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The iron chelator pyridoxal isonicotinoyl hydrazone (PIH) and its analogues prevent damage to 2-deoxyribose mediated by ferric iron plus ascorbate

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Abstract

Iron chelating agents are essential for treating iron overload in diseases such as β-thalassemia and are potentially useful for therapy in non-iron overload conditions, including free radical mediated tissue injury. Deferoxamine (DFO), the only drug available for iron chelation therapy, has a number of disadvantages (e.g., lack of intestinal absorption and high cost). The tridentate chelator pyridoxal isonicotinoyl hydrazone (PIH) has high iron chelation efficacy in vitro and in vivo with high selectivity and affinity for iron. It is relatively non-toxic, economical to synthesize and orally effective. We previously demonstrated that submillimolar levels of PIH and some of its analogues inhibit lipid peroxidation, ascorbate oxidation, 2-deoxyribose degradation, plasmid DNA strand breaks and 5,5-dimethylpyrroline-*N*-oxide (DMPO) hydroxylation mediated by either Fe(II) plus H_2O_2 or Fe(III)–EDTA plus ascorbate. To further characterize the mechanism of PIH action, we studied the effects of PIH and some of its analogues on the degradation of 2-deoxyribose induced by Fe(III)–EDTA plus ascorbate. Compared with hydroxyl radical scavengers (DMSO, salicylate and mannitol), PIH was about two orders of magnitude more active in protecting 2-deoxyribose from degradation, which was comparable with some of its analogues and DFO. Competition experiments using two different concentrations of 2-deoxyribose (15 vs. 1.5 mM) revealed that hydroxyl radical scavengers (at 20 or 60 mM) were significantly less effective in preventing degradation of 2-deoxyribose at 15 mM than 2-deoxyribose at 1.5 mM. In contrast, 400 μM PIH was equally effective in preventing degradation of both 15 mM and 1.5 mM 2-deoxyribose. At a fixed Fe(III) concentration, increasing the concentration of ligands (either EDTA or NTA) caused a significant reduction in the protective effect of PIH towards 2-deoxyribose degradation. We also observed that PIH and DFO prevent 2-deoxyribose degradation induced by hypoxanthine, xanthine oxidase and Fe(III)–EDTA. The efficacy of PIH or DFO was inversely related to the EDTA concentration. Taken together, these results indicate that PIH (and its analogues) works by a mechanism different than the hydroxyl radical scavengers. It is likely that PIH removes Fe(III) from the chelates (either Fe(III)–EDTA or Fe(III)–NTA) and forms a Fe(III)–PIH_2 complex that does not catalyze oxyradical formation.

Keywords: Chelator; Iron; Iron overload; Free radical; Antioxidant; Deferoxamine; Pyridoxal isonicotinoyl hydrazone

1. Introduction

Iron overload resulting from pathologies such as β-thalassemia are treated with the iron chelator deferoxamine (DFO). However, DFO is costly, requires long subcutaneous infusions (12–24 h/day, 5–6 times per week) and has a short plasma half-life [1–3]. Pyridoxal isonicotinoyl hydrazone (PIH), a condensation product of isonicotinic acid hydrazide and pyridoxal [4], may be a possible alternative for iron chelation therapy [1–3,5–10] since in vivo studies demonstrated that it can be administered orally, has low toxicity and induces excretion of excess iron and negative iron balance [2,10–12].

Iron-mediated oxidative stress is thought to be involved in several pathologies, including hemochromatosis, β-thalassemia, ischemic heart disease and cancer [13–16]. Iron is directly involved in cell damage by oxyradicals since it catalyzes the formation of highly reactive $^{\bullet}OH$ radicals through Haber–Weiss reactions [17–19]. Therefore, substances that are able to trap 'free iron' and make it unavailable for Haber–Weiss reactions act as antioxidants

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[14,20,21]. Thus, PIH may be a useful antioxidant because of its iron chelating characteristics. It has been demonstrated in vitro that PIH prevents iron-dependent lipid peroxidation, ascorbate oxidation, 2-deoxyribose degradation [22–24], plasmid DNA strand breaks [25] and 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) hydroxylation [24]. Bhattacharya et al. [26] also demonstrated that PIH has in vivo antioxidant activity in preventing retinal lipid peroxidation in newborn pigs.

