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Pyridoxal isonicotinoyl hydrazone inhibits iron-induced ascorbate oxidation and ascorbyl radical formation

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Abstract

Previous work from our laboratory demonstrated that pyridoxal isonicotinoyl hydrazone (PIH) has in vitro antioxidant activity against iron plus ascorbate-induced 2-deoxyribose degradation due to its ability to chelate iron; the resulting Fe(III)–PIH_2 complex is supposedly unable to catalyze oxyradical formation. A putative step in the antioxidant action of PIH is the inhibition of Fe(III)-mediated ascorbate oxidation, which yields the Fenton reagent Fe(II) [Biochim. Biophys. Acta 1523 (2000) 154]. In this work, we demonstrate that PIH inhibits Fe(III)–EDTA-mediated ascorbate oxidation (measured at 265 nm) and the formation of ascorbyl radical (in electron paramagnetic resonance (EPR) studies). The efficiency of PIH against ascorbate oxidation, ascorbyl radical formation and 2-deoxyribose degradation was dose dependent and directly proportional to the period of preincubation of PIH with Fe(III)–EDTA. The efficiency of PIH in inhibiting ascorbate oxidation and ascorbyl radical formation was also inversely proportional to the Fe(III)–EDTA concentration in the media. When EDTA was replaced by the weaker iron ligand nitrilotriacetic acid (NTA), PIH was much more effective in preventing ascorbate oxidation, ascorbyl radical formation and 2-deoxyribose degradation. Moreover, the replacement of EDTA with citrate, a physiological chelator with a low affinity for iron, also resulted in PIH having a higher efficiency in inhibiting iron-mediated ascorbate oxidation and 2-deoxyribose degradation. These results demonstrate that PIH removes iron from EDTA (or from either NTA or citrate), forming an iron–PIH complex that cannot induce ascorbate oxidation effectively, thus inhibiting iron-mediated oxyradical formation. These results are of pharmacological relevance because PIH has been considered for experimental chelating therapy in iron-overload diseases.

Keywords: Iron chelator; Pyridoxal isonicotinoyl hydrazone; Antioxidant; Ascorbate; Ascorbyl radical

1. Introduction

Iron ions catalyze the formation of oxygen radicals and can accumulate at high hepatic levels in iron-overload diseases, including hemochromatosis and β-thalassemia [1–4]. Iron-chelating agents were introduced in the 1960s for the treatment of iron-overload diseases, and currently deferoxamine is still the only iron chelator clinically used. However, the costs of the treatment and lack of intestinal absorption of deferoxamine has prompted research in the search for alternatives [5]. Moreover, iron is also a mediator for free radical effects in several other pathological conditions including ischemic heart disease and cancer [1–3,6,7].

Pyridoxal isonicotinoyl hydrazone (PIH) was discovered by Ponka in the late 1970s and has been demonstrated to be useful in producing negative iron balance in laboratory animals and inducing iron excretion in humans in preclinical trials [5,8–11]. Several years ago, Schulman et al. [12] observed that PIH has substantial in vitro antioxidant activity against iron-induced lipid peroxidation and 2-deoxyribose degradation, possibly due to its ability to chelate iron (see the structure of PIH and its proposed sites for iron coordination in Scheme 1). In addition, Hermes-Lima et al. [13,14] showed that PIH inhibits hydroxyl radical (·OH) formation by the

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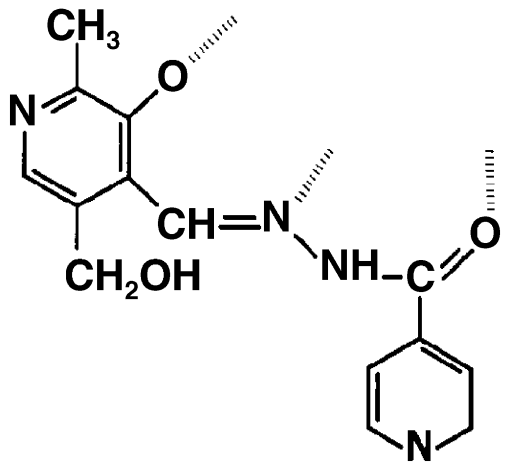

Scheme 1. PIH structure and its proposed sites for iron coordination. Two molecules of PIH forms a complex with Fe(III) (see Ref. [5]).

Fenton reaction. We demonstrated that PIH causes a rapid oxidation of Fe(II), diminishing the availability of Fe(II) for the Fenton reaction and thus preventing plasmid DNA strand breaks, 2-deoxyribose degradation and 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) hydroxylation, in electron paramagnetic resonance (EPR) studies [13–15]. PIH was also found to be effective against iron-mediated lipid peroxidation in isolated rat liver mitochondria [15] and against retinal oxidative stress in newborn pigs under conditions of asphyxia and reoxygenation [16].

More recently, we provided further evidence that the in vitro antioxidant mechanism of PIH is related to its iron-chelating properties and not merely due to $^{\cdot}OH$ scavenging activity [17]. We proposed that in a system where $^{\cdot}OH$ is generated by Fe(III)–EDTA, ascorbate and dissolved oxygen (see reactions (1)–(4)), PIH removes iron from EDTA and forms an iron–PIH complex (reaction (5)) that is unable to catalyze oxyradical formation. We further proposed that the in vitro antioxidant properties of PIH are also due to its capacity to inhibit ascorbate-mediated Fe(III) reduction to Fe(II) [17]. The use of an iron co-chelator (EDTA, nitrilotriacetic acid (NTA) or citrate) in these experiments was needed because free ferric ions are insoluble at near-neutral pH.

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

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

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

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

$$\mathrm{Fe(III)-EDTA + 2PIH \rightarrow EDTA + Fe(III)-PIH_2} \quad (5)$$

The goal of the present study was to provide additional proof that the inhibition of the ascorbate-mediated reduction of Fe(III) (as Fe(III)–EDTA, Fe(III)–citrate or Fe(III)–NTA) is indeed a key component of the in vitro antioxidant mechanism of PIH. By inhibiting iron-mediated ascorbate oxidation and ascorbyl radical formation (reaction (1)), PIH would diminish the efficiency of the Fenton reaction (reaction (4)) and thus $^{\cdot}OH$ formation.

