribose was abolished in the presence of 100 µM PIH during the 20-min observation period. Moreover, 10 µM PIH was highly effective in inhibiting 2-deoxyribose degradation.

Titration of PIH in the 2-deoxyribose assay (8 min of incubation) was performed in comparison with DFO, SIH, PBH and d-penicillamine (Figs 2A and 2B). An I_{50} value of 6 µM was found for PIH. The I_{50} values obtained for PBH, SIH, d-penicillamine and DFO were 5.3, 5.5, 10 and 11 µM, respectively. The low I_{50} values obtained for the chelators are inconsistent with the classical antioxidant mechanism of ·OH scavenging activity because ·OH scavengers are effective only at mM levels in aqueous solutions [4, 27, 29]. Indeed, the classical ·OH scavengers ethanol and DMSO induced half-inhibition of copper/ascorbate-mediated 2-deoxyribose oxidative degradation at 19 and 38 mM, respectively (data not shown). Thus, PIH and its analogues (as well as DFO and d-penicillamine) may prevent 2-deoxyribose degradation by a mechanism other than via ·OH trapping.

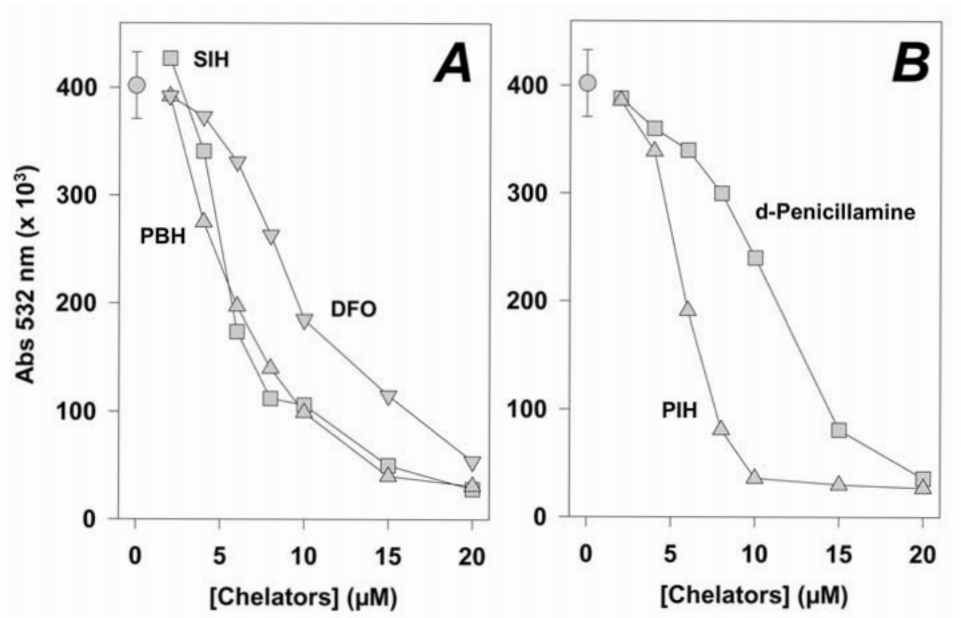

Fig. 2. Effect of SIH, PBH, DFO (Panel A), PIH and d-penicillamine (Panel B) concentrations on the oxidative degradation of 5 mM 2-deoxyribose caused by 10 µM Cu(II) plus 3 mM ascorbate in 20 mM phosphate buffer (pH 7.2). Reaction time was 8 min at room temperature. The figure shows the average values of 6 experiments. The values of I_{50} described in the text were calculated using software designed by Dr. S.P.J. Brooks [47].

Another relevant point is that almost complete protection against 2-deoxyribose degradation was attained at 10 μM PIH or at 20 μM d-penicillamine (Fig. 2B), which corresponds to a 1:1 and 2:1 ratio, respectively, between the chelators and Cu(II). This suggests that 10 μM PIH or 20 μM d-penicillamine induce complexation of 10 μM copper in such a way as to remove all the 'free' Cu(II) from the solution.[1] The resulting copper complexes with PIH and d-penicillamine appear unable to catalyze ·OH formation and 2-deoxyribose degradation in the presence of ascorbate and dissolved O_2.