The aim of the present work is to further document that PIH acts as an antioxidant mainly through its chelating properties and not via $^{\bullet}OH$ scavenging activity. We demonstrate that PIH removes Fe(III) from EDTA or NTA and forms a Fe(III)–PIH_2 complex that is unable to catalyze $^{\bullet}OH$ formation and the degradation of 2-deoxyribose.

2. Materials and methods

2-Deoxyribose, deferoxamine mesylate (DFO), dimethyl sulfoxide (DMSO), EDTA, hypoxanthine, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (Hepes), isonicotinic acid hydrazide, mannitol, nitrilotriacetic acid (NTA), salicylate, thiobarbituric acid (TBA) and xanthine oxidase were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytical purity.

The synthesis of PIH (3-hydroxy-5-(hydroxymethyl)-2-methyl-4-pyridine-carboxaldehyde 4-pyridinecarbonylhydrazone) and its analogues was performed by a Schiff base condensation of either pyridoxal or salicylaldehyde with a series of various acid hydrazides. Details of this procedure [8,27] and of the chemical identification of the products have been previously published [28,29]. The hydrazides used to react with pyridoxal were: isonicotinic acid hydrazide (forming PIH), benzoic acid hydrazide (forming PBH), *m*-fluorobenzoic acid hydrazide (forming compound 109), 2-thiophenecarboxylic acid hydrazide (forming compound 115). The hydrazide used to react with salicylaldehyde was isonicotinic acid hydrazide (forming SIH). Stock solutions (1.5 mM) of the iron chelators were prepared daily in 1 mM Hepes buffer, pH 7.2 [24,27].

Stock solutions of EDTA were neutralized with HCl/NaOH. Ferric chloride stock solutions (1 mM) were prepared daily in 10 mM HCl. Stock solutions of 1% TBA were prepared in 50 mM NaOH and used within one week. All solutions were made with milli-Q deionized water.

The formation of $^{\bullet}OH$ radicals was measured using 2-deoxyribose oxidative degradation. The principle of the assay is the quantification of the main 2-deoxyribose degradation product, malonaldehyde (MDA), by its condensation with TBA [18,21]. Typical reactions were started by addition of ascorbate (2 mM final concentration) to 0.5 ml of a solution containing 20 mM Hepes buffer (pH 7.2), 15 mM 2-deoxyribose, 25, 50 or 100 μM EDTA, 10 μM Fe(III) and 400 μM PIH (or no PIH). Ascorbate was added approximately 10 min after the addition of PIH. Reactions using enzymatic generation of oxyradicals are described in Table 2. Reactions were carried out for 30–60 min at 37°C in a shaking bath to ensure continuous flow of O_2 into the tubes and 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 at 532 nm of solutions were recorded. 'Zero time' absorbance values (where ascorbate is added to solutions after phosphoric acid and TBA) were subtracted from the values obtained for each experimental condition. PIH (300 μM) did not interfere with the reaction of MDA (formed from 10 μM Fe(III)–EDTA plus ascorbate) with TBA (data not shown).

The results shown as 'Damage to 2-deoxyribose (% of controls)' were calculated as: [(sample A_{532}−'zero time' A_{532})/(control A_{532}−'zero time' A_{532})]×100.

The kinetics of Fe(III)–PIH_2 complex formation was followed at 476 nm in solutions buffered with 20 mM Hepes (pH 7.2) [23]. PIH was added to a final concentration of 400 μM and A_{476} was followed at room temperature.

