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The iron chelator pyridoxal isonicotinoyl hydrazone inhibits mitochondrial lipid peroxidation induced by Fe(II)–citrate

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Abstract

Pyridoxal isonicotinoyl hydrazone (PIH) is able to prevent iron-mediated hydroxyl radical formation by means of iron chelation and inhibition of redox cycling of the metal. In this study, we investigated the effect of PIH on Fe(II)–citrate-mediated lipid peroxidation and damage to isolated rat liver mitochondria. Lipid peroxidation was quantified by the production of thiobarbituric acid-reactive substances (TBARS) and by antimycin A-insensitive oxygen consumption. PIH at 300 μM induced full protection against 50 μM Fe(II)–citrate-induced loss of mitochondrial transmembrane potential ($\Delta\psi$) and mitochondrial swelling. In addition, PIH prevented the Fe(II)–citrate-dependent formation of TBARS and antimycin A-insensitive oxygen consumption. The antioxidant effectiveness of 100 μM PIH (on TBARS formation and mitochondrial swelling) was greater in the presence of 20 or 50 μM Fe(II)–citrate than in the presence of 100 μM Fe(II)–citrate, suggesting that the mechanism of PIH antioxidant action is linked with its Fe(II) chelating property. Finally, PIH increased the rate of Fe(II) autoxidation by sequestering iron from the Fe(II)–citrate complex, forming a Fe(III)–PIH_2 complex that does not participate in Fenton-type reactions and lipid peroxidation. These results are of pharmacological relevance since PIH is a potential candidate for chelation therapy in diseases related to abnormal intracellular iron distribution and/or iron overload.

Keywords: Free radical; Iron chelator; Lipid peroxidation; Mitochondria; Oxidative stress; Pyridoxal isonicotinoyl hydrazone

1. Introduction

Iron is an essential element for life. However, it is involved in the in vivo catalysis of oxygen free radical formation. There are several human pathologies related to increased intracellular iron levels (iron overload) or "de-localization" of iron from heme- or nonheme proteins. These pathologies include hereditary hemochromatosis, β-thalassemia, Friedreich's ataxia, ischemic heart disease, Parkinson's disease and several types of cancer, as well as inflammatory processes (Britton et al., 1994; Stal, 1995; Gassen and Youdim, 1997; Meneghini, 1997; Smith et al., 1999; Gordon, 2000; Sheth and Brittenham, 2000). Iron chelators have been shown to be effective as antiproliferative agents, to reduce post-ischemic oxidative damage to tissues, and to diminish lipid peroxidation and DNA damage in models of iron overload (Britton et al., 1994; Hershko, 1994; Richardson and Milnes, 1997; Link et al., 1999). In the case of β-thalassemia, the treatment is based on iron chelation therapy with the siderophore deferoxamine. However, deferoxamine is costly, requires long subcutaneous infusions and has a short plasma half-life (Richardson and Ponka, 1998a).

Pyridoxal isonicotinoyl hydrazone (PIH), a condensation product of pyridoxal and isonicotinic acid hydrazine, may be an alternative candidate for low-cost iron chelation therapy (Webb and Vitolo, 1988; Richardson and Ponka, 1998b). It can be administered orally, has low toxicity, has low affinity for calcium and magnesium ions and induces the excretion of iron and a negative iron balance (Sookvanichsilp et al., 1991; Richardson and Ponka, 1998a,b). We have recently demonstrated that PIH has also in vitro antioxidant activity since it makes iron unavailable for catalysis of hydroxyl radical ($^{\bullet}OH$) formation (Schulman et al., 1995; Hermes-Lima et al., 2000). Antioxidant activity is a relevant characteristic of iron chelators for clinical use (Hershko, 1994; Hermes-Lima et al., 2000),

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since some chelators (such as EDTA) enhance iron solubility and increase oxyradical formation (Hermes-Lima et al., 1994). We have observed that PIH prevents iron-mediated ascorbate oxidation, 2-deoxyribose degradation, plasmid DNA strand breaks, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) hydroxylation and liposomal peroxidation (Schulman et al., 1995; Hermes-Lima et al., 1998, 1999, 2000). PIH is also effective against retinal lipid peroxidation in newborn pigs subjected to asphyxia and reoxygenation (Bhattacharya et al., 1997).

