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EPR spin trapping and 2-deoxyribose degradation studies of the effect of pyridoxal isonicotinoyl hydrazone (PIH) on •OH formation by the Fenton reaction

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Abstract

The search for effective iron chelating agents was primarily driven by the need to treat iron-loading refractory anemias such as β-thalassemia major. However, there is a potential for therapeutic use of iron chelators in non-iron overload conditions. Iron can, under appropriate conditions, catalyze the production of toxic oxygen radicals which have been implicated in numerous pathologies and, hence, iron chelators may be useful as inhibitors of free radical-mediated tissue damage. We have developed the orally effective iron chelator pyridoxal isonicotinoyl hydrazone (PIH) and demonstrated that it inhibits iron-mediated oxyradical formation and their effects (e.g. 2-deoxyribose oxidative degradation, lipid peroxidation and plasmid DNA breaks). In this study we further characterized the mechanism of the antioxidant action of PIH and some of its analogs against •OH formation from the Fenton reaction. Using electron paramagnetic resonance (EPR) with 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) as a spin trap for •OH we showed that PIH and salicylaldehyde isonicotinoyl hydrazone (SIH) inhibited Fe(II)-dependent production of •OH from H_2O_2. Moreover, PIH protected 2-deoxyribose against oxidative degradation induced by Fe(II) and H_2O_2. The protective effect of PIH against both DMPO hydroxylation and 2-deoxyribose degradation was inversely proportional to Fe(II) concentration. However, PIH did not change the primary products of the Fenton reaction as indicated by EPR experiments on •OH-mediated ethanol radical formation. Furthermore, PIH dramatically enhanced the rate of Fe(II) oxidation to Fe(III) in the presence of oxygen, suggesting that PIH decreases the concentration of Fe(II) available for the Fenton reaction. These results suggest that PIH and SIH deserve further investigation as inhibitors of free-radical mediated tissue damage.

Keywords: Iron; Pyridoxal isonicotinoyl hydrazone; Salicylaldehyde isonicotinoyl hydrazone; Free radical; Electron paramagnetic resonance; Fenton reaction

1. Introduction

Previous studies have shown that the iron chelator pyridoxal isonicotinoyl hydrazone (PIH) has antioxidant activity against iron-mediated oxyradical for-

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mation, including inhibition of their effects on 2-deoxyribose oxidation, lipid peroxidation and plasmid DNA strand breaks [1–3]. PIH is also effective in vivo since following its administration to newborn pigs it protected retinal function in animals exposed to stresses known to increase the generation of free radicals [2]. These results are of pharmacological relevance since PIH is being studied for use in iron overload therapy in β-thalassemia [4–7]. Desferal (DFO) therapy for iron overload in β-thalassemia is the only agent currently available; however, it is expensive, requires parenteral injection and is not without side effects [7,8]. On the other hand, PIH can be given orally and has low toxicity [5,7,9]. PIH has high affinity and specificity for iron ions and induces iron excretion in experimental iron overload [5–7,10].

It was proposed by Schulman and co-workers [1] that the formation of $^{\bullet}OH$ via an ascorbate/Fe(III)-EDTA/O_2 generating system is inhibited by PIH due to its iron chelating activity. Ferric-PIH_2 complexes [7] would not catalyze oxyradical formation and probably slow the rate of ascorbate-induced reduction of ferric iron (reaction 1).

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

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

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

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

The possibility that PIH could also interfere with the Fenton reaction was recently explored. Micromolar levels of PIH could prevent strand breaks in plasmid DNA induced by $^{\bullet}OH$ generated from Fe(II) and H_2O_2 [3], but the mechanism by which PIH inhibits the Fenton reaction was not fully investigated.

The present paper reports further studies on the mechanism of PIH inhibition of the Fenton reaction using electron paramagnetic resonance (EPR) with 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) as a spin trap for $^{\bullet}OH$ [11] and the 2-deoxyribose degradation assay [12]. Since iron overload diseases are connected to hepatic oxidative stress, including enhancement of hepatocarcinoma occurrence [3,13], these studies are of potential pharmacological importance.

2. Material and methods

2.1. Materials

2-Deoxyribose, DMPO, EDTA, H_2O_2, HEPES, ferrozine and thiobarbituric acid (TBA) were purchased from Sigma. DFO was obtained from Ciba-Geigy. PIH and SIH were synthesized as previously described [1,14]. All other reagents were of analytical grade and Milli-Q quality water was used in all solutions. Ferrous ammonium sulfate and DFO stock solution (1 mM) were freshly prepared in water just before use. TBA stock solution was prepared in 50 mM NaOH and DMPO stock solution was prepared in the presence of activated charcoal in order to remove impurities and the charcoal was removed by decantation. PIH and SIH stock solutions (1 mM) were freshly prepared in HEPES buffer (pH 7.2) as described by Baker et al. [14].