3. Results

Fig. 1 shows a comparison between PIH and classical $^{\bullet}OH$ scavengers in preventing 2-deoxyribose degradation induced by 10 μM Fe(III)–EDTA plus 2 mM ascorbate. Compared with the $^{\bullet}OH$ scavengers (DMSO, salicylate and mannitol), PIH was remarkably active in protecting

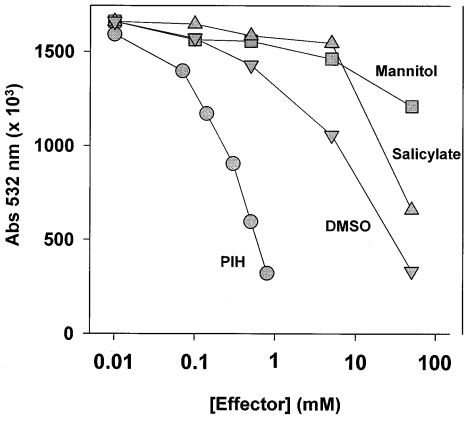

Fig. 1. Effect of $^{\bullet}OH$ scavengers (DMSO, salicylate or mannitol) and PIH on the oxidative degradation of 2-deoxyribose induced by Fe(III)–EDTA plus ascorbate. Solutions were incubated for 45 min at 37°C and contained 20 mM Hepes (pH 7.2), 15 mM 2-deoxyribose, 50 μM EDTA and 10 μM Fe(III). The concentrations of PIH or antioxidants are as indicated on the *x*-axis. Reactions were started by addition of ascorbate to a final concentration of 2 mM. The figure shows average values of 2–3 independent experiments. The A_{532} value for the control reaction (in the absence of PIH or scavengers) was 1.68±0.03, which represents ~3.8 μM malonaldehyde (using the extinction coefficient of 149 mM^{-1} [44] and the dilution factor of 3). The values of I_{50} described in the text were calculated using software designed by S. Brooks [45].

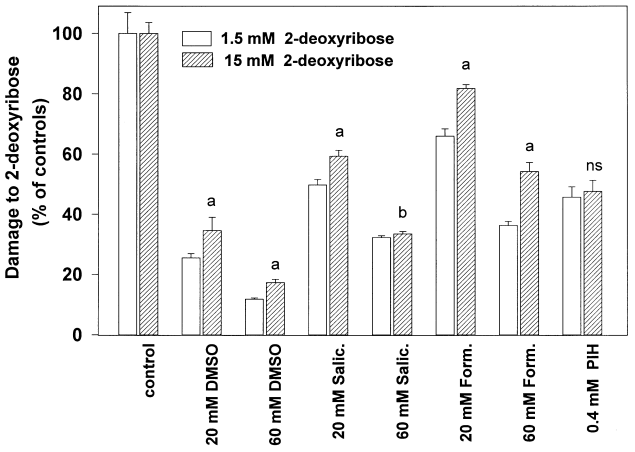

Fig. 2. Effect of PIH and •OH scavengers dimethyl sulfoxide (DMSO), salicylate (salic.) and formate (form.) on oxidative damage to 1.5 or 15 mM 2-deoxyribose. Experimental conditions are as described in the legend to Fig. 1, except that incubation times were 45 and 90 min for reactions containing 15 and 1.5 mM 2-deoxyribose, respectively. The figure shows means ± S.D. ($n = 4$). A_{532} values for controls were 0.41 ± 0.03 and 1.48 ± 0.05 for 1.5 and 15 mM 2-deoxyribose. Significance versus respective reaction with 1.5 mM 2-deoxyribose (one-tailed t-test): a, $P < 0.01$; b, $P < 0.025$; ns, not significant.

2-deoxyribose. PIH at 400 μM decreased 2-deoxyribose degradation by about 55% while at that concentration •OH scavengers decreased it by only 4–13%. These results are consistent with the fact that classical antioxidants are effective only in mM concentrations in aqueous solutions [21,30]. Moreover, PIH ($I_{50} = 0.3$ mM) was about two orders of magnitude more effective than DMSO ($I_{50} = 7$–11 mM), salicylate ($I_{50} = 40$–50 mM), mannitol ($I_{50} =$ about 100 mM) (Fig. 1) and ethanol ($I_{50} =$ about 100 mM; data not shown). This strongly suggests that PIH works as an antioxidant by a different mechanism than the •OH scavengers.