Even though the antioxidant action of PIH in aqueous solutions has been well investigated, its effects against lipid peroxidation are still poorly investigated (see Schulman et al., 1995; Bhattacharya et al., 1997). Iron-mediated lipid peroxidation is a key event leading to cell membrane damage (including mitochondrial membranes), DNA alterations, carcinogenic processes and cell death (Carini et al., 1992; Britton et al., 1994; Castilho et al., 1994, 1999; Hermes-Lima et al., 1995; Stal, 1995; Nair et al., 1998; Cardoso et al., 1999). Therefore, the aim of the present study is to investigate the antioxidant activity of PIH against lipid peroxidation of isolated rat liver mitochondria induced by Fe(II)–citrate. We also correlated the iron chelation properties of PIH with its activity against lipid peroxidation and mitochondrial damage.

2. Materials and methods

2.1. Reagents and solutions

ADP, antimycin A, butylated hydroxytoluene, citrate, deferoxamine, 2-deoxyribose, EGTA, *N*-(2-hydroxyethyl)-piperazine-*N*-2-ethanesulfonic acid (HEPES), 1,10-phenanthroline, rotenone, succinate, tetraphenyl phosphonium bromide (TPP^+) and thiobarbituric acid were purchased from Sigma (St. Louis, MO). PIH was a gift from Dr. Prem Ponka (Jewish General Hospital, Montreal). Ferrous ion $[Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O]$ solutions were prepared in Milli-Q water and immediately used. PIH stock solutions (1 or 6 mM) were freshly prepared as previously described (Schulman et al., 1995; Hermes-Lima et al., 2000).

2.2. Isolation of rat liver mitochondria

Mitochondria were isolated by conventional differential centrifugation from the livers of adult Wistar rats fasted overnight, as previously described (Hermes-Lima et al., 1995; Castilho et al., 1999). Homogenates were rapidly prepared in 250 mM sucrose, 0.5 mM EGTA and 10 mM HEPES (pH 7.2). The mitochondrial suspension was washed twice in the same medium containing 0.1 mM EGTA and the final pellet was diluted in 250 mM sucrose to a protein concentration of 80–100 mg/ml, as determined by the Biuret method.

2.3. Measurement of oxygen concentration

Oxygen concentration in mitochondrial suspensions was polarographically determined with a Clark-type electrode in a 1.3-ml glass chamber equipped with a magnetic stirrer at 30 °C. The respiratory control (respiratory rate of state 3/state 4) of mitochondrial preparations was more than 4.0, measured with 2 mM succinate as substrate. PIH at 300 μM did not change the respiratory control of isolated rat liver mitochondria nor the respiratory rate of state 4 (data not shown; $n = 3$). Determination of O_2 concentration in media without mitochondria was also performed with a Clark-type electrode.

2.4. Measurements of transmembrane electrical potential ($\Delta\psi$)

Determination of mitochondrial $\Delta\psi$ was performed with the TPP^+-selective electrode technique, as previously described (Hermes-Lima, 1995; Hermes-Lima et al., 1995). The electrical potential was calculated from the TPP^+ distribution between intra- and extramitochondrial compartments. Values were corrected to account for TPP^+ binding to the membranes, according to Jensen et al. (1986).

2.5. Mitochondrial swelling and measurement of thiobarbituric acid-reactive substances (TBARS)

Mitochondrial swelling was determined at 600 nm in a Carl Zeiss spectrophotometer (at 30 °C). TBARS values were determined at 532 nm, as previously described (Castilho et al., 1994, 1999). Isolated rat liver mitochondria were incubated for 20 min (at 30 °C) in the presence of Fe(II)–citrate (with or without PIH) and the reaction was subsequently interrupted by the addition of 0.5 mM butylated hydroxytoluene and thiobarbituric acid solution. TBARS values are the means of six determinations with two different mitochondrial preparations.

2.6. Determination of Fe(II) oxidation

Concentrations of Fe(II) in extramitochondrial media were quantified in supernatants of samples (0.5 ml) that had been centrifuged for 3 min in an Eppendorf centrifuge. Subsequently, 1 ml of 15 mM 1,10-phenanthroline was added. The red complex of Fe(II) with 1,10-phenanthroline was determined at 510 nm and compared with a Fe(II) standard curve (Hermes-Lima et al., 1995). In experiments under anoxic conditions, nitrogen gas was bubbled for 20 min in all solutions before determinations.

2.7. Determination of the Fe(II) complex with PIH

Fe(II) (50 μM, final concentration) was added to 0.95-ml solution containing citrate (0.5 mM) plus 100 μM PIH

(final concentrations at 1 ml) and an immediate wavelength scan (from 250 to 700 nm) was performed in a Hitachi 2001 spectrophotometer at room temperature. This allowed the observation of the characteristic peak of the Fe(II) complex with PIH (at near 420 nm), which oxidizes to the Fe(III)–PIH_2 complex. These determinations where performed in near-neutral solutions without HEPES because this buffer makes the reaction too fast to be observed without the use of stop-flow techniques (Santos, 1998). The use of 0.5 mM citrate, instead of 2 mM, was for the same reason.