These conclusions are corroborated by the study of the dependence of Cu(II) concentration on the 2-deoxyribose assay. In the absence of PIH, the increase in Cu(II) concentration caused an increase in 2-deoxyribose degradation, which saturates at about 20 μM Cu(II) (Fig. 3). In the presence of 7 μM PIH, 2-deoxyribose degradation was highly inhibited when Cu(II) concentrations were in the 1–7 μM range. However, the effectiveness of PIH (at 7 μM) decreased progressively with further increase in Cu(II) concentration (inset to Fig. 3). Possibly, most of the copper in reaction media is complexed to PIH (at 7 μM) when the concentration of metal in the solutions is less than 7 μM. These observations also indicate that the formation of copper complex with PIH at a 1:1 ratio inhibits 2-deoxyribose degradation mediated by Cu(II), ascorbate and dissolved O_2.

The concentration dependence of 2-deoxyribose on its oxidative degradation induced by 10 μM Cu(II) and 3 mM ascorbate (and dissolved O_2) in the absence or presence of 7 μM PIH is shown in Fig. 4. If PIH had acted mainly as an ·OH scavenger, we would have expected its effectiveness to diminish with increasing 2-deoxyribose concentrations (from 1 to 15 mM) because the ·OH detector molecule (2-deoxyribose) would have competed with PIH for ·OH radical trapping. However, the effectiveness of PIH was unaffected by increasing 2-deoxyribose concentrations (inset to Fig. 4). These data strongly suggest that PIH (at 7 μM) works as an antioxidant in preventing ·OH formation, possibly by means of copper chelation rather than trapping ·OH radicals. A similar result was observed for the antioxidant activity of tannic acid (at 10 μM) against the degradation of 2-deoxyribose (from 3 to 70 mM) induced by Fe(II) and H_2O_2 [27]. However, in the case of tannic acid, a small component of ·OH trapping activity was detected.

Figure 5 shows a typical bell-shaped curve indicating the dependence of ascorbate concentration on the copper-mediated degradation of 2-deoxyribose in the presence of O_2. This two-phase effect of ascorbate is explained by its pro-oxidant action at low concentrations (due to the reduction of Cu(II) to Cu(I), a Fenton reagent) and by its antioxidant action at higher concentrations due to ·OH scavenging activity [30]. A similar bell-shaped curve was observed for the experiments in the presence of 7 μM PIH. Interestingly, at

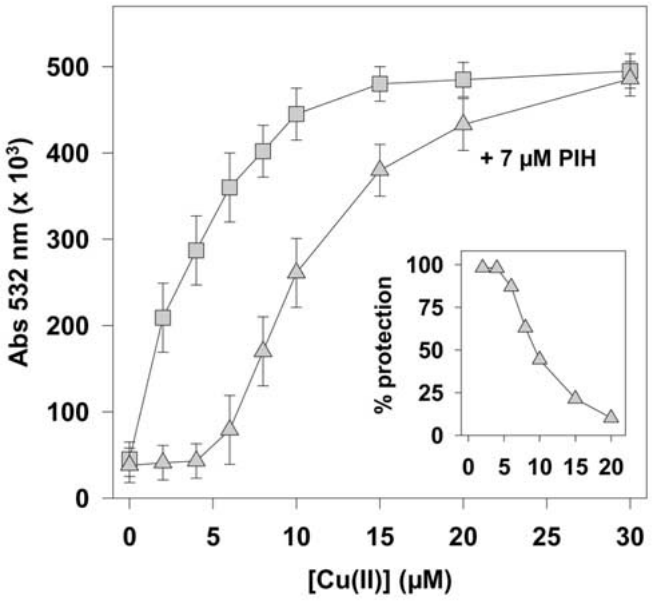

Fig. 3. Dependence of Cu(II) concentration (0–30 μM) on the oxidative degradation of 5 mM 2-deoxyribose induced by Cu(II) plus 3 mM ascorbate in 20 mM phosphate buffer (pH 7.2) and in the absence or presence of 7 μM PIH. Reaction time was 8 min at room temperature. Data are means ± S.D. (n = 4). Inset: the % protection of 2-deoxyribose by PIH at various Cu(II) concentrations.