Competition studies were performed in order to evaluate the effectiveness of PIH and three •OH scavengers (DMSO, salicylate and formate) in protecting 1.5 or 15 mM 2-deoxyribose from iron-mediated oxidative damage (Fig. 2). The •OH scavengers (at 20 or 60 mM) protected 15 mM 2-deoxyribose significantly less than 1.5 mM 2-deoxyribose. For example, 20 mM DMSO prevented oxidative degradation of 1.5 mM 2-deoxyribose by 74.5%, but of 15 mM 2-deoxyribose by 65.4% ($P < 0.01$). On the other hand, 400 μM PIH was equally effective in preventing oxidative degradation of both 1.5 and 15 mM 2-deoxyribose. These results indicate that PIH, in contrast to DMSO, salicylate and formate, does not interfere with the reaction between 2-deoxyribose and •OH radicals and support the proposal that PIH acts by preventing •OH formation from Fe(III)–EDTA plus ascorbate rather than by trapping •OH radicals.

Titration of PIH in the 2-deoxyribose assay was performed with three different concentrations of EDTA at a fixed Fe(III) concentration of 10 μM (Fig. 3). Increasing the concentration of EDTA from 25 μM to 100 μM caused a significant reduction in the effectiveness of PIH. For example, the protection of 2-deoxyribose by 400 μM PIH was reduced from 75% to 30%. A similar experiment was performed with complexes of NTA and Fe(III). As in the previous experiment, the effectiveness of PIH was higher with 500 μM NTA than with 2 mM NTA. (Fig. 4). These results also show that NTA allows for a greater protection of 2-deoxyribose by PIH than EDTA (see I_{50} values in Table 1). Since NTA forms a weaker complex with Fe(III) compared with EDTA (see legend to Table 1), PIH more easily removes the metal from NTA resulting in more effective protection against iron-mediated damage to 2-deoxyribose.

We also followed the kinetics of Fe(III)–PIH_2 complex

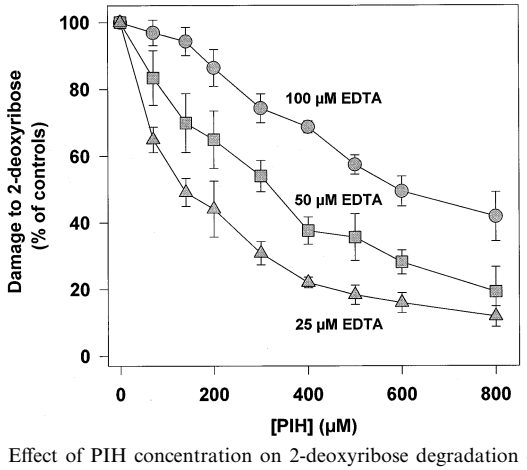

Fig. 3. Effect of PIH concentration on 2-deoxyribose degradation caused by Fe(III)–EDTA plus ascorbate. Solutions were incubated for 60 min at 37°C and contained 20 mM Hepes (pH 7.2), 15 mM 2-deoxyribose, 10 μM Fe(III)–EDTA (with 25, 50 or 100 μM EDTA), PIH (zero to 800 μM) and 2 mM ascorbate. The figure shows means ± S.D. ($n = 3$). A_{532} values for control reactions were 1.64 ± 0.15, 1.84 ± 0.03 and 1.85 ± 0.07 for 25, 50 and 100 μM EDTA, respectively.

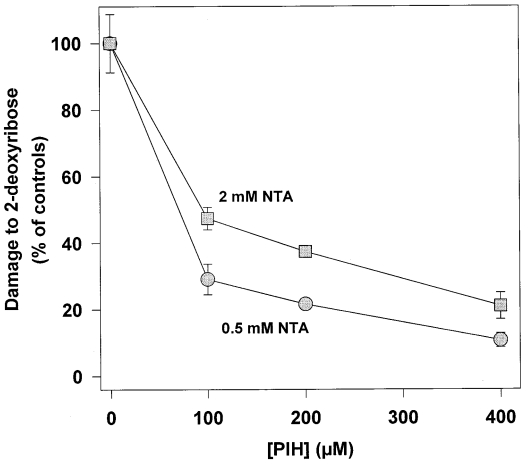

Fig. 4. Effect of PIH concentration on 2-deoxyribose degradation caused by Fe(III)–NTA plus ascorbate. Solutions and experimental conditions are as described in the legend to Fig. 1, except that the concentration of NTA was 0.5 mM or 2 mM. The figure shows means ± S.D. ($n = 3$–4). A_{532} values for control reactions were 0.87 ± 0.13 and 1.32 ± 0.12 for 0.5 and 2 mM NTA, respectively.