2. Materials and methods

Ascorbic acid, *N*-[2-hydroxyethyl]-piperazine-*N*′-[2-ethanesulfonic acid] (HEPES), citrate, 2-deoxyribose, EDTA and NTA were purchased from Sigma Chemical (St. Louis, MO). The synthesis of PIH (3-hydroxy-5-(hydroxymethyl)-2-methyl-4-pyridine-carboxaldehyde 4-pyridinecarbonylhydrazone) was performed by a Schiff base condensation of pyridoxal with isonicotinic acid hydrazide [17]. PIH was prepared by Dr. Prem Ponka (Lady Davis Institute for Medical Research, Jewish General Hospital, Montreal, Canada) and kindly donated to our research group. All the other reagents were of analytical purity.

Stock solutions of PIH (0.5 or 2 mM) were freshly prepared in 1 mM HEPES buffer at pH 7.2 [17]. Ferric chloride stock solutions (1 mM) were freshly prepared in 10 mM HCl. Stock solutions of Fe(III)–EDTA (1:1 ratio), Fe(III)–NTA (1:2 ratio) and Fe(III)–citrate (1:2 ratio) were prepared weekly in 10 mM HCl. We observed that these solutions gave the same rates of ascorbate oxidation as fresh solutions. All solutions were made with milli-Q deionized water.

The rate of oxidation of ascorbate (at 20 or 100 μM) was followed at 265 nm [12] in a Hitachi U-2001 spectrophotometer at room temperature. Fe(III)–EDTA, Fe(III)–citrate or Fe(III)–NTA were added to 20 mM HEPES-buffered media (pH 7.2) followed by the addition of PIH (or water for controls) and ascorbate in 1 ml solutions. The reaction was recorded for 3 min and the linear slope (with r^2>0.99) was then calculated. Absorbance was read against blanks: buffer or buffer plus PIH. The time interval between addition of PIH and ascorbate was indicated in the figure legends.

EPR measurements of ascorbyl radical [18] were performed at room temperature in a Bruker ESP 300 spectrometer, with a microwave frequency of 9.42 GHz, modulation frequency of 100 kHz, microwave power of 10 mW, modulation amplitude of 0.25 G and a gain of 6.3×10^5. Reactions were initiated in 0.5 ml solutions and samples were removed and evaluated on capillary tubes with a final volume of 0.05 ml. EPR signals were measured after 3.5 min ascorbate addition to reaction media. Because the EPR spectral line shape from ascorbyl radical was kept essentially the same in all experiments, we quantified ascorbyl radical formation (as arbitrary units) by measuring the peak height of the first resonance line. The baseline EPR signal in all determinations was 0.5 arbitrary units.

The formation of $^{\cdot}OH$ radicals was measured using the 2-deoxyribose oxidative degradation method. The principle of

the assay is the quantification of the main 2-deoxyribose degradation product, malonaldehyde, by its condensation with TBA [19,20]. Reactions were started by the addition of 0.01 ml 2-deoxyribose (5 mM final concentration) and 0.01 ml ascorbate (0.7 mM final concentration) to 0.48 ml solutions containing 10 mM buffer (either HEPES or phosphate, pH 7.2, depending on the experiment), and 10 μM Fe(III) (as Fe(III)–EDTA, Fe(III)–citrate or Fe(III)–NTA), with or without PIH. Reactions were incubated at room temperature and terminated by the addition of 0.5 ml 4% phosphoric acid (v/v) followed by 0.5 ml 1% TBA (w/v) in 50 mM NaOH. After boiling for 15 min, 532 nm absorbance values were recorded. Blank reactions (“zero time incubations”) were carried out as previously described [17] and were used to correct for background levels of 2-deoxyribose degradation. Previous results [17] indicated that PIH (300 μM) do not interfere with the reaction of malonaldehyde (formed from 10 μM Fe(III)–EDTA plus ascorbate) with TBA.

Reduction of Fe(III)–PIH_2 by ascorbate (0.1–50 mM) was monitored at 476 nm (absorbance peak for Fe(III)–PIH_2 [17]) for 45 min in 5 mM phosphate-buffered media (pH 7.2). Solutions were previously bubbled with nitrogen gas to prevent reoxidation of ferrous ions.

3. Results

3.1. Iron-mediated ascorbate oxidation

PIH at 100 μM caused an inhibitory effect on ascorbate oxidation in 20 mM HEPES buffered medium; however, its efficiency decreased with the augment in iron–EDTA (at 1:1 ratio) concentration. At 20 μM Fe(III)–EDTA, the inhibition of ascorbate oxidation by PIH was about 20% (Fig. 1A,B). The effectiveness of PIH was inversely proportional to the augment in iron concentration.

Changing the iron co-chelator from EDTA to NTA, a weaker iron ligand [17], resulted in an increased effectiveness of PIH (at 100 μM) in inhibiting ascorbate oxidation. A complete block of ascorbate oxidation was observed at up to 20 μM Fe(III)–NTA (1:2 ratio) (Fig. 2A). A marked decrease in PIH effectiveness was observed only at 50 μM Fe(III)–NTA, where the inhibition of ascorbate oxidation was lowered to about 30% (Fig. 2B).

Fig. 3A shows that the increase in the preincubation period of solutions containing 20 μM Fe(III)–EDTA (1:1 ratio) and 100 μM PIH caused an augment in the effectiveness of PIH. The percent inhibition of iron-mediated ascorbate oxidation by PIH increased from 22% with no preincubation to 42% with 85 min of preincubation (Fig. 3B). Preincubation of solutions containing Fe(III)–EDTA and PIH would cause the removal of iron from EDTA and the formation of an iron–PIH complex before the addition of ascorbate (reaction (5)). On the other hand, the preincubation (up to 85 min) of Fe(III)–NTA (20 μM iron) with 100 μM PIH did not change the rate of ascorbate oxidation in comparison with samples without preincubation ($n=3$, data not shown).

The dependence of PIH concentration on the rate of ascorbate oxidation was assayed in the presence of 10 μM FeIII–EDTA (1:1 ratio) and 100 μM ascorbate. When solutions were preincubated for 2 h, the value of I_{50} was 90 ± 15 μM PIH (Fig. 4A, open circles). In media preincubated for only 8 min, the effectiveness of PIH was reduced greatly when compared to media preincubated for 2