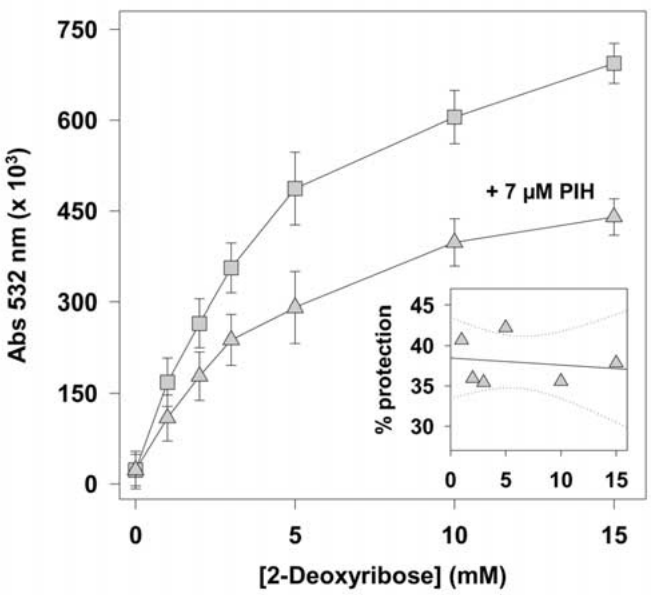

Fig. 4. Dependence of 2-deoxyribose concentration (0–15 mM) on the oxidative degradation of 2-deoxyribose induced by 10 μM Cu(II) plus 3 mM ascorbate in 20 mM phosphate buffer (pH 7.2) and in the absence or presence of 7 μM PIH. Reaction time was 8 min at room temperature. Data are means ± S.D. (n = 4). Inset: the % protection of 2-deoxyribose by PIH at various 2-deoxyribose concentrations. A first-order regression line is shown in the inset ($r^2 = 0.027$; p = not significant); dotted lines represent the 95% confidence interval.

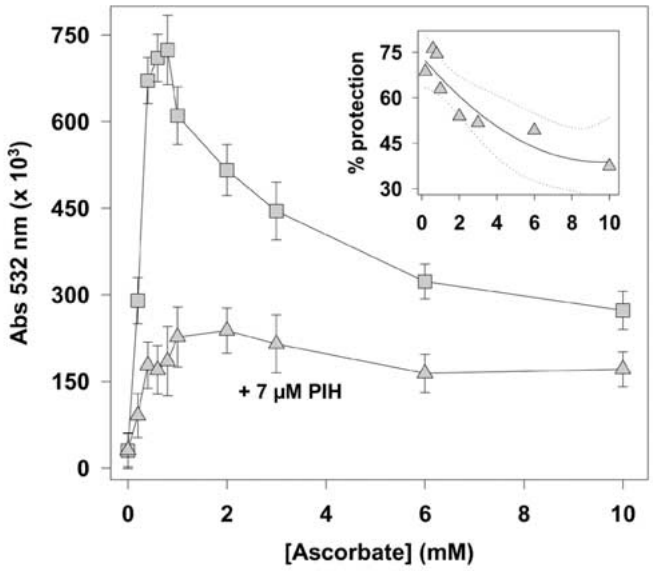

Fig. 5. Dependence of ascorbate concentration (0–10 mM) on the oxidative degradation of 5 mM 2-deoxyribose induced by 10 µM Cu(II) plus ascorbate in 20 mM phosphate buffer (pH 7.2) and in the absence or presence of 7 µM PIH. Reaction time was 8 min at room temperature. Data are means ± S.D. (n = 4). Inset: the % protection of 2-deoxyribose by PIH at various ascorbate concentrations. A second-order regression line is shown in the inset ($r^2 = 0.841$); dotted lines represent the 95% confidence interval.

low concentrations of ascorbate (from 0.2 to 1 mM), the antioxidant efficacy of PIH was greater than at high concentrations of ascorbate (inset to Fig. 5). This may be ascribed to a competition between ascorbate and PIH for the formation of complexes with copper. Indeed, ascorbate forms a complex with Cu(II) before the reduction of the metal to Cu(I) [31]. These differences in the efficiency of PIH may be relevant *in vivo*, since changes in intracellular ascorbate levels would affect the extent to which copper-mediated oxyradical formation is inhibited by PIH.