Table 1
PIH I_{50} values from titration experiments

Co-chelator	I_{50} (μM)
EDTA 25 μM	147 ± 23
EDTA 50 μM	315 ± 29
EDTA 100 μM	615 ± 20
NTA 500 μM	48 ± 24
NTA 2000 μM	99 ± 12

Values of I_{50} were calculated (see [45]) from data depicted in Figs. 3 and 4. The concentration of Fe(III) was 10 μM in all experiments. The formation constants (log β_{11}) for Fe(III)–NTA, Fe(III)–EDTA and Fe(III)–PIH_2 are 8.3, 25.5 and 24.8, respectively [9,46,47].

formation after addition of 400 μM PIH to solutions of 10 μM Fe(III)–EDTA (100 μM EDTA) or 10 μM Fe(III)–NTA (500 μM NTA). Fe(III)–PIH_2 complexes were formed much faster from Fe(III)–NTA (complexation completed within 30 s) than from Fe(III)–EDTA (completed within 30–40 min; $t_{1/2} = 10$ min) (data not shown). Differences in the formation constants for Fe(III)–NTA and Fe(III)–EDTA (see legend of Table 1) may account for these results. These experiments support the proposal that PIH prevents 2-deoxyribose degradation by removing iron from Fe(III)–EDTA or Fe(III)–NTA. The resulting complex, Fe(III)–PIH_2, does not catalyze oxyradical formation. Schulman et al. [22] observed that the efficiency of 150 μM PIH in protecting 2-deoxyribose from degradation induced by Fe(III)–EDTA plus ascorbate was reduced from 91% to 11% when the concentration of Fe(III)–EDTA was increased from 5 to 100 μM while the iron:EDTA ratio was kept at 1:1. This also indicates a competition between PIH and EDTA for Fe(III).

Although iron–EDTA and iron–NTA are not physiological iron complexes, they are models of intracellular low molecular mass iron complexes such as iron–citrate

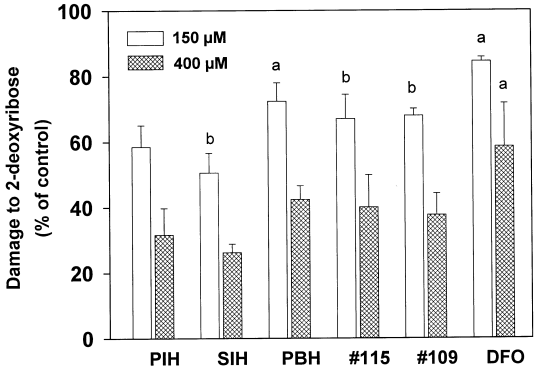

Fig. 5. Comparison of the effects of DFO, PIH and its analogues on the oxidative degradation of 2-deoxyribose. Experimental protocols are as described in the legend to Fig. 1, except that EDTA was 25 μM. Values are mean ± S.D. ($n = 3$, except for the experiments with 400 μM SIH, 400 μM PIH and 150 μM PIH where $n = 2$, 5 and 6, respectively). The A_{532} value for the control reaction was 1.54 ± 0.12. Significance versus corresponding results with PIH: a, $P < 0.01$; b, $P < 0.05$ (post-ANOVA one-tailed Dunnett's test).