2.2. The EPR method

The EPR spectra were obtained at room temperature using a quartz liquid flat cell (Wilmad WG-812-Q) in a Te_{102} cavity of a Bruker ESP 300E spectrometer. The spectrometer was operated at a microwave frequency of 9.68 GHz, with microwave power of 16 mW, modulation frequency of 100 kHz and modulation amplitude of 3.2 G. For the spectra, center field was 3480 G, sweep width 200 G, sweep time 42 s (336 s for ethanol experiments) and time constant 1.28 ms (81.92 ms for ethanol experiments). Typical experiments were started with addition of 50 μl Fe(II) to a flat cell containing 10 mM HEPES buffer (pH 7.2), DMPO (50 mM), H_2O_2 and chelator (final reaction volume of 1 ml) followed by a 3 min period for collection of the EPR signal. In the ethanol experiments 10 mM phosphate buffer (pH 7.2) was used instead of HEPES.

2.3. 2-Deoxyribose oxidative degradation and ferrozine assays

2-Deoxyribose oxidative degradation was deter-

mined as previously described [12], with slight modifications. Reactions were started by addition of Fe(II) to 0.5 ml incubation media containing 15 mM 2-deoxyribose, H_2O_2 (0–450 μM), iron chelator (0–200 μM) and HEPES 20 mM (pH 7.2). Reactions were carried out for 5 min at room temperature. Addition of 0.5 ml of 3% H_3PO_4 followed by 0.5 ml of 1% TBA (w/v, in 50 mM NaOH) was used to stop the reactions. After boiling for 15 min, absorbance at 532 nm was determined. Fe(II) was measured by addition of 0.5 ml of 15 mM ferrozine to 0.5 ml reaction media. The Fe(II)-ferrozine complex was then quantified at 562 nm.

3. Results

3.1. Effect of PIH on the Fenton reaction: EPR spin trapping studies

The hydroxylation of DMPO by $^{\bullet}OH$ (reactions 5 and 6) was started by addition of Fe(II) (50 μM final concentration) to the incubation medium containing 50 mM DMPO, 10 mM HEPES (pH 7.2) and 300 μM H_2O_2. A 4-lined spectrum was obtained (Fig. 1, Exp. A) and showed hyperfine splitting constants of $a_N = a_H = 14.9$ G, that is typical of the spin adduct DMPO-OH [11,15]. Iron alone could not induce DMPO hydroxylation and neither could the addition of DMPO 10 s after the inclusion of Fe(II) in the reaction (Fig. 1, Exp. B and C, respectively). PIH at 50 μM reduced the signal intensity approx. 57% without changes in a_N and a_H values (see Exp. D in Fig. 1). The effect of 50 μM DFO on DMPO hydroxylation was comparable to that of PIH (60% reduction in signal intensity; see Exp. F in Fig. 1).

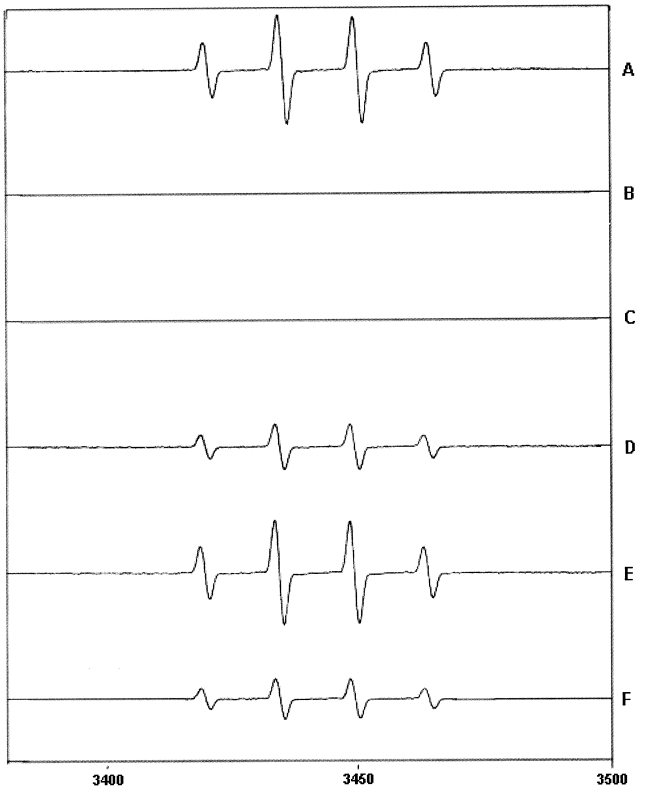

Fig. 1. EPR spectra of DMPO-OH adducts produced by Fenton reagents (50 μM Fe(II) plus 300 μM H_2O_2) in 10 mM HEPES buffered media (pH 7.2) containing 50 mM DMPO (Exp. A); B, H_2O_2 not included; C, DMPO added 10 s after Fenton reagents; D, 50 μM PIH plus Fenton reagents; E, 50 μM PIH added 10 s after Fenton reagents; F, 50 μM DFO plus Fenton reagents. These are representative spectra of experiments done at least twice.