PIH inhibits copper-mediated oxidation of ascorbate

The oxidation of ascorbate with concomitant reduction of Cu(II) to Cu(I) is the first step in a series of reactions leading to ·OH formation and 2-deoxyribose damage (reactions 1–6) [30]. The differences in the effectiveness of PIH against 2-deoxyribose degradation with different ascorbate concentrations (see inset to Fig. 5) suggest that PIH may also influence the rate of ascorbate oxidation mediated by copper. Schulman *et al*. [1] have previously observed that PIH and SIH are effective in inhibiting ascorbate oxidation mediated by Fe(III)-EDTA. Thus, we decided to examine the effects of PIH in the Cu(II)-mediated oxidation of ascorbate.

$$\text{Cu(II)} + \text{ascorbate} \rightarrow \text{Cu(II)-ascorbate} \rightarrow \text{Cu(I)} + \text{ascorbyl} \quad (1)$$

$$\text{Cu(I)} + O_2 \rightarrow \text{Cu(II)} + O_2^- \quad (2)$$

$$\text{Cu(I)} + O_2^- + 2H^+ \rightarrow H_2O_2 + \text{Cu(II)} \quad (3)$$

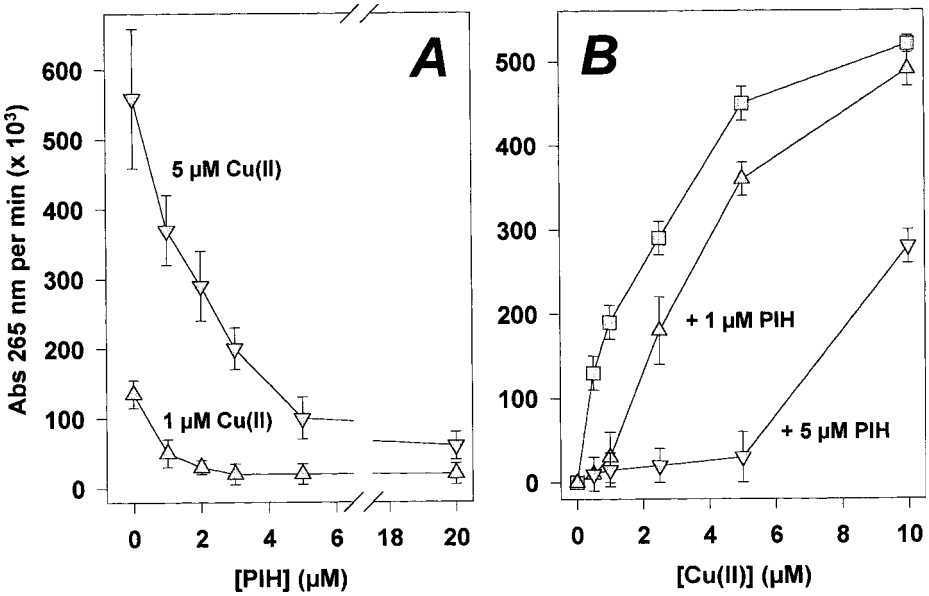

Fig. 6. (A) Effect of PIH concentration on the rate of ascorbate oxidation. Reaction media contained 20 mM phosphate buffer (pH 7.2), 1 or 5 µM Cu(II), PIH (0–20 µM) and 100 µM ascorbate. (B) Effect of Cu(II) concentration on the rate of ascorbate concentration. Reaction media contained 20 mM phosphate buffer (pH 7.2), Cu(II) (0–10 µM), PIH (0, 1 or 5 µM) and 100 µM ascorbate. The initial ascorbate oxidation rate in the presence of 5 µM Cu(II) was 0.5–0.6 µM per sec, based on the molar extinction coefficient of 14,500 $M^{-1}cm^{-1}$ [32]. Data in (A) and (B) are means ± S.D. (n = 4).

$$2\,O_2^{-} + 2H^{+} \rightarrow H_2O_2 + O_2 \quad (4)$$

$$Cu(I) + H_2O_2 \rightarrow Cu(II) + OH^{-} + {}^{\cdot}OH \quad (5)$$

$${}^{\cdot}OH + \text{2-deoxyribose} \rightarrow \text{degradation products} \quad (6)$$

Figure 6A shows a titration of PIH on the ascorbate oxidation rate induced by either 1 μM or 5 μM Cu(II). PIH was more effective in inhibiting the rate of ascorbate oxidation with 1 μM Cu(II) (I_{50} = 0.8 μM PIH) than with 5 μM Cu(II) (I_{50} = 2.4 μM PIH), indicating that PIH competes with ascorbate for complexation of Cu(II) and thus prevents ascorbate oxidation (reaction 1) and the following formation of ·OH radicals (reactions 2–5). In addition, strong inhibition of ascorbate oxidation occurred with concentrations of PIH close to the 1:1 ratio with Cu(II).