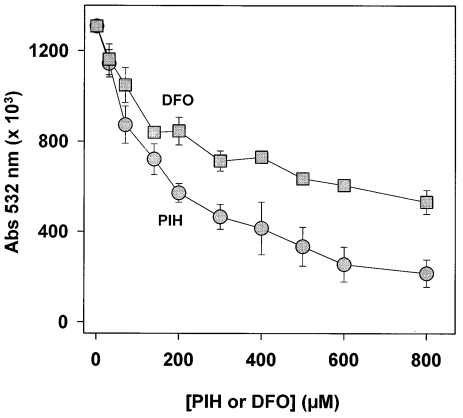

Fig. 6. Effect of concentration of PIH and DFO on the oxidative degradation of 2-deoxyribose induced by Fe(III)–EDTA plus ascorbate. Experimental protocols are as described in the legend to Fig. 1, except that EDTA was 25 μM. Values are mean ± S.D. ($n = 3$).

and iron–ATP [13,31,32]. PIH at 10 μM inhibits the degradation of 2-deoxyribose induced by 10 μM Fe(III)–citrate (with 2 mM citrate) plus 2 mM ascorbate by 66% and at 20 μM PIH by 77% (data not shown).

The antioxidant efficiency of PIH in the 2-deoxyribose assay was compared with that of other iron chelators. The effectiveness of 150 μM PIH was significantly higher than that of three of its analogues (PBH, #115 and #109) and DFO (Fig. 5). SIH at 150 μM was slightly more effective than PIH. However, at concentrations of 400 μM only DFO was less effective than PIH. Moreover, the effectiveness of 400 μM PIH was much higher than isonicotinic acid hydrazide (at 400 μM), a component of PIH structure that has less affinity for iron, which produced only 20% protection against 2-deoxyribose degradation (data not shown). Fig. 6 shows that DFO is less effective than PIH in a large range of concentrations (70 μM to 800 μM). Interestingly, Schulman et al. [22] observed that DFO was slightly more efficient than PIH in preventing Fe(III)-dependent 2-deoxyribose damage when phosphate rather Hepes was used in the assay. Since all the ferric iron chelators tested had comparable effectiveness, i.e., in the same order of magnitude, it would seem that DFO, PIH and PIH analogues have a similar mechanism of antioxidant action in preventing $^{\bullet}OH$ formation and 2-deoxyribose damage.

PIH also protects 2-deoxyribose from oxidative damage induced by hypoxanthine, xanthine oxidase and Fe(III)–EDTA. Superoxide radicals ($O_2^{\bullet-}$) formed from the hypoxanthine/xanthine oxidase system substitute for ascorbate in reducing Fe(III) to Fe(II). Three different concentrations of EDTA were tested (25, 50 and 100 μM) with 10 μM Fe(III) (Table 2). The protective efficiency of PIH (at 400 or 600 μM) or DFO (at 400 μM) was inversely related to the EDTA concentration. DFO was much less efficient than PIH at all EDTA concentrations tested ($P < 0.01$, one-tailed t-test), suggesting that the antioxidant actions of PIH and DFO are not only based on their ability to

Table 2
Effect of PIH on the oxidative degradation of 2-deoxyribose induced by 200 μM hypoxantine, 25 mU xanthine oxidase and 10 μM Fe(III)–EDTA (EDTA was 25 μM, 50 μM or 100 μM)

Conditions[a]	EDTA (μM)	Damage to 2-deoxyribose (% of respective controls)
400 μM DFO	100	88.9 ± 0.6
	50	80.5 ± 2.1[b]
	25	79.4 ± 14.0
400 μM PIH	100	30.5 ± 2.4
	50	26.2 ± 3.2
	25	21.3 ± 2.3[c,d]
600 μM PIH	100	23.0 ± 6.6
	50	17.0 ± 2.9
	25	10.2 ± 2.8[c,d]

[a]Reactions were started by adding xanthine oxidase and were incubated at 37°C for 30 min. A_{532} of samples in the absence of PIH or DFO were 0.532 ± 0.101 (at 25 μM EDTA), 0.599 ± 0.025 (50 μM EDTA) and 0.505 ± 0.066 (100 μM EDTA). Values are mean ± S.D. ($n = 3$).
[b]Significantly different from 100 μM EDTA, $P < 0.05$ (one-tailed t-test).
[c]Significantly different from 100 μM EDTA, $P < 0.01$ (one-tailed t-test).
[d]Significantly different from 50 μM EDTA, $P < 0.05$ (one-tailed t-test).

remove Fe(III) from EDTA but may be related to impairment of the reduction of Fe(III) by ascorbate or O_2^- radicals.