Table 1
Effect of iron chelators on the EPR signal of DMPO-OH induced by 50 μM Fe(II) and 300 μM H_2O_2

Conditions[a]	Peak height[b]	Protection (%)
No chelator	7.44 ± 0.52 (7)[c]	–
PIH	3.12 ± 0.37 (8)	58
SIH	3.09 ± 0.49 (2)	58
DFO	2.72 ± 0.27 (4)	63
EDTA	2.77 ± 0.03 (3)	63

[a]Experimental conditions are as described in Section 2. Concentration of iron chelators was 50 μM.
[b]The intensity of the DMPO-OH signal was calculated as described in the legend to Fig. 2.
[c]Values are means ± S.D.; *n* values are in parentheses.

$$H_2O_2 + Fe(II) \rightarrow Fe(III) + OH + {}^{\bullet}OH \quad (5)$$

$$DMPO + {}^{\bullet}OH \rightarrow DMPO\text{-}OH \quad (6)$$

As a control, PIH was added 3 min after incubation of H_2O_2 and Fe(II) and no significant decrease in the control EPR signal was observed (Fig. 1, Exp. E). This demonstrated that PIH does not catalyze the decomposition of DMPO-OH but inhibits DMPO-OH formation (reaction 6) from Fenton reagents.

We analyzed the effect of PIH on DMPO hydroxylation by titrating the iron chelator (Fig. 2). Increasing PIH concentration in the presence of 300 μM H_2O_2 and 50 μM Fe(II) diminished the intensity of

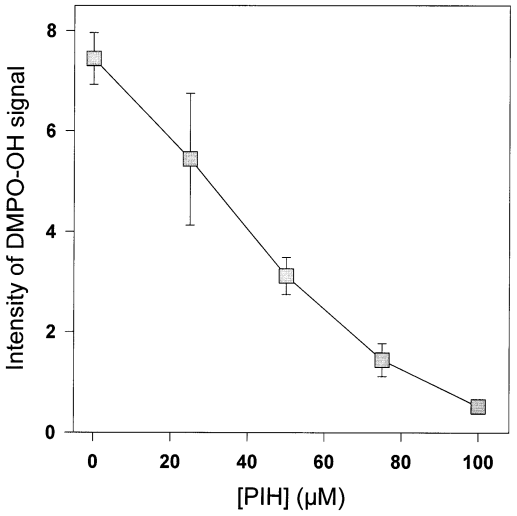

Fig. 2. Effect of PIH concentration on the intensity of DMPO-OH signal represented in arbitrary units. Hydroxylation of 50 mM DMPO was induced by 300 μM H_2O_2 plus 50 μM Fe(II) in HEPES buffered media, pH 7.2 (see Section 2). The signal intensities (peak height) were obtained from the amplitude of the first line for each 4-lined signal. Values are means ± S.D. ($n = 7$ for control, 8 for 50 μM PIH, and 3–2 for the others). The I_{50} for this experiment was calculated using a software designed by Brooks [25].

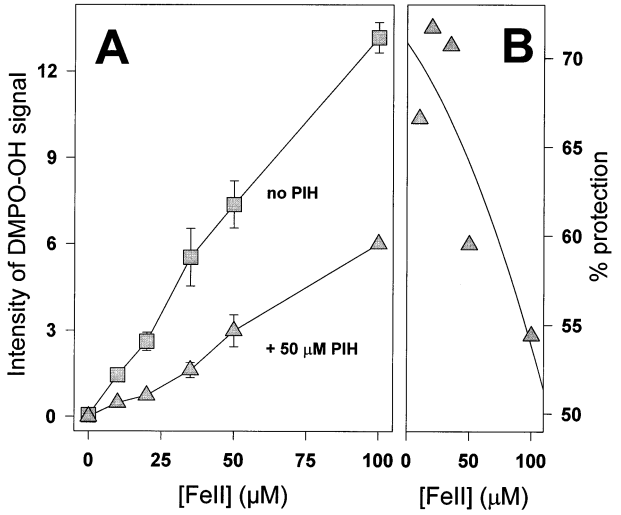

Fig. 3. Effect of Fe(II) concentration on the intensity of DMPO-OH signal represented in arbitrary units (A). Hydroxylation of 50 mM DMPO was induced by 300 μM H_2O_2 plus Fe(II) (from 0 to 100 μM), in the absence or in the presence of 50 μM PIH. Values are means ± S.D. ($n = 2$–3). Further information is in the legend to Fig. 2. Panel B is a replot of data from panel A and depicts the % protection induced by PIH against DMPO hydroxylation. The line in the figure is a 2nd order regression.