3. Results

3.1. Effect of PIH on iron-induced lipid peroxidation

PIH inhibited TBARS formation induced by a 20-min incubation with 50 μM Fe(II)–citrate in rat liver mitochondria. Fig. 1 shows a titration curve for PIH on TBARS, the I_{50} value was 60 μM. Full protection against peroxidation was observed with 200 μM PIH, or with 10 μM butylated hydroxytoluene, a lipid peroxidation chain breaker.

Lipid peroxidation was also determined by measuring O_2 consumption of mitochondria in the presence of 2 μM antimycin A. The arrest of mitochondrial respiration by antimycin A suggests that O_2 uptake under these conditions is caused by lipid peroxidation (Hermes-Lima et al., 1995). Moreover, O_2 uptake induced by Fe(II)–citrate [and Fe(II)–ATP] was almost totally prevented by butylated hydroxytoluene, a well-known chain-breaking antioxidant

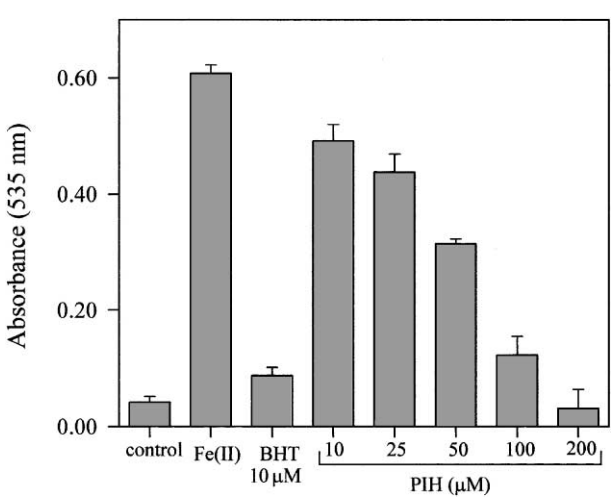

Fig. 1. PIH inhibits TBARS formation induced by 50 μM Fe(II)–citrate. Rat liver mitochondria (RLM; 0.5 mg/ml) were incubated in reaction medium containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES buffer (pH 7.2), 2 mM succinate, 4 μM rotenone and 2 mM citrate, with or without butylated hydroxytoluene (10 μM) or PIH (10–200 μM). All experiments, except the control, were started by the addition of 50 μM Fe(II). Incubation period was 20 min at 30 °C. Values are the means ± S.D. ($n = 6$).

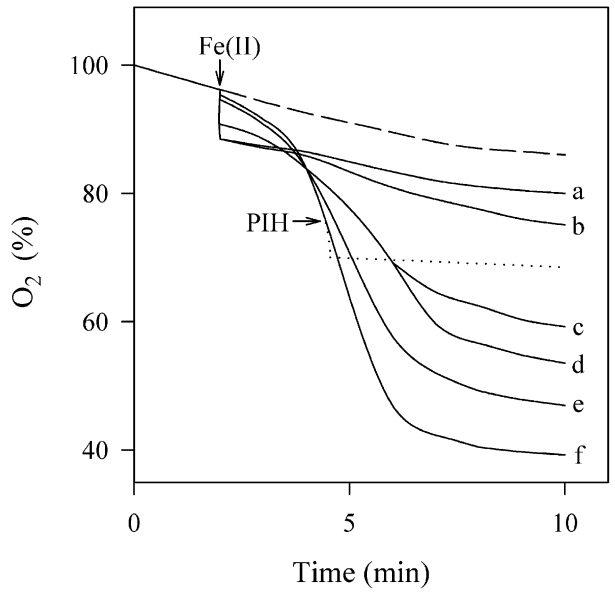

Fig. 2. PIH inhibits mitochondrial lipid peroxidation measured as O_2 consumption. Experimental conditions are as described in the legend of Fig. 1, except that 2 μM antimycin A was present (iron was 50 μM). RLM (0.5 mg/ml) were incubated in reaction medium containing: (a) 300 μM PIH, (b) 100 μM PIH, (c) 50 μM PIH, (d) 25 μM PIH, (e) 10 μM PIH, (f) no PIH. Fe(II) (50 μM) was added where indicated by the arrow (lines a–f). Dotted line indicates addition of 300 μM PIH after 2 min of Fe(II) addition. Dashed line indicates O_2 consumption in mitochondrial suspension without Fe(II) addition. Results are representative of three determinations.