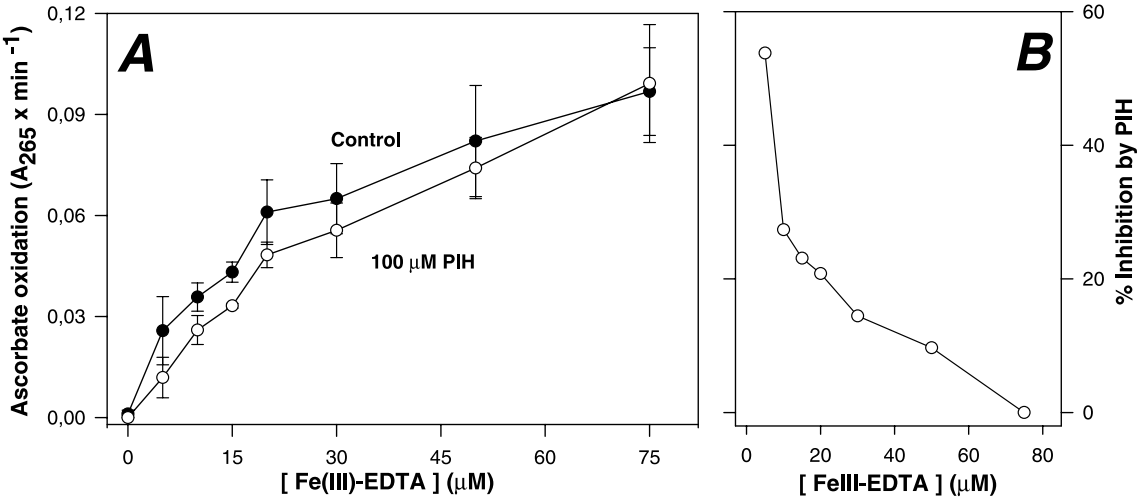

Fig. 1. Panel A: Dependence of Fe(III)–EDTA concentration on the rate of ascorbate oxidation. Reactions were carried out in the absence or presence of 100 μM PIH, containing the following final concentrations of reagents: HEPES buffer (20 mM, pH 7.2), ascorbate (100 μM) and varying Fe(III)–EDTA concentrations (0–75 μM, with 1:1 ratio iron–EDTA). The rate of ascorbate oxidation (as loss of A_{265} per minute) was calculated as described in Materials and methods. Based on the extinction coefficient of ascorbate at 265 nm (at neutral pH), the loss of 0.1 absorbance corresponds to the oxidation of 6.9 μM ascorbate [33]. Values in Panel A are means ± S.D. ($n=3$). Panel B is a replot of data from Panel A showing the percent inhibition of ascorbate oxidation by PIH.

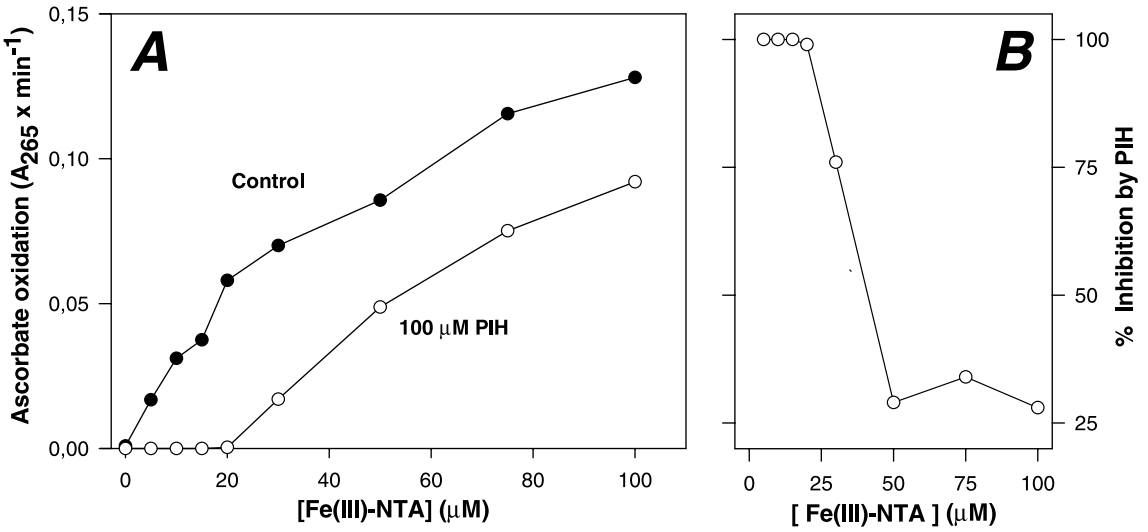

Fig. 2. Panel A: Dependence of Fe(III)–NTA concentration on the rate of ascorbate oxidation. Experimental conditions are as described in the legend to Fig. 1, except that the iron co-chelator was changed from EDTA to NTA (Fe(III)–NTA ratio = 1:2). Data are the average of three independent experiments. Panel B is a replot of data from Panel A and depicts the percent inhibition of ascorbate oxidation by PIH.

h. The apparent I_{50} under these conditions was ~ 0.5 mM PIH (Fig. 4A, closed circles).

Similar qualitative results on the effect of preincubation were obtained when testing the systems with a lower amount of ascorbate (20 μM) (Fig. 4B). It suggests that the effectiveness of PIH in inhibiting ascorbate oxidation is mainly dependent on the competition between PIH and EDTA for iron chelation. Ascorbate concentration, in a 20–100 μM range, does not influence the effect of PIH.

Another relevant factor for PIH effectiveness against iron-mediated ascorbate oxidation is the ratio between iron (10 μM) and EDTA. A titration of PIH was performed in solutions containing two different iron–EDTA ratios (1:1 and 1:5) (Fig. 5). Lower rates of ascorbate oxidation (in the absence of PIH) were obtained in media containing less EDTA (i.e., 10 μM). The value of I_{50} was 90 ± 15 μM PIH for reactions with Fe(III)–EDTA at a 1:1 ratio. Reactions with iron–EDTA at a 1:5 ratio (EDTA = 50 μM) showed an I_{50} value of 230 ± 30 μM of PIH. Since these experiments were performed with a 2-h preincubation period (see legend to Fig. 3), it is possible that a longer period of preincubation would decrease the I_{50} value for Fe(III)–EDTA at a 1:5 ratio.