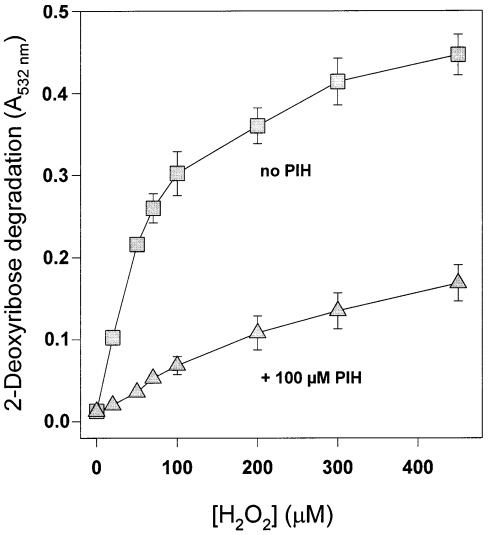

Fig. 4. Dependence of H_2O_2 concentration on the oxidative degradation of 2-deoxyribose (as Abs 532 nm), in the absence or in the presence of 200 μM PIH. Fe(II) and 2-deoxyribose were 30 μM and 15 mM, respectively. Experiments were conducted at room temperature as described in Section 2. Values are means ± S.D. ($n = 3$).

the EPR signal. The I_{50} value for PIH was 40 ± 3 μM (see legend to Fig. 2). The antioxidant activity of PIH at 50 μM was not significantly different from that of SIH (a structural analogue of PIH), EDTA and DFO (Table 1).

Fig. 3A shows that the intensity of the EPR signal was augmented with increases in iron levels, from zero to 100 μM, in a fixed concentration of H_2O_2 (300 μM). Fifty μM PIH reduced EPR peak height in all iron concentrations. The effectiveness of PIH was inversely related to the concentration of Fe(II) (Fig. 3B); protection by PIH was nearly 70% at 10–35 μM Fe(II) and 60–55% at 50–100 μM Fe(II).

3.2. *PIH effect on the Fenton reaction: 2-deoxyribose degradation studies*

Oxidative degradation of 2-deoxyribose with formation of malonaldehyde is a reliable assay for $^{\bullet}OH$ formation [1,12]. Fig. 4 shows that 100 μM PIH protects 2-deoxyribose (15 mM) from oxidative degradation induced by 30 μM Fe(II) and increasing concentrations of H_2O_2 in 20 mM HEPES (pH 7.2).

PIH was titrated in the 2-deoxyribose oxidative degradation reaction induced by 100 μM H_2O_2 and

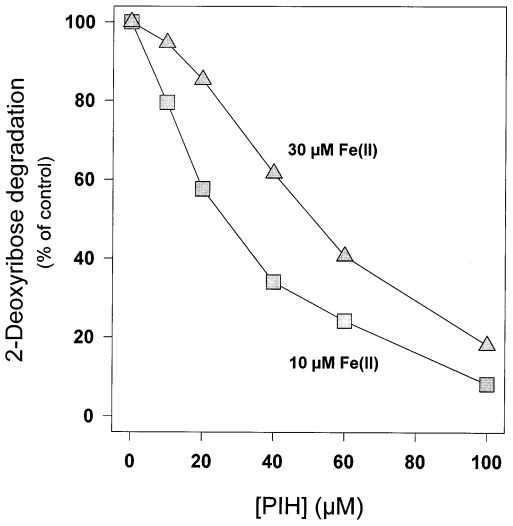

Fig. 5. Dependence of PIH concentration on 2-deoxyribose degradation (% of control) induced by 100 μM H_2O_2 and Fe(II) (10 or 30 μM). Values on the *y*-axis represent the average of 5–6 determinations. The I_{50} values were determined according to Brooks [25].

10 or 30 μM Fe(II) (Fig. 5). The data show that PIH is a more effective antioxidant with 10 μM Fe(II) ($I_{50} = 26 \pm 5$ μM) than with 30 μM Fe(II) ($I_{50} = 51 \pm 3$ μM). The effect of 100 μM PIH on 2-deoxyribose degradation mediated by 100 μM H_2O_2 and 30 μM Fe(II) was compared to other chelators at 100 μM. The protective effect of PIH (78%) was similar to that of EDTA (77%), but higher than those of SIH (57%) and DFO (58%) (data not shown).

Fig. 6A shows the dependence of iron concentration on the oxidative degradation of 2-deoxyribose, in media containing 100 μM H_2O_2 and PIH (0, 20 or 100 μM). The effectiveness of PIH was inversely related to Fe(II) concentration (Fig. 6B). Interestingly, the lowest efficiency of 100 μM PIH was reached at 100 μM iron, and the lowest level of protection by 20 μM PIH was reached at about 20 μM iron.