Figure 6B shows the dependence of Cu(II) concentration on the oxidation of ascorbate. It can be observed that PIH at 1 or 5 μM induced almost total inhibition of ascorbate oxidation when the copper concentration was 0.5–1 μM or 0.5–5 μM Cu(II), respectively. This is also indicative of the formation of a copper complex with PIH at 1:1 ratio, which cannot induce ascorbate oxidation. Moreover, the effectiveness of 1 μM PIH was significantly higher than d-penicillamine, DFO, EDTA and neocuproine (all at 1 μM) in the ascorbate oxidation assay in the presence of 5 μM copper (40 vs. 18–24% decrease in the rate of ascorbate oxidation; data not shown).

O_2 uptake from the reaction of ascorbate, Cu(II) and O_2: Effects of PIH and catalase

Figure 7A shows that PIH, depending on the concentration (1–15 μM), inhibits O_2 uptake from the reaction of 10 μM Cu(II), 3 mM ascorbate and dissolved O_2 (220 μM). The rate of O_2 uptake (1.76 ± 0.06 nmol/sec) was inhibited by 79% in the presence of 10 μM PIH. Full inhibition of O_2 uptake was observed only at 15 μM PIH. The inhibition of ascorbate oxidation induced by PIH (see Fig. 6A) may prevent the formation of Cu(I), which is the copper species that reacts with O_2 (see reaction 2, above). However, O_2 may be generated from the spontaneous dismutation of O_2^{-} (reaction 4) and a considerable rate of O_2 uptake occurs in solutions containing 3 mM ascorbate but no added copper (0.31 ± 0.02 nmol/sec; data not shown). This O_2 uptake, which is fully inhibited by 10 μM PIH (data not shown), is possibly caused by μM contamination of metallic ions from phosphate buffer [28, 32]. Moreover, it is possible that the formation of a Cu(I)-PIH complex (see results below) could account for some O_2 uptake. These facts suggest that the observed effect of PIH on copper-mediated O_2 uptake may be underestimated. Taking these points into consideration, it is possible to assume that PIH inhibits O_2 uptake at a 1:1 ratio with copper.

Release of O_2 is observed after the addition of 1,000 U/mL catalase (after 3 min of reaction) to media containing 3 mM ascorbate, 10 μM Cu(II) and dissolved O_2 (Fig. 7B). As