that can stop the propagation phase of lipid peroxidation reactions (Castilho et al., 1994; Hermes-Lima et al., 1995). These results strongly indicate that Fe(II)–citrate-induced antimycin A-insensitive O_2 uptake is primary caused by the peroxidation of mitochondrial membrane lipids. Addition of 50 μM Fe(II) to a mitochondrial suspension containing 2 mM citrate, as previously observed (Castilho et al., 1994), induced extensive O_2 consumption (Fig. 2, line f), preceded by a lag phase (Cadenas and Sies, 1998). This extensive O_2 consumption was inhibited by 300 μM deferoxamine (data not shown). PIH was also able to inhibit iron-induced O_2 consumption in a concentration-dependent manner (lines a–e). At 300 μM PIH, the rate of O_2 consumption was the same as in controls, that is, in the absence of iron (dashed line). Addition of 300 μM PIH to the ongoing process of lipid peroxidation [at 2 min after Fe(II) addition] immediately stopped O_2 consumption (dotted line). The small O_2 consumption observed just after addition of PIH was due to Fe(II) autoxidation (see Fig. 6 for a detailed study).

3.2. Effect of PIH on iron-mediated mitochondrial swelling and loss of $\Delta\psi$

Iron-induced damage to the inner mitochondrial membrane can be assessed by the classic swelling technique, which monitors the net influx of the osmotic support

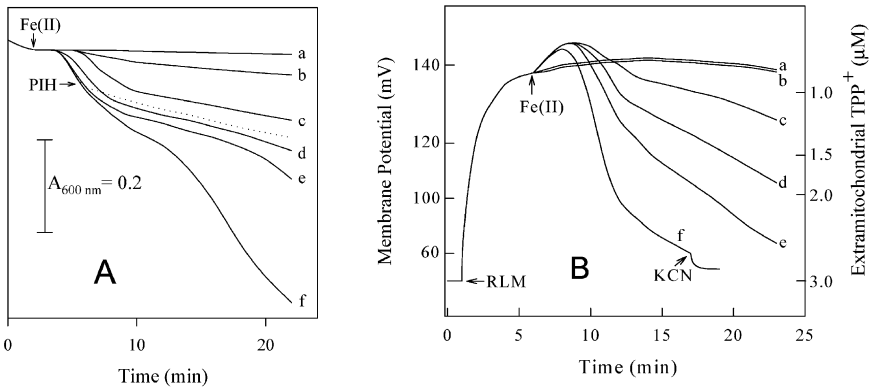

Fig. 3. PIH inhibits both mitochondrial swelling (Panel A) and loss of mitochondrial $\Delta\psi$ (Panel B) induced by 50 μM Fe(II)–citrate. Experimental conditions are as described in the legend of Fig. 1, except that 3 μM TPP^+ was present when $\Delta\psi$ was measured. Panel A represents measurements of mitochondrial swelling in reaction medium containing: (a) 300 μM PIH, (b) 100 μM PIH, (c) 50 μM PIH, (d) 25 μM PIH, (e) 10 μM PIH, (f) no PIH. Fe(II) (50 μM) was added where indicated by the arrow (lines a–f). Dotted line indicates addition of 300 μM PIH after 2 min of Fe(II) addition. Line a also represents mitochondrial swelling without Fe(II) addition. Panel B represents measurements of mitochondrial $\Delta\psi$ in reaction medium containing: (a) no PIH (without iron addition), (b) 100 or 200 μM PIH, (c) 50 μM PIH, (d) 25 μM PIH, (e) 10 μM PIH, (f) no PIH. Fe(II) (50 μM) was added where indicated by the arrow (lines b–f). Potassium cyanide (KCN; 1 mM) was added where indicated by the arrow. Results from Panels A and B are representative of three determinations.

(sucrose, KCl) associated with a nonspecific increase in membrane permeability. Castilho et al. (1994) have previously shown that Fe(II)–citrate induces mitochondrial swelling (which is butylated hydroxytoluene sensitive) due to lipid peroxidation. PIH inhibited the swelling process in a concentration-dependent manner and near-full protection was attained at 300 μM (Fig. 3A). Based on the rates of mitochondrial swelling in the absence or presence of PIH, we could estimate an I_{50} value of 50 μM for PIH. The iron chelators deferoxamine and 1,10-phenanthroline at 300 μM were also able to induce near-full protection against mitochondrial swelling (data not shown). Addition of PIH (300 μM) to ongoing iron-mediated swelling (at 2 min) caused a significant reduction in the rate of swelling (Fig. 3A, dotted line). When PIH was added at different moments (at 12–15 min) during swelling, no protection was detected (data not shown). Even though PIH was able to immediately halt lipid peroxidation at any time (by measuring O_2 consumption), the damage to the membrane at a given moment was so great that PIH could not block the membrane permeabilization and fragmentation. Similar results were observed with either butylated hydroxytoluene or deferoxamine in the case of mitochondrial swelling induced by Fe(II)–ATP (Hermes-Lima et al., 1995).