We next compared the effect of PIH against ascorbate (100 μM) oxidation induced by either Fe(III)–citrate, a physio-

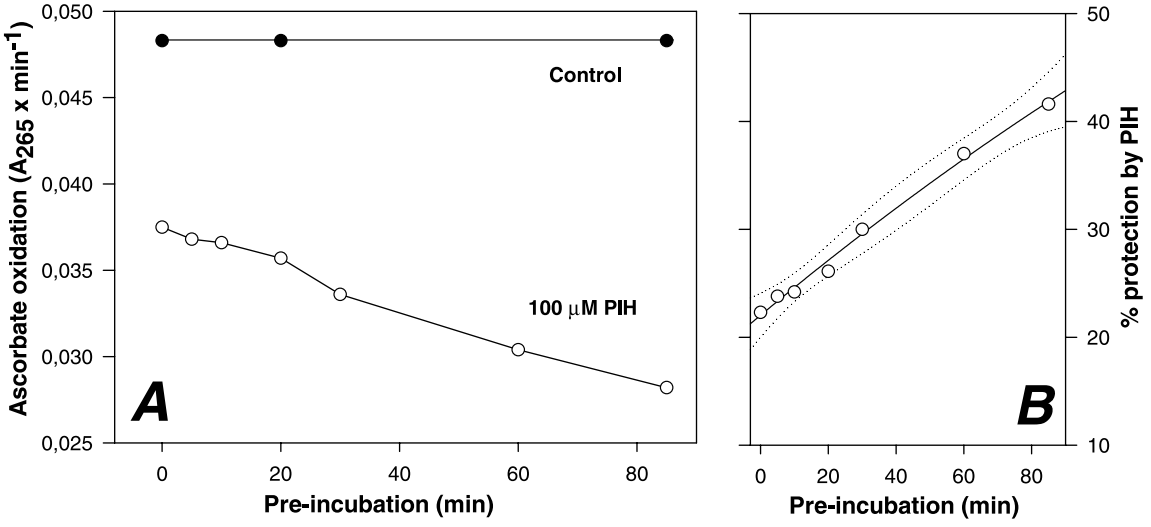

Fig. 3. Panel A: Effect of preincubation of Fe(III)–EDTA and PIH on the rate of ascorbate oxidation. Solutions were preincubated at room temperature for periods from 0 to 85 min in the absence or presence of 100 μM PIH, in 20 mM HEPES-buffered media (pH 7.2) containing 20 μM Fe(III)–EDTA (1:1 ratio). After the preincubation period, ascorbate (100 μM) was added and the rate of ascorbate oxidation was determined. Data are the average of three independent experiments. Panel B is a replot of data from Panel A showing the percent inhibition of ascorbate oxidation by PIH. A second-order regression line is shown ($r^2 = 0.994$; $P < 0.01$); dotted lines represent the 99% confidence interval.

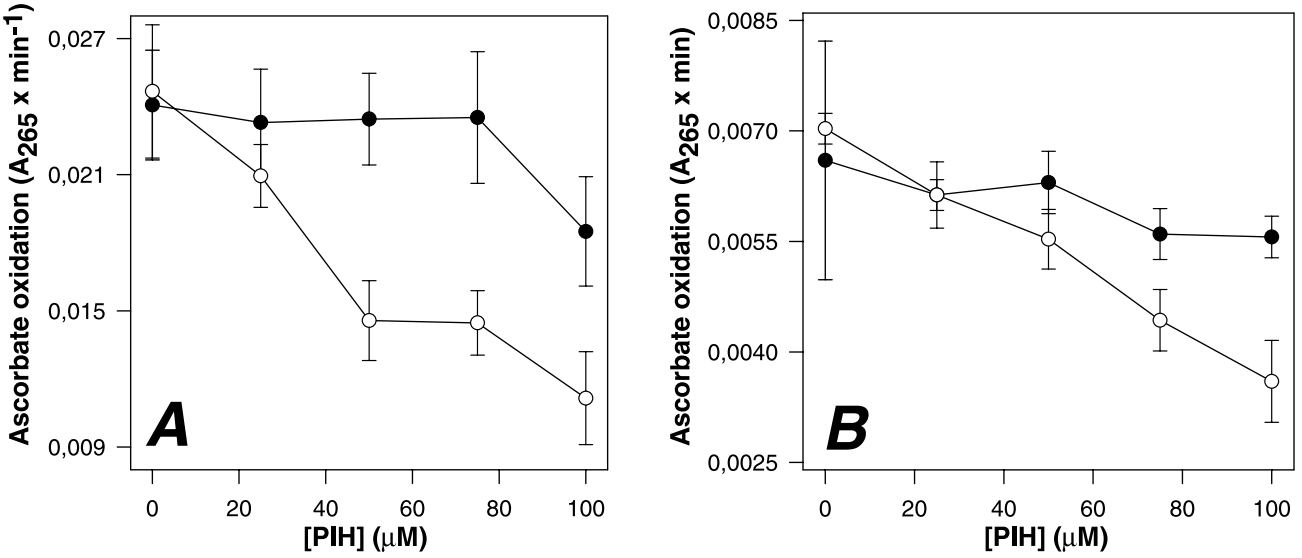

Fig. 4. Effect of PIH concentration on the rate of ascorbate oxidation employing two different periods of preincubation (at room temperature) for PIH and Fe(III)–EDTA. Reaction media contained 20 mM HEPES (pH 7.2), 10 μM Fe(III)–EDTA (1:1 ratio) and varying PIH concentrations (0–100 μM) were preincubated for 8 min (closed circles) or 2 h (open circles), followed by addition of 100 μM ascorbate (Panel A) or 20 μM ascorbate (Panel B). Values are means ± S.D. (n = 2–3). Values of I_{50} in this study (shown in the text) were calculated according to Ref. [41].

logical iron chelator [2,15], Fe(III)–NTA or Fe(III)–EDTA. Both citrate and NTA form a complex with Fe(III) that is weaker than the complex of Fe(III) with EDTA [17]. The rate of ascorbate oxidation induced by 10 μM Fe(III)–citrate or Fe(III)–NTA was strongly inhibited by 20 μM PIH (by 100% and 74.7%, respectively). On the other hand, 20 μM PIH inhibited the oxidation of ascorbate mediated by 10 μM Fe(III)–EDTA by only 33% (n = 3–4; data not shown). In the presence of 100 μM PIH, the rate of Fe(III)–EDTA-mediated ascorbate oxidation was lowered by 51%, while ascorbate oxidation was fully blocked when Fe(III) was initially bound to citrate or NTA (n = 3–4; data not shown). In these experiments, solutions containing either of the iron complexes were preincubated for 10 min in the presence PIH.