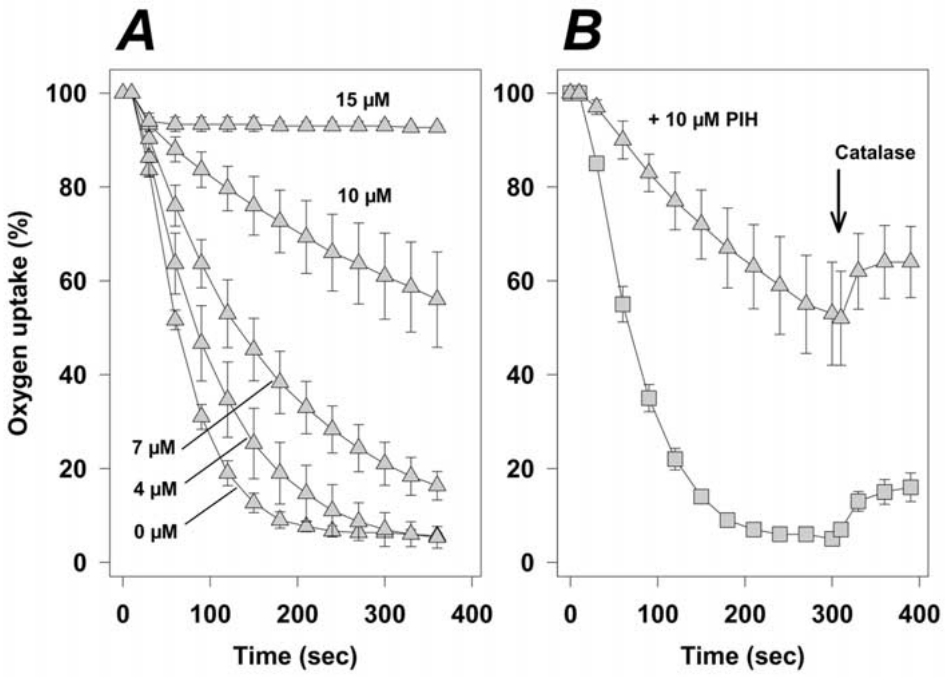

Fig. 7. (A) Time course of O_2 uptake of solutions containing 20 mM phosphate buffer (pH 7.2), 10 μM Cu(II), PIH (0–15 μM, as shown in (A)) and 3 mM ascorbate. (B) Effect of the addition of catalase (1,000 U/mL) to solutions in the absence or presence of 10 μM PIH. Data in (A) and (B) are means ± S.D. (n = 4).

expected, H_2O_2 is generated as an intermediary of these reactions (see reactions 3 and 4, above). Based on the percentage of O_2 recovery, we estimated that approximately 40 μM H_2O_2 is present in reaction media, both in the absence and in the presence of 10 μM PIH.

Characteristics of the copper-PIH complex

Figure 8 shows a UV-VIS spectra of PIH (20 μM) in the presence of various concentrations of Cu(II) in 20 mM phosphate buffer (pH 7.2). The PIH absorbance peaks at 295 and 385 nm decreased with increased Cu(II) concentrations up to 20 μM (a 15 nm red-shift occurred with the A_{295} peak with 20 μM copper). Moreover, the absorbance at 445 nm intensified with the increase in copper concentration, up to 20 μM. The absorption spectra of the copper complex with PIH was unchanged during a 30 min observation period. When A_{445} is measured as a function of Cu(II) concentration, in the presence of 20 μM PIH, there is a linear increase in absorbance up to 20 μM Cu(II) (inset to Fig. 8). However, the absorbance values at 445 nm remained unchanged at higher concentrations of copper (25–40 μM). Moreover, increasing PIH concentration from 5 to 50 μM, in phosphate-buffered solutions (pH 7.2) and with a fixed concentration of Cu(II) (20 μM), also induced a linear increase in A_{445} up to 20 μM PIH (data not shown). These data provide further evidence that a complex with a 1:1 ratio is formed between PIH and Cu(II) in aqueous solutions.

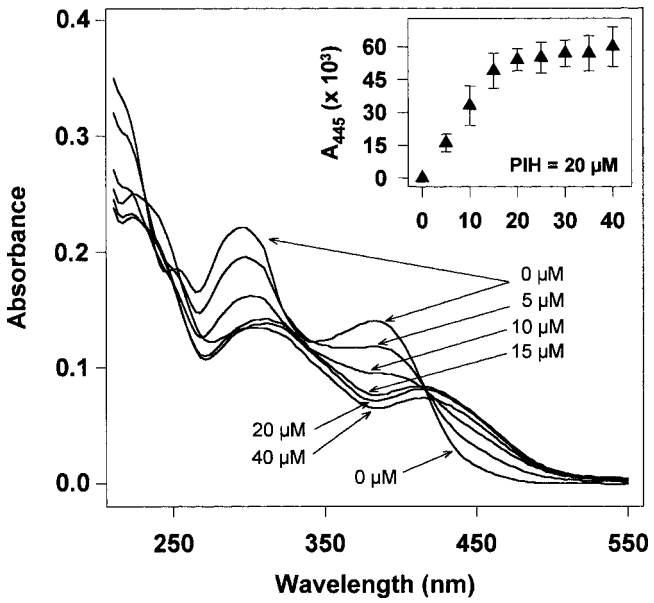

Fig. 8. Spectra of the complexes formed between 20 μM PIH and several concentrations of Cu(II) (0–40 μM) in 20 mM phosphate buffer (pH 7.2) recorded with buffer as the blank. Spectra were recorded with phosphate buffer as the blank. Spectra shown are representative of four independent determinations. Inset: Dependence of Cu(II) concentration on the absorbance, at 445 nm, of solutions containing 20 mM phosphate buffer (pH 7.2), 20 μM PIH and copper (0–40 μM, as indicated on the *x*-axis). A_{445} values were recorded with PIH plus phosphate buffer as the blank. Data are means ± S.D. (n = 4).