Measurement of mitochondrial $\Delta\psi$, using a TPP^+-selective electrode, showed that PIH prevented the loss of $\Delta\psi$ caused by 50 μM Fe(II)–citrate (Fig. 3B). The protective effect of PIH was concentration dependent, and full

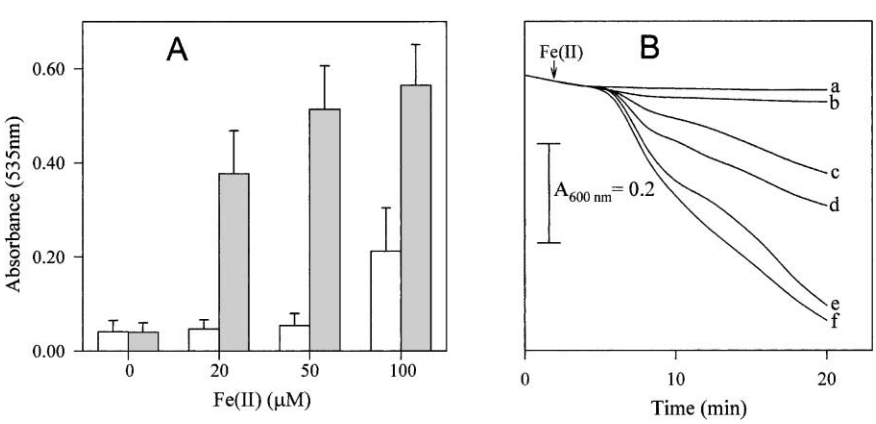

Fig. 4. Effect of PIH on TBARS formation (Panel A) and mitochondrial swelling (Panel B) induced by different Fe(II) concentrations. Experimental conditions are as described in the legend of Fig. 1 (2 mM citrate is present in all experiments). Panel A: Fe(II) was 0–100 μM (empty and hatched bars) and PIH 100 μM (empty bars). Values are the means ± S.D. ($n = 6$). Panel B represents measurements of mitochondrial swelling under the following conditions: (a) 100 μM PIH (without iron), (b) 100 μM PIH plus 20 or 50 μM Fe(II), (c) 100 μM PIH plus 100 μM Fe(II), (d) 20 μM Fe(II), (e) 50 μM Fe(II), (f) 100 μM Fe(II). Fe(II) was added where indicated by the arrow (lines a–f). Results from Panel B are representative of three determinations.

protection was observed at 100 μM PIH. Moreover, PIH at 300 μM had no effect on mitochondrial $\Delta\psi$ in suspensions in the absence of iron. This is another indicator (as observed for the respiratory control, see Materials and methods section) of the nontoxicity of PIH to rat liver mitochondria (Wallace and Starkov, 2000).

3.3. Titration of iron versus effectiveness of PIH against mitochondrial damage

Fig. 4 shows the effect of 20, 50 and 100 μM Fe(II)–citrate on TBARS (Panel A) and mitochondrial swelling (Panel B). In both cases, 50 μM iron had near-saturating effects on mitochondrial damage. Addition of 100 μM PIH caused nearly full protection (against both TBARS formation and mitochondrial swelling) in mitochondrial suspensions containing either 20 or 50 μM Fe(II)–citrate. However, PIH conferred only partial protection in the presence of 100 μM Fe(II)–citrate: 67% and 60% inhibition of TBARS formation and mitochondrial swelling rate, respectively.

These results show that the effectiveness of PIH was inversely related to the iron concentration present in the suspensions. The data suggested that the antiperoxidative mechanism of PIH is linked to its Fe(II) chelating property and ability to remove iron from the citrate complex. In the presence of 100 μM Fe(II)–citrate (citrate = 2 mM), only a portion of iron forms a complex with 100 μM PIH at a 1:2 ratio. This is the stable form of the Fe(III) complex with PIH (Richardson and Ponka, 1998b; Hermes-Lima et al., 2000). The iron–PIH complexes (see Eq. (1)) do not induce mitochondrial lipid peroxidation.