3.3. EPR studies of the ethanol effect on PIH antioxidant activity

Ethanol can be oxidized by $^{\bullet}OH$, generated from Fenton reagents, to an ethanol radical, which can be trapped by DMPO (reactions 7 and 8). Ethanol is assumed not to change the primary products of the Fenton reaction [16]. The DMPO-$CH(OH)CH_3$ adduct (DMPO-Et) has a typical 6-lined spectrum with parameters of $a_N = 16.0$ G, $a_H = 23.1$ G [17]. The EPR spectrum of the DMPO-Et adduct, together with the DMPO-OH spectrum (see reaction 6), could be detected in 10 mM phosphate buffer (pH 7.2), in media containing 2 M ethanol and Fenton reagents (Fig. 7, Exp. A). The DMPO-Et spectrum was undetected in media containing HEPES buffer (data not shown).

$$^{\bullet}OH + CH_2(OH)CH_3 \rightarrow H_2O + {}^{\bullet}CH(OH)CH_3 \quad (7)$$

$$^{\bullet}CH(OH)CH_3 + DMPO \rightarrow$$

$$DMPO\text{-}CH(OH)CH_3\ (DMPO - Et) \quad (8)$$

PIH at 100 μM diminished by approx. 64% the DMPO-Et/DMPO-OH signals (Fig. 7, Exp. B). The chelator also reduced the intensity of the DMPO-OH signal obtained in phosphate buffer to a comparable extent (67% reduction; data not shown). Similar results were observed for the antioxidant activity of DFO at 100 μM (Fig. 7, Exp. C). This indicates that both PIH and DFO did not directly inhibit DMPO-Et formation (see reaction 8), but rather they inhibited $^{\bullet}OH$-mediated ethanol radical formation (reaction 7) by blocking $^{\bullet}OH$ formation.

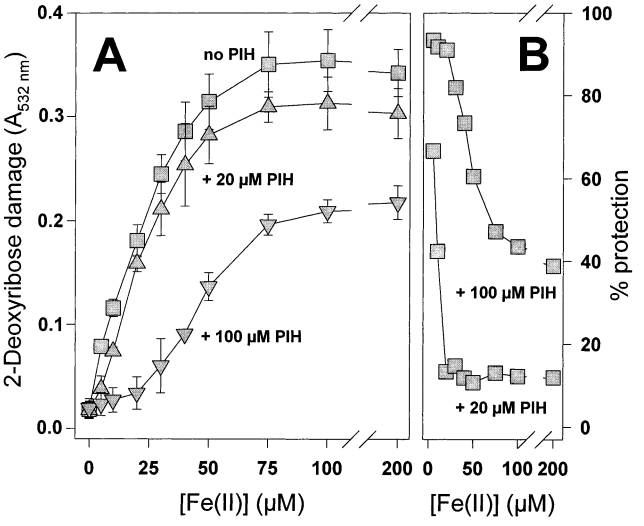

Fig. 6. Effect of different Fe(II) concentrations on the degradation of 2-deoxyribose, in the absence or presence of 20 or 100 μM PIH (A). H_2O_2 was 100 μM. Values are means ± S.D. ($n = 6$). Panel B is a replot of data from panel A and depicts the % reduction in 2-deoxyribose degradation induced by PIH.

3.4. Effect of PIH on the autoxidation of Fe(II)

Fig. 8 shows the effect of PIH on the rate of Fe(II) oxidation to Fe(III) in HEPES buffered media, as measured by the time dependent disappearance of Fe(II). Oxidation of Fe(II) was manifold faster in the presence of 50 μM PIH than in its absence and even higher in the presence of 100 μM PIH. Moreover, O_2 uptake (measured with a Clark electrode) during oxidation of Fe(II) (50 μM) to Fe(III) showed an increase in the rate of O_2 disappearance from media containing PIH at 300 μM [18].

However, in media previously saturated with N_2, to remove O_2 from solution, no detectable Fe(II) oxidation occurred within 20 min either in the absence or presence of 100 μM PIH (Fig. 8). These results demonstrate that PIH speeds up the oxidation of Fe(II) to Fe(III) by enhancing the rate of Fe(II) oxidation by O_2.

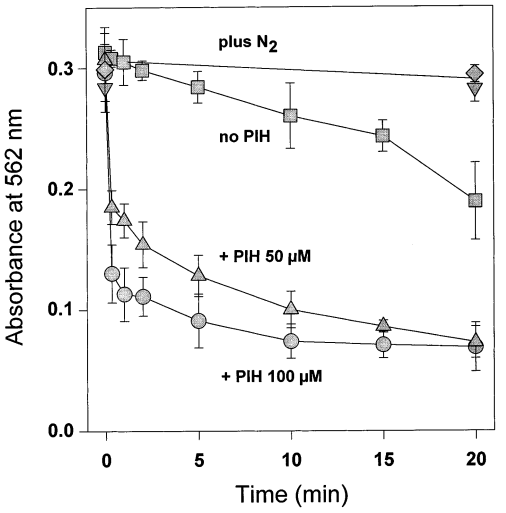

Fig. 8. Effect of PIH on the autoxidation of 30 μM Fe(II) in 20 mM HEPES buffer (pH 7.2). Fe(II) concentration was measured as Abs 562 nm (see Section 2). As indicated in the figure ('plus N_2'), two experiments were run in media previously bubbled with N_2 gas (in the absence or presence of 100 μM PIH). Values are means ± S.D. ($n = 4$–6).