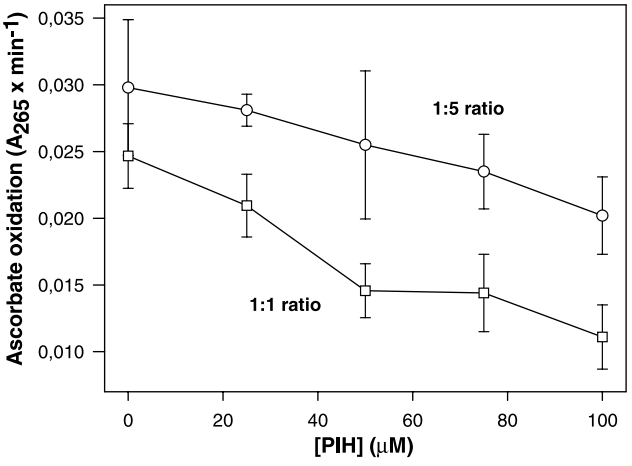

Fig. 5. Effect of PIH concentration on the rate of ascorbate oxidation at two ratios of Fe(III)–EDTA ratios (n = 3). Experimental conditions are as described in the legend to Fig. 4. Preincubation time of PIH and Fe(III)–EDTA was 2 h; EDTA concentration was either 10 μM (1:1 ratio Fe(III)–EDTA; squares) or 50 μM (1:5 ratio Fe(III)–EDTA; circles); ascorbate was 100 μM. Values are means ± S.D. (n = 3). Values of I_{50} in this study (shown in the text) were calculated according to Ref. [41].

3.2. Iron-mediated ascorbyl radical formation

We also determined the effect of PIH on the EPR signal of ascorbyl radical formed from Fe(III)–EDTA (1:1 ratio) and 1 mM ascorbate. Preliminary tests showed that PIH was able to diminish the intensity of the EPR signal without changing the typical hyperfine splitting constants of the EPR signal for ascorbyl radical. The efficiency of PIH in inhibiting ascorbyl formation was inversely correlated with the Fe(III)–EDTA concentration (Fig. 6). PIH efficiency decreased from 26%, at 2.5 μM Fe(III)–EDTA, to 11% at 50 μM Fe(III)–EDTA (inset to Fig. 6).

Fig. 7 shows that the effectiveness of PIH (200 μM) in inhibiting the EPR signal intensity was dependent on the time of preincubation of Fe(III)–EDTA (10 μM) with PIH, before the addition of ascorbate. A much greater PIH-induced inhibition of ascorbyl radical formation was observed with 90 min preincubation than with no preincubation (72% vs. 8% efficiency of PIH, respectively). This result is in full agreement with that observed for the effect of preincubation on ascorbate oxidation (see Fig. 3).

The effect of PIH on the formation of ascorbyl radical was dose dependent (apparent I_{50} was 375 ± 45 μM PIH; see Table 1). This was determined employing a constant preincubation period (30 min) and 5 μM Fe(III)–EDTA (1:1 ratio) (Fig. 8C and D depict the effect of 200 μM PIH). However, when using 5 μM Fe(III)–NTA (1:2 ratio) to induce ascorbyl radical formation, the effect of PIH was much stronger than

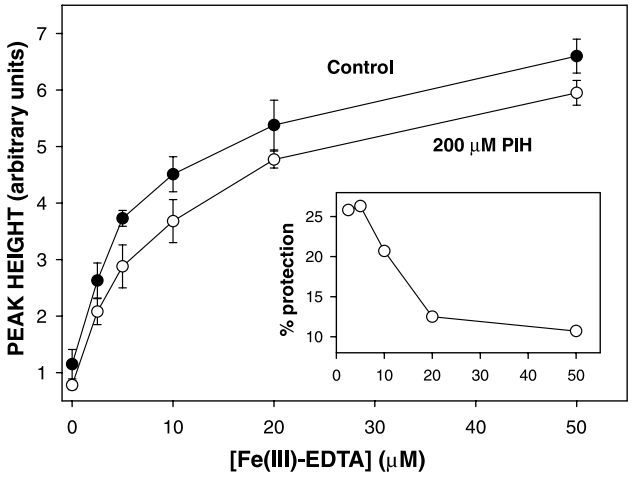

Fig. 6. Dependence of Fe(III)–EDTA concentration on the intensity of the EPR signal of ascorbyl radical. Solutions contained 20 mM HEPES buffer (pH 7.2), with varying Fe(III)–EDTA (1:1 ratio) concentrations (0–50 μM), in the absence or presence of 200 μM PIH. Reaction media were preincubated at room temperature for 30 min before the addition of 1 mM ascorbate. EPR signals were determined 3.5 min after the addition of ascorbate. Values are means ± S.D. ($n=4$). Inset: Replot of data from the principal panel showing the percent inhibition of the ascorbyl EPR signal by PIH.

that observed for Fe(III)–EDTA (Fig. 8A,B; see also legend to Fig. 8). This is in agreement with the determinations of ascorbate oxidation at 265 nm (see Figs. 1 and 2).

3.3. Iron-mediated oxidative degradation of 2-deoxyribose

Oxyradical formation from ascorbate, 10 μM Fe(III)–EDTA (1:1 ratio) and dissolved oxygen was detected by the oxidative degradation of 5 mM 2-deoxyribose, which produces malonaldehyde [19,20]. Preliminary tests indicated that iron-dependent 2-deoxyribose degradation is linear for 60 min at room temperature ($n=3$, data not shown). Previous results [12,17] demonstrated that PIH is able to inhibit Fe(III)–EDTA plus ascorbate-mediated 2-deoxyribose degradation. However, the effect of preincubation of Fe(III)–EDTA with PIH has not yet been determined.

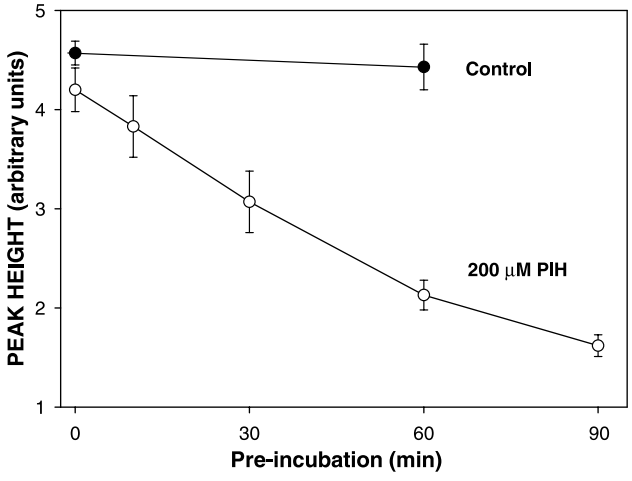

Fig. 7. Effect of preincubation of Fe(III)–EDTA and PIH on the intensity of the ascorbyl EPR signal. Solutions contained 20 mM HEPES buffer (pH 7.2), 10 μM Fe(III)–EDTA (1:1 ratio), in the absence or presence of 200 μM PIH. Reaction media were preincubated at room temperature for 0–120 min before the addition of 1 mM ascorbate. EPR signals were determined 3.5 min after the addition of ascorbate. Values are means ± S.D. ($n=3$–5).