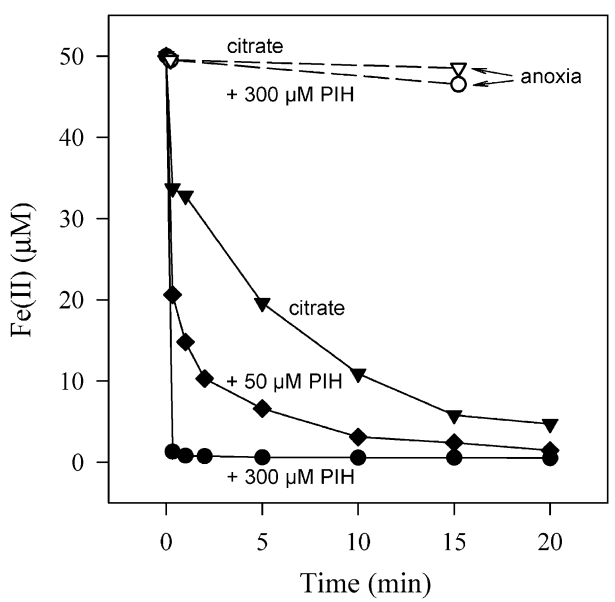

Fig. 5. Effect of PIH on Fe(II) autoxidation, in the absence of rat liver mitochondria. Experimental conditions: 125 mM sucrose, 65 mM KCl, 10 mM HEPES buffer (pH 7.2), with or without PIH. Experiments at 30 °C were started by the addition of 50 μM Fe(II). Anoxic conditions: (▽) 2 mM citrate, without PIH; (○) 2 mM citrate plus 300 μM PIH. Normoxic conditions: (▼) 2 mM citrate, without PIH; (◆) 2 mM citrate plus 50 μM PIH; (●) 2 mM citrate plus 300 μM PIH. Values are the average of three determinations.

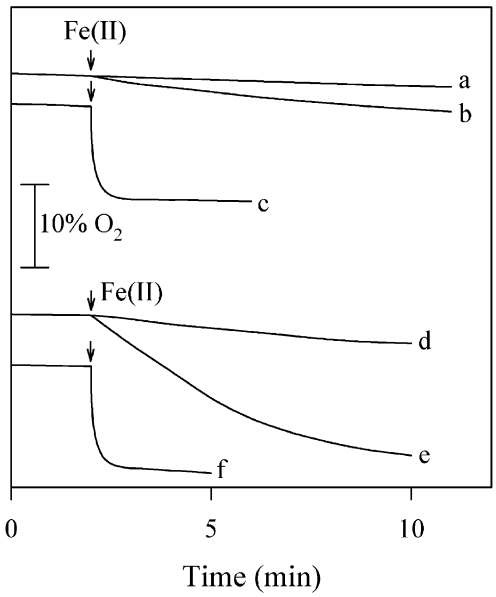

Fig. 6. Effect of PIH on O_2 consumption due to Fe(II) autoxidation, in the absence of rat liver mitochondria. Experimental conditions: 125 mM sucrose, 65 mM KCl, 10 mM HEPES buffer (pH 7.2), with or without 300 μM PIH and/or 2 mM citrate. Experiments were conducted at 30 °C, Fe(II) (50 μM) was added where indicated by the arrows. Experiments without citrate: (a) control (no iron), (b) 50 μM Fe(II), (c) 50 μM Fe(II) plus 300 μM PIH. Experiments with 2 mM citrate: (d) control (no iron), (e) 50 μM Fe(II), (f) 50 μM Fe(II) plus 300 μM PIH. Results are representative of three determinations.

$$\text{Fe(II)–citrate} + 2\text{PIH} \rightarrow \text{citrate} + \text{Fe(III)–PIH}_2 \qquad (1)$$

3.4. Fe(II)–citrate autoxidation and O_2 consumption induced by PIH

The rate of oxidation of Fe(II) to Fe(III) in the presence of 2 mM citrate (in the absence of mitochondria) was greatly increased by 50 μM PIH (Fig. 5). The rate of Fe(II) oxidation was even faster in the presence of 300 μM PIH. Hermes-Lima et al. (1998, 1999) have previously observed that PIH increases the rate of Fe(II) oxidation in the absence of citrate. These results indicate that PIH removes Fe(II) from citrate and oxidizes it to a ferric form (see Eq. (1) above). An unstable complex of Fe(II) with PIH is possibly formed before the oxidation of Fe(II) to Fe(III) and formation of a Fe(III)–PIH_2 complex (see Hermes-Lima et al., 2000; Webb and Vitolo, 1988). Indeed, addition of 50 μM Fe(II) to neutral solutions containing citrate (0.5 mM) and 100 μM PIH led to the appearance of an absorbance peak characteristic of the green complex between PIH and Fe(II) (near 420 nm) (Santos, 1998; Hermes-Lima et al., 1998, 1999). This peak

quickly disappeared within 2 min, concomitantly with the appearance of a 476-nm peak characteristic of Fe(III)–PIH_2 ($n = 3$; data not shown).