4. Discussion

We observed that PIH efficiently inhibits 2-deoxyribose degradation by Fe(III)–EDTA (or Fe(III)–NTA) plus ascorbate. The process of $^{\bullet}OH$ formation and 2-deoxyribose damage may occur via the following sequence of reactions [18,19,22]:

$$\text{Fe(III)} - \text{EDTA} + \text{ascorbate} \rightarrow \text{Fe(II)} - \text{EDTA} + \text{ascorbyl} \quad (1)$$

$$\text{Fe(II)} - \text{EDTA} + O_2 \rightarrow \text{Fe(III)} - \text{EDTA} + O_2^- \quad (2)$$

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (3)$$

$$H_2O_2 + \text{Fe(II)} - \text{EDTA} \rightarrow \text{Fe(III)} - \text{EDTA} + OH^- + {}^{\bullet}OH \quad (4)$$

$$^{\bullet}OH + \text{2-deoxyribose} \rightarrow \text{degradation products} \quad (5)$$

Hermes-Lima and coworkers [24,25] demonstrated that PIH prevents DMPO hydroxylation, 2-deoxyribose degradation and plasmid DNA damage caused by Fe(II) plus H_2O_2. It was proposed that PIH forms a transient complex with Fe(II) that is quickly autoxidized to Fe(III)–PIH_2, diminishing the concentration of Fe(II) available for the Fenton reaction and $^{\bullet}OH$ formation (Eq. 6).

$$\text{Fe(II)} + 2\text{PIH} \rightarrow \text{Fe(II)} - \text{PIH}_2 + O_2 \rightarrow \text{Fe(III)} - \text{PIH}_2 + O_2^- \quad (6)$$

The rate of O_2 consumption induced by Fe(II), in Hepes-buffered media (Eq. 2), is increased in the presence of PIH [23,24], suggesting that O_2^- is formed as a product of Eq. 6. This proposed mechanism for the antioxidant activity of PIH is different from that for the Fe(II) chelator 1,10-phenanthroline, which prevents $^{\bullet}OH$ formation from Fenton reactions by forming a complex with Fe(II) that cannot react with H_2O_2 [33].

Previous results [22–25] did not exclude the possibility that PIH reacts directly with $^{\bullet}OH$ (possibly by hydroxylation of one of the aromatic rings of PIH) and prevents 2-deoxyribose oxidation or DMPO hydroxylation. However, the present study provides evidence that 10–400 μM PIH does not prevent 2-deoxyribose degradation by trapping $^{\bullet}OH$. This is based on (i) the competition experiments using two concentrations of 2-deoxyribose (see Fig. 2), (ii) the PIH titration experiments using different concentrations of NTA or EDTA, with a fixed amount of Fe(III) (see Figs. 3 and 4), (iii) the quantitative difference (about two orders of magnitude) in the antioxidant activities of PIH and classical $^{\bullet}OH$ scavengers (see Fig. 1), and (iv) the similarity in effectiveness of PIH and other chelators (see Figs. 5 and 6) (the efficiency of 150 μM PIH was also similar to 1,10-phenanthroline, data not shown). These data indicate that PIH removes Fe(III) from Fe(III)–EDTA or Fe(III)–NTA and forms Fe(III)–PIH_2 that does not catalyze oxyradical formation (Eqs. 7, 8).

$$\text{Fe(III)} - \text{EDTA} + 2\text{PIH} \rightarrow \text{Fe(III)} - \text{PIH}_2 + \text{EDTA} \quad (7)$$

$$\text{Fe(III)} - \text{NTA} + 2\text{PIH} \rightarrow \text{Fe(III)} - \text{PIH}_2 + \text{NTA} \quad (8)$$