Table 1
Effect of PIH concentration on the intensity of ascorbyl EPR signal

PIH concentration (μM)	Intensity of EPR signal
0	3.75 ± 0.41
100	3.11 ± 0.17
200	2.79 ± 0.36
300	2.21 ± 0.17
390	1.93 ± 0.15

Solutions contained 20 mM HEPES buffer (pH 7.2), 5 μM Fe(III)–EDTA (1:1 ratio) and varying concentrations of PIH. The reaction medium was preincubated for 30 min before the addition of 1 mM ascorbate. EPR signals (as arbitrary units) were determined 3.5 min after the addition of ascorbate. Values are means ± S.D. ($n=4$). The value of I_{50} in this study (375 ± 45 μM PIH) was calculated according to Ref. [41].

By fixing the incubation period to 20 min, we observed (Fig. 9) that the increase in the period of preincubation (of PIH plus Fe(IIII)–EDTA in HEPES buffer), from 0 to 2 h, caused a marked increase in the antioxidant efficiency of PIH (at 200 μM). This is in agreement with the effect of PIH (in preincubation experiments) on ascorbate oxidation and ascorbyl formation.

Furthermore, it is known that HEPES is able to quench oxyradicals, forming a HEPES radical [21]. Moreover, Fe(III)–EDTA may also oxidize HEPES, forming radical species. These phenomena could have interfered somehow with the antioxidant action of PIH in the preincubation experiments. To isolate the effect of preincubation itself on the antioxidant action of PIH against 2-deoxyribose degradation, the experiments were repeated using phosphate buffer in place of HEPES (same protocol as in Materials and methods; 10 μM Fe(III)–EDTA, 0.7 mM ascorbate, 20-min incubation). Similar to the observations depicted in Fig. 9, the antioxidant efficiency of PIH (200 or 400 μM) was significantly higher with 60 min preincubation than with no preincubation: 82% vs. 15% protection by 200 μM PIH, respectively; 94% vs. 35% protection by 400 μM PIH, respectively ($n=3$; data not shown).

We also tested the effect of PIH against 2-deoxyribose (5 mM) degradation induced by Fe(III)–citrate, in comparison with Fe(III)–EDTA and Fe(III)–NTA. In these assays, phosphate-buffered solutions containing PIH (20 or 100 μM) were preincubated for 10 min before the addition of ascorbate (0.7 mM) and then incubated further for 20 min. The effectiveness of PIH (20 μM) against 2-deoxyribose degradation induced by 10 μM Fe(III)–NTA or Fe(III)–citrate was 48% and 32%, respectively. When EDTA was employed, the antioxidant efficiency of PIH (20 μM) was much lower (14%) than with NTA or citrate ($n=4$; data not shown). In the presence of 100 μM PIH, Fe(III)–EDTA-

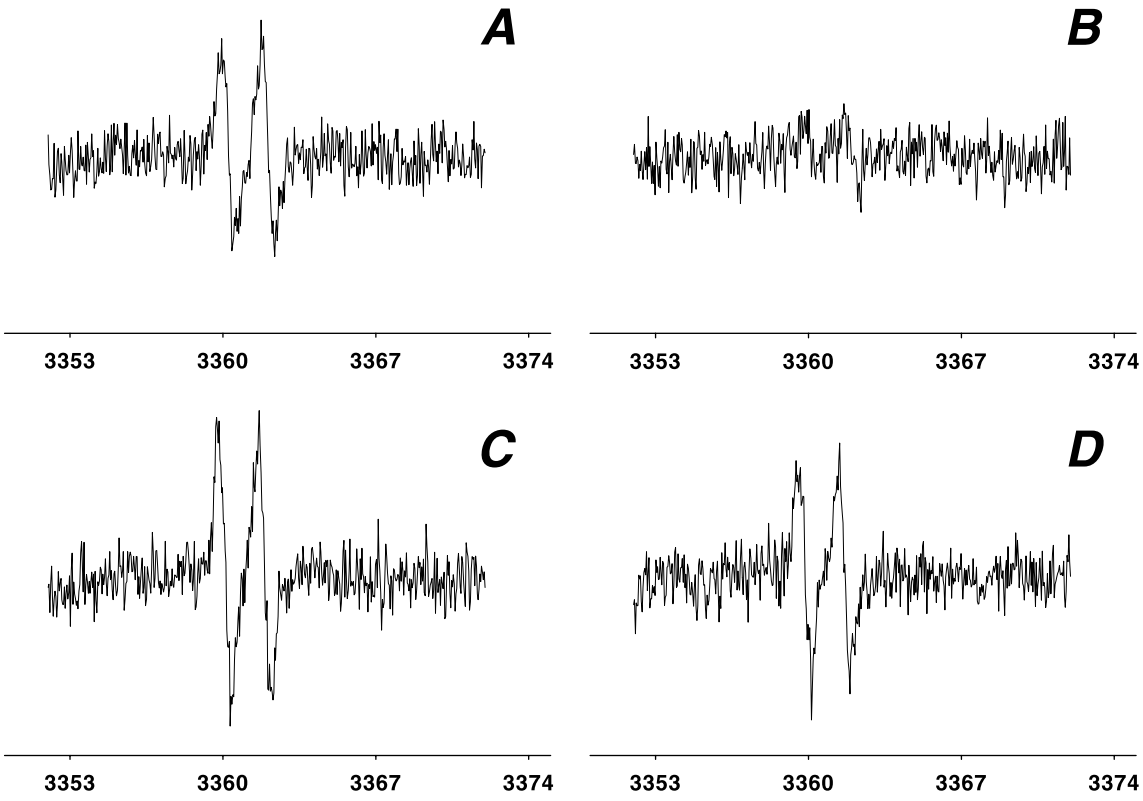

Fig. 8. The effect of PIH in reducing the intensity of the EPR signal of ascorbyl radical formation is dependent on the nature of the iron co-chelator. The presence of 200 μM PIH in a 20 mM HEPES-buffered media (pH 7.2), containing 1 mM ascorbate and 5 μM Fe(III)–NTA (1:2 ratio), caused a marked decrease in the intensity of EPR signal: from 2.33 ± 0.03 ($n=5$) to 0.90 ± 0.08 arbitrary units ($n=4$) (Panels A and B show representative EPR spectra of these reactions). PIH (at 200 μM) was less effective in reducing the ascorbyl EPR signal formed from 5 μM Fe(III)–EDTA. Control reactions produced 3.75 ± 0.41 ($n=4$) arbitrary units while the presence of PIH produced 2.79 ± 0.36 ($n=4$) arbitrary units (Panels C and D, respectively). The preincubation period for PIH and iron–EDTA (or iron–NTA) was 30 min at room temperature. Values in the x-axis represent the magnetic field (Gauss).