Since no iron oxidation was observed under anoxic conditions (up to 15 min; Fig. 5), the PIH-dependent conversion of Fe(II) to Fe(III) is a process that requires O_2 as an electron acceptor (see Eqs. (2) and (3)). Accordingly, the rate of O_2 consumption induced by the autoxidation of 50 μM Fe(II) or 50 μM Fe(II)–citrate (citrate = 2 mM), in the absence of mitochondria, was greatly increased by 300 μM PIH (Fig. 6). By increasing the rate of Fe(II) oxidation to Fe(III), PIH diminished the amount of Fe(II) (bound to citrate) involved in the formation of reactive species, which can initiate the peroxidation of the mitochondrial membrane.

$$\text{Fe(II)–citrate} + 2\text{PIH} \rightarrow \text{citrate} + \text{Fe(II)–PIH}_2 \quad (2)$$

$$\text{Fe(II)–PIH}_2 + \text{O}_2 \rightarrow \text{Fe(III)–PIH}_2 + \text{O}_2^- \quad (3)$$

4. Discussion

The present study investigated mitochondrial oxidative damage induced by iron. We observed that PIH protects the mitochondrial membrane against lipid peroxidation and consequently protects against a loss of membrane integrity. Fe(II)–citrate induces membrane lipid peroxidation with consequent mitochondrial depolarization and organelle swelling due to nonspecific permeabilization of the inner mitochondrial membrane (Castilho et al., 1994). Addition of 10–300 μM PIH to the mitochondrial suspensions inhibited Fe(II)–citrate-mediated mitochondrial swelling, loss of transmembrane potential, TBARS formation, and O_2 consumption during lipid peroxidation (Figs. 1–4). Interestingly, the in vitro antioxidant action of PIH against mitochondrial damage was quantitatively similar to that of deferoxamine (Hermes-Lima et al., 1995).

Previous results have demonstrated that PIH inhibits lipid peroxidation in liposomes [induced by iron plus ascorbate (Schulman et al., 1995)] and retinas [induced by postasphyxia stress in newborn pigs (Bhattacharya et al., 1997)]. However, the explanations for the antioxidant mechanism of PIH were only speculative. The results showing that the effectiveness of PIH (on TBARS formation and mitochondrial swelling, Fig. 4) was inversely related to the iron concentration suggest that PIH works as an iron chelator with antiperoxidative activity rather than as a chain-breaking antioxidant. Schulman et al. (1995) also observed that the effectiveness of PIH against degradation of 2-deoxyribose mediated by Fe(III)–EDTA plus ascorbate was inversely correlated with the Fe(III)–EDTA concentration. Moreover, the effectiveness of PIH against 2-deoxyribose degradation and DMPO hydroxylation (in electron paramagnetic resonance experiments) mediated by Fe(II) and H_2O_2 was also inversely related to the initial concentration of Fe(II) in solutions (Hermes-Lima et al., 1999). The results of the present study (Figs. 4–6) suggest that PIH removes iron from the Fe(II)–citrate complex and forms an unstable Fe(II) complex with PIH, followed by the oxidation of Fe(II) to Fe(III) and formation of a Fe(III)–PIH_2 complex (Figs. 5 and 6) that is not able to initiate and/or propagate mitochondrial lipid peroxidation. Recently, Hermes-Lima et al. (2000) have demonstrated that PIH inhibits $^{\bullet}OH$ formation (measuring 2-deoxyribose degradation) induced by ascorbate and Fe(III)–EDTA or Fe(III)–nitrilotriacetic acid by means of removing iron from the complex with either EDTA or nitrilotriacetic acid. The resulting complex Fe(III)–PIH_2 cannot participate in the Haber Weiss-type reactions involved in $^{\bullet}OH$ formation.