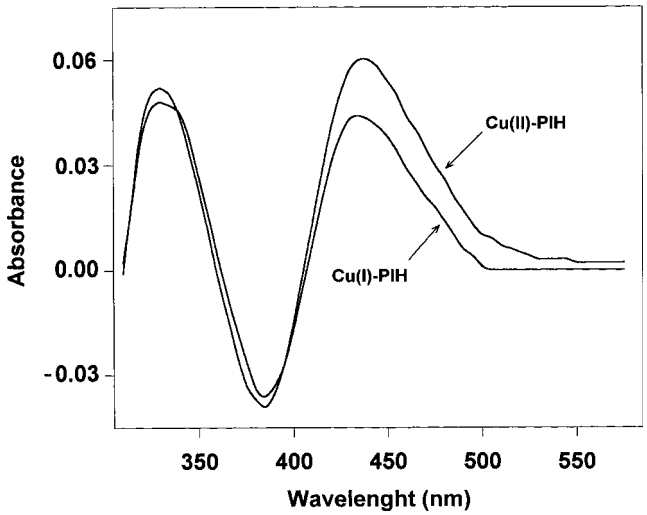

Fig. 9. Differential spectra of the copper-PIH complexes formed in 20 mM phosphate buffer (pH 7.2) after 5 min incubation of 20 μM Cu(II), in the absence or in the presence of 200 μM ascorbate, followed by the addition of 20 μM PIH. All the solutions were previously bubbled with N_2 gas for 20 min. Spectra were recorded with PIH plus phosphate buffer as the blank. Spectra shown are representative of three independent determinations.

Finally, 20 μM Cu(II) and 200 μM ascorbate were incubated in phosphate buffer for 5 min (in solutions previously bubbled with N_2 gas to prevent O_2-mediated redox-cycling of ascorbate [32] and copper) followed by the addition of 20 μM PIH. The observed differential spectra (i.e. using PIH as blank) on the 310–590 nm range was essentially the same as the one obtained in the absence of ascorbate (Fig. 9). The spectra in the 200–310 nm range was not analyzed due to the major interference of ascorbate, which shows a major absorbance in this region (the molar extinction coefficient of ascorbate at A_{265} in near-neutral pH is 14,500 $M^{-1}.cm^{-1}$ [32]) and undergoes fast oxidation in the presence of copper (see Fig. 6). These data strongly suggest that PIH can also form a complex with Cu(I). The Cu(I) complex with PIH seems to have the same absorption spectra in the 310–590 nm range as does Cu(II)-PIH.

Conclusions

The results of this work demonstrate that the copper-dependent formation of ·OH and 2-deoxyribose degradation (see reactions 1–6, above) is significantly decreased by PIH. This

may be a consequence of the inhibitory effect of PIH on copper-mediated ascorbate oxidation (see Fig. 6A), which is the first step toward ·OH formation. Thus, the complexation of Cu(II) by PIH makes the former unavailable for reduction by ascorbate with the consequent formation of Cu(I), a substrate of reactions 2, 3 and 5.

Copper plus ascorbate-mediated O_2 uptake (reaction 2) is, indeed, inhibited by PIH (Fig. 7A), possibly because the Cu (II) complex with PIH is not reactive with O_2. Moreover, Cu(I) complexed to PIH (Fig. 9) may be either poorly reactive with O_2 or present in a small concentration, which is why 'autoxidation' (see reaction 7, below) would contribute only in a minor way to O_2 uptake.

PIH may also directly inhibit the Fenton reaction with Cu(I) (reaction 5). This might occur if a Cu(I)-PIH complex were formed that is either (i) not reactive with H_2O_2 (μM levels of H_2O_2 are formed in reaction media in the absence or presence of PIH, see Fig. 7B) or (ii) that is oxidized to Cu(II)-PIH, thus decreasing the amount of Cu(I) that could react with H_2O_2. Oxidation of Cu(I)-PIH might occur via a mechanism that is O_2-dependent ('autoxidation'; see reaction 7), similarly as hypothesized for the case of Fe(II)-PIH [3]. When Fe(II) reacts with H_2O_2 there is evidence that PIH inhibits the formation of ·OH by a mechanism similar to (ii) [2, 3]. Be it as it may, the results of this study indicate that PIH diminishes the concentration of 'free' Cu(I) (a substrate for Fenton reaction) by inhibiting reaction 1.