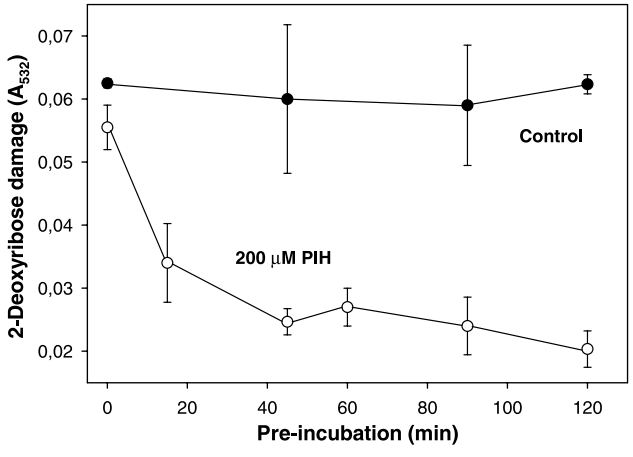

Fig. 9. Effect of preincubation of Fe(III)–EDTA and PIH on 2-deoxyribose degradation. Solutions contained 10 mM HEPES (pH 7.2), 10 μM Fe(III)–EDTA (1:1 ratio), in the absence or presence of PIH (200 μM). The reaction medium was preincubated at room temperature from 0 to 2 h before the addition of 5 mM 2-deoxyribose and 0.7 mM ascorbate. Reactions were then carried out for 20 min and terminated as described in Materials and methods. Values are means ± S.D. ($n=3$). Based on the extinction coefficient of malonaldehyde (149 mM^{-1}, as TBA-reactive product [20]), and the dilution factor of three, 0.05 A_{532} represents the formation of ~ 1 μM malonaldehyde in the 0.5 ml reaction media.

mediated 2-deoxyribose degradation was inhibited by 32%, while the extent of 2-deoxyribose degradation was substantially diminished when the iron co-chelators were NTA or citrate (83% and 93% inhibition, respectively) ($n=4$; data not shown).

3.4. Effect of ascorbate on the complex Fe(III)–PIH_2

Since PIH slows down the action of ascorbate in reducing the complexes Fe(III)–EDTA, Fe(III)–NTA or Fe(III)–citrate, the reaction of Fe(III)–PIH_2 with ascorbate may occur at a very slow rate. Indeed, 0.1–2.5 mM ascorbate was barely able to reduce Fe(III) bound to PIH (50 μM iron plus 100 μM PIH) after 30-min incubation. Notably, only 16% of Fe(III) bound to PIH was reduced after 15 min with 2.5 mM ascorbate ($n=4$, data not shown). However, significant reduction of the complex Fe(III)–PIH_2 occurred with 5, 10 and 50 mM ascorbate: 70%, 92% and 100% reduction, respectively, within 0.5 min ($n=6$, data not shown). This contrasts with the high rate of reaction of Fe(III) (as iron–EDTA, iron–NTA or iron–citrate) with 0.1 mM ascorbate: 5–7 μmol/min in the presence of 50 μM iron (see legend to Fig. 1).

4. Discussion

Previous results from Schulman et al. [12] have shown that PIH is able to prevent in vitro ascorbate oxidation. However, that study only tested the effect of PIH concentration, in comparison with salicylaldehyde isonicotinoyl hydrazone (SIH, a PIH analogue [5]) and deferoxamine. These results revealed that the three chelators were similarly effective in inhibiting the oxidation of ascorbate induced by 20 μM Fe(III)–EDTA. These observations led us to propose [12] that the effect of PIH on ascorbate oxidation (reaction (1)) could be relevant in explaining the overall in vitro antioxidant mechanism of PIH (see Introduction and Ref. [17]). The present study presented three main evidences indicating this was indeed true.

Firstly, the inhibitory effect of PIH on iron-mediated ascorbate oxidation and ascorbyl radical formation and on iron plus ascorbate-mediated 2-deoxyribose degradation was dependent on the period of preincubation of PIH and Fe(III)–EDTA (Figs. 3, 7 and 9). Any putative interfering effect of HEPES on the antioxidant action of PIH in 2-deoxyribose degradation was discounted because the same qualitative results were obtained with the use of phosphate buffer (see Results). Thus, as the preincubation period increases, there is an augmented removal of iron from EDTA, forming a complex iron–PIH_2 that reacts with ascorbate with low efficiency (see comments below) and that cannot catalyze oxyradical formation and 2-deoxyribose degradation (see Refs. [12,17]).

Secondly, previous data have shown that the antioxidant effect of PIH on 2-deoxyribose degradation is inversely dependent on the Fe(III)–EDTA concentration [12] and Fe–EDTA ratio [17]. This agreed with the current observations on the inhibitory action of PIH in ascorbate oxidation and/or ascorbyl radical formation. PIH was more effective in preventing ascorbate oxidation when the iron–EDTA ratio was 1:1 when compared with a ratio of 1:5 (Fig. 5). The presence of 4 mol of free EDTA per mole of Fe(III)–EDTA (in solutions with 1:5 ratio iron–EDTA) would make the formation of the Fe(III)–PIH_2 complex difficult. Moreover, the efficiency of PIH was inversely dependent on the concentration of Fe(III)–EDTA (or Fe(III)–NTA) in the solutions. This was observed in the case of ascorbate oxidation (Figs. 1 and 2) and ascorbyl radical formation (Fig. 6).

Thirdly, we observed that the efficiency of PIH against ascorbate oxidation and ascorbyl radical formation was greater when iron was complexed with NTA than with EDTA (Figs. 1B, 2B and 8). NTA forms a weaker complex with iron when compared with EDTA (formation constants (log β_{11}) for Fe(III)–NTA and Fe(III)–EDTA are 8.3 and 25.5, respectively [22]). This is also in agreement with previous determinations of the antioxidant activity of PIH against 2-deoxyribose degradation mediated by ascorbate plus iron–NTA (I_{50} = 48 μM PIH; iron–NTA with 1:50 ratio) or iron–EDTA (I_{50} = 315 μM PIH; iron–EDTA with 1:5 ratio) [17] and with the present results of 2-deoxyribose degradation mediated by iron–NTA (see Results). The similarity between EDTA and PIH in log β_{11} values (log β_{11} for Fe(III)–PIH_2 is 24.8 [9,23]) explains the need of relatively high concentrations of PIH to produce an efficient antioxidant effect when using iron–EDTA.