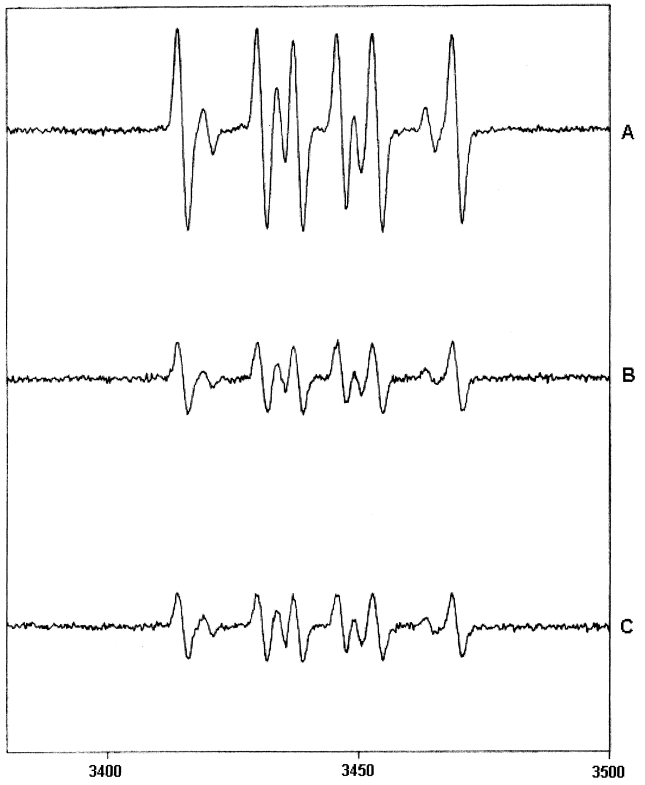

Fig. 7. EPR spectra showing the effect of ethanol and iron chelators (PIH or DFO at 100 μM) on the Fenton reaction. A, reactions were performed in 10 mM phosphate buffer, pH 7.2, in the presence of 50 mM DMPO, 2 M ethanol, 50 μM Fe(II) and 300 μM H_2O_2; B, PIH+H_2O_2+Fe(II)+ethanol; C, DFO+H_2O_2+Fe(II)+ethanol. These are representative spectra of experiments done twice.

4. Discussion

The present paper provides evidence that the iron chelator PIH diminishes the formation of $^{\bullet}OH$ in vitro by inhibiting the Fenton reaction. This was demonstrated with experiments on DMPO hydroxylation (Figs. 1–3) and 2-deoxyribose degradation (Figs. 4–6) and was corroborated by the observation that PIH inhibits $^{\bullet}OH$-mediated ethanol radical formation (Fig. 7). Ethanol radical can be formed by oxidizing species other than $^{\bullet}OH$, such as FeO^{2+} [18,19]. However, in the ethanol experiments PIH reduced the height of the lines of the EPR signals from both DMPO-OH and DMPO-Et adducts with the same effectiveness (see Exp. B on Fig. 7). This suggests that PIH does not change the primary products of the Fenton reaction.

The effectiveness of PIH, in both EPR spin trapping and 2-deoxyribose degradation experiments, was inversely related to iron concentration, indicating that PIH acts as an antioxidant by chelating iron ions. The efficiency of PIH in preventing DMPO-OH

formation started to decrease when a 1:1 molar ratio between iron and PIH was approached (Fig. 3). Similarly, the effectiveness of PIH in inhibiting 2-deoxyribose degradation reached its lowest level at an iron:PIH ratio of 1:1 (Fig. 5). These data, taken together, indicate that a Fe(II)-PIH (1:1) complex is formed during the PIH-dependent inhibition of $^{\bullet}OH$ formation. Schulman et al. [1] also observed an inverse relationship between the effectiveness of PIH in preventing 2-deoxyribose degradation (caused by Fe(III)-EDTA/ascorbate/O_2) and the concentration of Fe(III)-EDTA.