PIH may prevent both the initiation and the propagation of mitochondrial lipid peroxidation induced by Fe(II)–citrate. The autoxidation of Fe(II)–citrate (Eq. (4); Fig. 5) may generate reactive species, such as $^{\bullet}OH$ (Kachur et al., 1998), which can initiate the peroxidation of mitochondrial phospholipids (LH) (Eqs. (5) and (6)). Minotti and Aust (1987) observed that liposomal peroxidation is initiated by Fe(II)–citrate autoxidation. Preliminary results from our laboratory (R. Araujo and M. Hermes-Lima, unpublished) indicated that PIH inhibits $^{\bullet}OH$ formation (measured as 2-deoxyribose degradation) from the autoxidation of Fe(II) in phosphate-buffered media. Moreover, Fe(II)–citrate and Fe(III)–citrate may react with preformed lipid peroxides (LOOH), producing either peroxyl ($LOO^{\bullet}$) or alkoxyl species ($LO^{\bullet}$) that feed the propagation process (Eqs. (7)–(10)). Possibly, Fe(II) and/or Fe(III) chelated by PIH are not involved in the propagation steps of mitochondrial peroxidation, which may partially explain the antiperoxidative effect of PIH.

$$\text{Fe(II)–citrate} + \text{O}_2 \rightarrow \rightarrow \rightarrow \text{Fe(III)–citrate} + {}^{\bullet}\text{OH} \quad (4)$$

$$\text{LH} + {}^{\bullet}\text{OH} \rightarrow \text{L}^{\bullet} + \text{H}_2\text{O} \quad (5)$$

$$\text{L}^{\bullet} + \text{O}_2 \rightarrow \text{LOO}^{\bullet} + \text{LH} \quad (6)$$

$$\text{LOO}^{\bullet} + \text{LH} \rightarrow \text{LOOH} + \text{L}^{\bullet} \quad (7)$$

$$\text{Fe(II)–citrate} + \text{LOOH} \rightarrow \text{Fe(III)–citrate} + \text{LO}^{\bullet} + \text{HO}^- \quad (8)$$

$$\text{Fe(III)–citrate} + \text{LOOH} \rightarrow \text{Fe(II)–citrate} + \text{LOO}^{\bullet} + \text{H}^+ \quad (9)$$

$$\text{LO}^{\bullet} + \text{LH} \rightarrow \text{LOH} + \text{L}^{\bullet} \quad (10)$$

The results presented in this manuscript are of pharmacological importance since the mitochondria are important targets of iron-promoted free radical formation and lipid peroxidation in diseases related to abnormal intracellular iron distribution and/or iron overload, such as hereditary hemochromatosis, β-thalassemia, Friedreich's ataxia (Bindoli, 1988; Britton et al., 1994; Stal, 1995; Nair et al., 1998; Link et al., 1999; Gordon, 2000) and possibly sideroblastic anemia (Sheth and Brittenham, 2000). Iron

chelators, such as PIH, are thus important tools to reduce oxidative stress in these pathologies. Iron-induced peroxidation of mitochondrial membrane lipids may result in nonspecific inner membrane permeabilization, with an uncoupling of respiration from ATP synthesis, organelle swelling, disruption of the outer membrane (Bindoli, 1988; Castilho et al., 1994, 1999; Hermes-Lima et al., 1995; Friberg et al., 1998; Link et al., 1999) and release of different apoptogenic factors into the cytosol (for a review, see Green and Reed, 1998). These factors include cytochrome *c*, apoptosis inducing factor (AIF) and procaspases, which result in apoptosis (Green and Reed, 1998). Iron-mediated oxyradical formation may also induce damage to proteins and DNA from mitochondria (Castilho et al., 1994; Link et al., 1999; Itoh et al., 1994), effects that may be prevented by PIH.

The fact that 100 μM PIH was able to induce 80–90% protection against mitochondrial damage mediated by 50 μM iron (this work) is also of pharmacological relevance. Since in vivo levels of free or loosely bound iron are hardly higher than 1 μM, even in iron overload (Halliwell and Gutteridge, 1999), less than 2 μM PIH [taking into consideration the 1:2 ratio of the iron complex with PIH (Richardson and Ponka, 1998b)] would afford protection against iron-mediated mitochondrial damage. Furthermore, the relatively high permeability of PIH through biological membranes (Richardson and Ponka, 1998b), its action against iron-mediated free radical formation and lipid peroxidation (Schulman et al., 1995; Hermes-Lima et al., 1998, 2000; this work) and its low toxicity (Sookvanichsilp et al., 1991; Richardson and Ponka, 1998b) may suggest that the observed in vitro effects of this compound in rat liver mitochondria could be relevant to in vivo conditions of iron overload. Further work on the putative beneficial effects of PIH against hepatic oxidative stress in rats under chronic iron overload is a necessary next step of investigation.

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