The differences between Fe(III)–EDTA and Fe(III)–NTA in the efficiency of PIH can also be explained by the slow and fast rates of iron removal from EDTA or NTA by PIH, respectively. Hermes-Lima et al. [17] determined that Fe(III)–PIH_2 complex was formed (at 476 nm) within a few seconds or 30–40 min ($t_{1/2}$ = 10 min) when 400 μM PIH was added to HEPES-buffered solutions containing Fe(III)–NTA (10 μM Fe plus 500 μM NTA) or Fe(III)–EDTA (10 μM Fe(III) plus 100 μM EDTA), respectively.

We also tested the effect of PIH on ascorbate oxidation and 2-deoxyribose degradation mediated by Fe(III)–citrate. Unlike EDTA and NTA, citrate is a physiological iron chelator, and it is believed to be an important constituent of the intracellular pool of low molecular weight iron complexes [2,15]. The effectiveness of PIH in the system using Fe(III)–citrate was comparable with Fe(III)–NTA, although much greater than with Fe(III)–EDTA (see Results). This is explained—as in the case of NTA, discussed above—by the small formation constant of Fe(III)–citrate (log β_{11} = 11 [22]). Thus PIH can easily remove iron from the complex with citrate, preventing in vitro iron-mediated $^{\cdot}OH$ formation (see Results) and mitochondrial lipid peroxidation [15].

The observed behavioral similarities of PIH as an inhibitor of 2-deoxyribose degradation, ascorbate oxidation and ascorbyl formation clearly indicate that the inhibition of reaction (1) (see Introduction) is a determinant step in the antioxidant mechanism of PIH. By diminishing the rate of ascorbate oxidation, PIH also decreases the rate of Fe(III) reduction to Fe(II). This will affect ‘‘downstream’’ reactions: less Fe(II) will be available for autoxidation (see reaction (2), which is a key event in the chain of reactions leading to H_2O_2 formation [24][2]), the Fenton reaction and $^{\cdot}OH$ formation (reaction (4)). A similar antioxidant mechanism was proposed in the case of free radical formation mediated by copper plus ascorbate, where PIH also blocks $^{\cdot}OH$ formation (detection by means of the 2-deoxyribose assay) [25]. In these studies, PIH inhibited ascorbate oxidation, which is fundamental in the reduction of Cu(II) to Cu(I), forming a Fenton reagent.

The present results strongly indicate that PIH inhibits ascorbate oxidation and ascorbyl formation by the removal of iron from the EDTA (or either NTA or citrate) complex, forming a Fe(III)–PIH_2 complex that is poorly capable of oxidizing ascorbate. This may be explained by the fact that

[2] Recent results indicated that 200 μM PIH inhibits oxygen uptake (see Ref. [25] for methodology) induced by 0.7 mM ascorbate and 10 μM Fe(III)–EDTA, in phosphate-buffered solutions, pH 7.2 (C.S. Gomes and M. Hermes-Lima, unpublished).

PIH occupies the six coordination sites of iron [26,27] (which does not occur with EDTA, NTA or citrate), causing a strong inhibition of electron transfer between ascorbate and Fe(III). The nature of the three functional groups of PIH involved in the chelation of Fe(III) (see Scheme 1 and Ref. [5]) may be responsible for the inhibition of electron transfer from ascorbate. Ascorbate oxidation by Fe(III) bound to PIH (reaction (6)) only takes place at relevant rates at a very high ascorbate/PIH ratio (50:1 and above; see Results).

$$\mathrm{Fe(III)} - \mathrm{PIH_2} + \text{ascorbate} \rightarrow \mathrm{Fe(II)} - \mathrm{PIH_2} + \text{ascorbyl}^{\cdot} \quad \text{(slow reaction)} \qquad (6)$$

Ramanathan and Das [28] also concluded that the polyphenol tannic acid acts as a antioxidant against lipid peroxidation induced by Cu(II) and ascorbate by inhibiting Cu(II)-induced ascorbate oxidation. Recent data from our laboratory showed that ascorbate oxidation and ascorbyl radical formation from Cu(II) (or Fe(III)–EDTA) were inhibited by tannic acid at neutral pH [29,30]. Inhibition of metal-mediated ascorbate oxidation and ascorbyl radical formation was considered a chief component of the antioxidant mechanism of tannic acid against $^{\cdot}OH$ generation in totally aqueous media [29,30]. Thus metal chelators that effectively inhibit ascorbate oxidation, and consequently ascorbyl radical formation, may have a substantial capacity to control $^{\cdot}OH$ formation and oxidative stress. On the other hand, the iron chelator 1,2-dimethyl-3-hydroxypyrid-4-one (L1) is able to increase the rate of ascorbate oxidation under certain conditions. This was linked to the DNA damaging action of L1 in cultured iron-loaded liver cells [31].

Reducing agents other than ascorbate, such as superoxide radicals and cysteine, also participate in the redox-cycling of transition metals and free radical formation. Hermes-Lima et al. [17] showed that PIH inhibits superoxide-mediated $^{\cdot}OH$ formation in the presence of Fe(III)–EDTA. This indicated that the inhibition of superoxide-mediated Fe(III) reduction to Fe(II) is a key factor in the antioxidant action of PIH [17].

The augment in ascorbyl radical concentration in body fluids is considered an important noninvasive marker of oxidative stress [32–34]. The decrease in plasma ascorbate levels (as well as other antioxidants) in patients with hemochromatosis or β-thalassemia is attributed to oxidative stress, with increase in lipid peroxidation [35,36], and possibly of hydroxyl and ascorbyl radicals. Thus chelating interventions may reduce ascorbyl levels in iron overload and other pathologies related to abnormal iron distribution. The currently observed in vitro effects of PIH inhibition of iron-mediated ascorbate oxidation could be relevant in vivo. The fact that intracellular levels of iron bound to low molecular weight compounds (mostly citrate and organic phosphates [15,37]) are hardly higher than 1–2 μM, even in iron overload [37], suggests that low micromolar levels of PIH could inhibit in vivo iron-mediated ascorbate oxidation and oxidative stress. This is particularly relevant when considering the very high effectiveness of PIH observed against iron–citrate-mediated ascorbate oxidation and $^{\cdot}OH$ formation (see Results). Moreover, the low toxicity and high specificity of PIH for transition metals [5,38,39], its ability to cross membranes [39,40] and its antioxidant capacity (Refs. [12–17,25,39]; this work) may constitute good indicators for the future pharmacological usefulness of PIH.

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