$$\text{Cu(I)} + \text{PIH} \rightarrow \text{Cu(I)-PIH} + O_2 \rightarrow \text{Cu(II)-PIH} + O_2^- \qquad (7)$$

Several copper complexes show superoxide scavenging activity and/or superoxide dismutase-like activity [33, 34]. Thus, it is possible that these mechanisms occur in the case of the copper complex with PIH. Further studies are needed to determine whether PIH directly affects the rate of reactions 3 and 4. Moreover, we have presented evidence that PIH (at μM levels) does not have an antioxidant mechanism based on ·OH scavenging activity, i.e. inhibition of reaction 6 (see Fig. 4). We have previously determined that PIH (at 400 μM) inhibits 2-deoxyribose degradation mediated by Fe(III)-EDTA plus ascorbate by a mechanism that is not based on ·OH scavenging activity. Rather, Fe(III) forms a complex with PIH that cannot participate in Haber–Weiss reactions [1, 4].

DFO and d-penicillamine are able to quickly reduce Cu(II) to Cu(I) [35, 36], forming a stable complex with Cu(I) (of mixed valence in the case of d-penicillanime [37, 38]) that does not mediate ascorbate oxidation (data not shown), ·OH formation and 2-deoxyribose degradation (Fig. 2). This antioxidant mechanism, which is also based on copper chelation, is different from the one proposed for PIH against the copper-mediated formation of free radicals.

We have also presented evidence that Cu(II) forms a complex with PIH in aqueous solutions with a 1:1 ratio, as revealed by experiments on 2-deoxyribose degradation (see Figs 2 and 3) and ascorbate oxidation (Fig. 6) and the spectrometric analysis of the copper-PIH interaction (Fig. 8). Accordingly, preliminary voltametric studies have shown that the electrical signal of Cu(II) is extinguished by the addition of equimolar amounts of PIH in neutral solutions [39; M.S. Gonçalves M. Hermes-Lima and J.R. Souza, unpublished], also indicating the formation of a 1:1 complex. These results confirm Rao and Sing's [23] x-ray diffraction studies of the Cu(II) complex with PIH in the solid state.

Although d-penicillamine and TRIEN are the drugs of choice for the treatment of Wilson's disease, they both have advantages and disadvantages at the biochemical level. They can both inhibit carbon-centered radical adducts from alpha-(4-pyridyl-1-oxide)-N-tert-butylnitrone (4-POBN) formed from Cu(I) plus H_2O_2, in the presence of ethanol or DMSO [40]. However, O_2^- and H_2O_2 are formed by the aerobic oxidation of d-penicillamine mediated by copper [41], which could inflict cell damage, including DNA strand breaks [42]. In addition, thiyl radicals derived from d-penicillamine are formed in the presence of O_2 [43, 44] and could also be harmful to cells [44]. Since the structure of PIH lacks sulfur groups, thiyl radicals cannot possibly be formed. Furthermore, TRIEN is an inhibitor of superoxide dismutase [20] and may reduce the enzymatic antioxidant potential of tissues. TRIEN also increases bleomycin-induced mutagenesis in Chinese hamster ovary cells [20]. On the other hand, PIH is able to protect plasmid DNA against strand breaks induced by Fenton reagents [2].

The low toxicity of PIH, along with its low cost of synthesis, ability to cross biological membranes [5–7, 45], capacity to form a complex with copper [23, 24, 39, this work] (but not with calcium or magnesium [6]) and relevant antioxidant activity [1–4, 46, this work], suggest that PIH could be considered in possible therapeutic approaches for the management of oxidative stress associated with Wilson's disease. In this regard, further studies will be required to test the antioxidant activity and protective effects of PIH in more complexes *in vitro* systems and in animals/cells with copper-overload. It is also important to determine whether PIH induces excretion of excess copper and negative copper balance in laboratory animals.

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Notes

1. Determination of the characteristics of the d-penicillamine and Cu(II) complex under our experimental conditions is outside the scope of this work. However, it has been demonstrated that a mixed valence polymeric complex, which is strongly influenced by the ratio d-penicillamine: copper, pH and the concentration of O_2, is formed in aqueous solutions (in the absence of ascorbate) [37, 38].

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