Moreover, the PIH tritation study demonstrated that the antioxidant activity of PIH in the 2-deoxyribose degradation assay was less efficient in the presence of high Fe(II) levels (30 μM; see Fig. 5). This suggests that PIH and H_2O_2 compete for reaction with Fe(II). Fe(II) may form a transient complex with PIH that is quickly oxidized by O_2 to a stable Fe(III)-PIH_2 complex. PIH could increase the rate of Fe(II) autoxidation because of its much greater affinity for Fe(III) than for Fe(II). The formation constant ($\log \beta_{11}$) for the complexes between PIH and Fe(II), and between PIH and Fe(III), at neutral pH are 12.5 and 24.8, respectively [9,20]. Thus, PIH may change the equilibrium of the autoxidation reaction and enhance the rate of Fe(III) formation (in the scheme below the rate constant K_2 is much higher than K_1).

$$\mathrm{Fe(II)} + \mathrm{O_2} \xrightarrow{K_1} \mathrm{Fe(III)} + \mathrm{O_2^-} \quad (9)$$

$$\mathrm{Fe(II)} + \mathrm{PIH} \rightarrow \mathrm{PIH\text{-}Fe(II)}$$

$$+\mathrm{O_2} + \mathrm{PIH} \xrightarrow{K_2} \mathrm{Fe(III)\text{-}PIH_2} + \mathrm{O_2^-} \quad (10)$$

By accelerating Fe(II) autoxidation (reaction 10), PIH would decrease the concentration of Fe(II) available for the Fenton reaction, thus reducing the amount of $^{\bullet}OH$ formed. EDTA and DFO are also able to increase the rate of Fe(II) oxidation [3,21] suggesting that these chelators act as antioxidants in the Fenton reaction with a mechanism similar to PIH. However, it has been observed that DFO, but not EDTA, induces H_2O_2 formation and DMPO hydroxylation during the autoxidation of Fe(II) [21]. On the other hand, PIH is able to block 2-deoxyribose degradation during the autoxidation of Fe(II), at neutral pH, indicating that no $^{\bullet}OH$-like species are formed (R. Araujo, M. Hermes-Lima, unpublished). Moreover, EDTA enhances the rate of $^{\bullet}OH$ formation from Fe(III) in the presence of a reducing agent [12]. In contrast, the Fe(III)-PIH_2 complex does not catalyze oxyradical formation [1] and therefore can be considered as a safe form of iron to be excreted in iron overload therapy.

The antioxidant efficiency of PIH toward the Fenton reaction was similar to DFO in the EPR experiments (Table 1), but slightly higher in the 2-deoxyribose assay (see Section 3), differences which cannot be explained at the moment. However, these results may be of pharmacological relevance, since PIH might be a better antioxidant than DFO in preventing iron-catalyzed oxyradical formation in vivo. Moreover, PIH is lipophilic, while DFO is highly hydrophilic [22]. Therefore, PIH transverses biological membranes and accesses intracellular iron pools more efficiently [7,23].

Finally, these studies add further insights on the mechanism of the antioxidant activity of PIH. When $^{\bullet}OH$ radicals are formed from Fe(III) plus ascorbate (see reactions 1–5), a process that supposedly occurs in vivo [12,24], PIH can inhibit both ascorbate oxidation (reaction 1) [1] and Fenton reactions (reactions 4 and 5). Furthermore, PIH accelerates Fe(II) autoxidation (reactions 2, 9 and 10) and thus decreases the availability of Fe(II) to participate in $^{\bullet}OH$-producing Fenton reactions. Thus, PIH is an iron chelator with antioxidant activity [1–3] that might be useful for experimental therapy in β-thalassemia and other pathologies related to iron-mediated oxidative stress.

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References

[1] H.M. Schulman, M. Hermes-Lima, E.M. Wang, P. Ponka, In vitro antioxidant properties of the iron chelator pyridoxal isonicotinoyl hydrazone (PIH) and some of its analogs, Redox Rep. 1 (1995) 373–378.

[2] M. Bhattacharya, P. Ponka, P. Hardy, N. Hanna, D.R. Varma, P. Lachapelle, S. Chemtob, Prevention of postasphyxia electroretinal dysfunction with a pyridoxal hydrazone, Free Radic. Biol. Med. 22 (1997) 11–16.

[3] M. Hermes-Lima, E. Nagy, P. Ponka, H.M. Schulman, The iron chelator pyridoxal isonicotinoyl hydrazone (PIH) protects plasmid pUC-18 DNA against $^{\bullet}OH$-mediated strand breaks, Free Radic. Biol. Med. (1998) in press.

[4] P. Ponka, J. Borova, J. Neuwirt, O. Fuchs, Mobilization of iron from reticulocytes. Identification of pyridoxal isonicotinoyl hydrazone as a new iron chelating agent, FEBS Lett. 97 (1979) 317–321.

[5] G.M. Brittenham, Pyridoxal isonicotinoyl hydrazone. Effective iron chelation after oral administration, Ann. NY Acad. Sci. 612 (1990) 315–326.

[6] P. Ponka, Physiology and pathophysiology of iron metabolism: implications for iron chelation therapy in iron overload, in: J.J.M. Bergeron, G.M. Brittenham (Eds.), The Development of Iron Chelators for Clinical Use, CRC Press, Boca Raton, FL, 1994, pp. 1–32.