It is possible that PIH blocks the first step (Eq. 1) in the chain of reactions leading to $^{\bullet}OH$ formation from Fe(III)–EDTA plus ascorbate since Schulman et al. [22] observed that ascorbate oxidation induced by 20 μM Fe(III)–EDTA (Fe:EDTA, 1:1) is inhibited by PIH, SIH and DFO. The I_{50} value for PIH on the ascorbate oxidation reaction (90 μM) was compatible with the I_{50} values for the inhibition of $^{\bullet}OH$ formation from 20 μM Fe(III)–EDTA (Fe:EDTA, 1:1) plus 5 mM ascorbate in the 2-deoxyribose assay ($I_{50} = 65$ μM; [22]) or in the 2-keto-4-methylthiobutyric acid (KMB) assay ($I_{50} = 95$ μM; [22]), which measures ethylene produced from KMB oxidation. In addition, Mauricio and Hermes-Lima [34] observed that the effectiveness of PIH against ascorbate oxidation mediated by Fe(III)–EDTA is inversely related to the EDTA concentration, with a fixed amount of iron. Furthermore, PIH also inhibits the Fenton reaction by oxidizing Fe(II) to Fe(III) as described above (see Eq. 6).

We also demonstrated that when ascorbate is replaced by O_2^- (formed from hypoxanthine and xanthine oxidase) as the reducing agent for Fe(III)–EDTA, PIH is also effective in preventing 2-deoxyribose degradation. This suggests that PIH, by forming a complex with iron, slows the rate of Fe(III) reduction by O_2^-. Further research is needed to clarify the reaction between O_2^- and Fe(III)–PIH_2. Hermes-Lima and coworkers [25] found that PIH

also inhibits plasmid DNA strand breaks caused by hypoxanthine, xanthine oxidase and Fe(II).

Ferric iron forms a complex with two molecules of PIH in which the six coordination sites may become occupied [35,36], making Fe(III) less accessible for reduction by ascorbate and O_2^-. This may inhibit ferric iron-mediated $^{\bullet}OH$ formation and damage to target molecules. A similar mechanism has been proposed for the antioxidant activity of DFO [20].

Recently, Hermes-Lima and coworkers determined that PIH (1 to 10 μM) prevents Cu(II)-mediated ascorbate oxidation and 2-deoxyribose degradation induced by copper (1–10 μM) and 3 mM ascorbate due to the formation of Cu(II)–PIH that does not catalyze free radical formation [37,38]. Complexes of Cu(II) and PIH have been identified by X-ray diffraction [39] and amperometric techniques [37].

It is possible that the antioxidant mechanism proposed here for PIH may also operate in vivo. PIH can remove iron from low molecular mass iron complexes, such as iron–citrate [23], and from ferritin [40], forming Fe(III)–PIH_2 that cannot participate in Haber–Weiss reactions. Thus, PIH might attenuate oxidative stress, including lipid peroxidation [22] and DNA damage [25], in sites where iron is 'delocalized' or accumulated, such as hepatocytes of patients with iron overload [13] or organs suffering ischemia and reperfusion injury [14,41]. Indeed, Bhattacharya and coworkers [26] demonstrated that PIH is a functional antioxidant in vivo. They observed that PIH (at 10 mg/kg) prevents lipid peroxidation in retinas of newborn pigs subjected to asphyxia followed by reoxygenation. During reoxygenation, it is possible that iron plays a role in the induction of retinal oxidative stress. More recently, we demonstrated that Fe(II)–citrate-mediated lipid peroxidation in isolated rat liver mitochondria [31,32] can be prevented or attenuated by 10–300 μM PIH [23]. In this case, PIH seems to remove Fe(II) from citrate, forming a complex that does not induce lipid peroxidation [23].

The observations that PIH is relatively non-toxic for humans and rodents [1,2,42], transverses biological membranes [6,7,43], forms an excretable iron chelate [11,12], and has potent antioxidant activity in vitro ([22–25,37]; this work) and in vivo [26] suggest that it may be a powerful drug for treating illnesses related to iron-mediated oxidative stress.

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