[7] D.R. Richardson, P. Ponka, Pyridoxal isonicotinoyl hydrazone and its analogues: potential orally effective iron chelating agents for the treatment of iron overload disease, J. Lab. Clin. Med. 131 (1998) 306–314.

[8] C. Hershko, Iron chelators, in: J.H. Brock, J.W. Halliday, M.J. Pippard, L.W. Powell (Eds.), Iron Metabolism in Health and Disease, W.B. Saunders, London, 1994, pp. 391–426.

[9] J. Webb, M.L. Vitolo, Pyridoxal isonicotinoyl hydrazone (PIH): a promising new iron chelator, Birth Defects Orig. Art. Ser. 23, (5B) (1998) 63–70.

[10] D.R. Richardson, G.T. Hefter, P.M. May, J. Webb, E. Baker, Iron chelators of the pyridoxal isonicotinoyl hydrazone class III. Formation constants with calcium(II), magnesium(II) and zinc(II), Biol. Metals 2 (1989) 161–167.

[11] G.R. Buettner, Spin trapping: ESP parameters of spin adducts, Free Radic. Biol. Med. 3 (1987) 259–303.

[12] M. Hermes-Lima, E.M. Wang, H.M. Schulman, K.B. Storey, P. Ponka, Deoxyribose degradation catalyzed by Fe(III)EDTA: kinetic aspects and potential usefulness for submicromolar iron measurements, Mol. Cell. Biochem. 137 (1994) 65–73.

[13] R.S. Britton, B.R. Bacon, A.S. Tavill, Mechanisms of iron toxicity, in: J.H. Brock, J.W. Halliday, M.J. Pippard, L.W. Powell (Eds.), Iron Metabolism in Health and Disease, W.B. Saunders, London, 1994, pp. 311–351.

[14] E. Baker, D. Richardson, S. Gross, P. Ponka, Evaluation of the iron chelation potential of hydrazones of pyridoxal, salicylaldehyde and 2-hydroxy-1-naphthylaldehyde using the hepatocyte in culture, Hepatology 15 (1992) 492–501.

[15] S.P. Sanders, S.J. Harrison, P. Kuppusamy, J.T. Sylvester, J.L. Zweier, A comparative study of EPR spin trapping and cytochrome C reduction techniques for the measurement of superoxide anions, Free Radic. Biol. Med. 16 (1994) 753–761.

[16] J. Jiang, J.F. Bank, C.P. Scholes, Subsecond time-resolved spin trapping followed by stopped-flow EPR of Fenton reaction products, J. Am. Chem. Soc. 115 (1993) 4742–4746.

[17] L.A. Reinke, J.M. Rau, P.B. McCay, Characteristics of an oxidant formed during iron(II) autoxidation, Free Radic. Biol. Med. 16 (1994) 485–492.

[18] N.C.F. Santos, Quantificação da Ação Antioxidante do Piridoxal Isonicotinoil Hidrazona (PIH) contra o Estresse Oxidativo Induzido por Íons Ferro, M.Sc. Thesis, University of Brasilia, Brasilia, 1998.

[19] I. Yamazaki, L.H. Piette, ESR spin-trapping studies on the reaction of Fe^{2+} ions with H_2O_2-reactive species in oxygen toxicity in biology, J. Biol. Chem. 23 (1990) 13589–13594.

[20] L.M.W. Vitolo, G.T. Hefter, B.W. Clare, J. Webb, Iron chelators of the pyridoxal isonicotinoyl hydrazone class. Part 2. Formation constants with iron(III) and iron(II), Inorg. Chim. Acta 170 (1990) 171–174.

[21] S.J. Klebanoff, A.M. Waltersdorph, B.R. Michel, H. Rosen, Oxygen-based free radical generation by ferrous ions and deferroxamine, J. Biol. Chem. 264 (1989) 19765–19771.

[22] D.R. Richardson, M.L. Wis Vitolo, G.T. Hefter, P.M. May, B.W. Clare, J. Webb, P. Wilairat, Iron chelators of the pyridoxal isonicotinoyl hydrazone class. Part I. Ionization characteristics of the ligands and their relevance to biological properties, Inorg. Chim. Acta 170 (1990) 165–170.

[23] Z.I. Cabantchik, H. Glickstein, P. Milgram, W. Breuer, A fluorescence assay for assessing chelation of intracellular iron in a membrane model system and in mammalian cells, Anal. Biochem. 233 (1996) 221–227.

[24] R.S. Britton, Metal-induced hepatotoxicity, Semin. Liver Dis. 16 (1996) 3–12.

[25] S.P.J. Brooks, A simple computer program with statistical tests for the analysis of enzymatic kinetics, Biotechniques 13 (1992) 906–911.