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Role of Fe(III) in Fe(II)citrate-mediated peroxidation of mitochondrial membrane lipids

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Abstract

In this report we study the effect of Fe(III) on lipid peroxidation induced by Fe(II)citrate in mitochondrial membranes, as assessed by the production of thiobarbituric acid-reactive substances and antimycin A-insensitive oxygen uptake. The presence of Fe(III) stimulates initiation of lipid peroxidation when low citrate:Fe(II) ratios are used (≤ 4:1). For a citrate:total iron ratio of 1:1 the maximal stimulation of lipid peroxidation by Fe(III) was observed when the Fe(II):Fe(III) ratio was in the range of 1:1 to 1:2. The lag phase that accompanies oxygen uptake was greatly diminished by increasing concentrations of Fe(III) when the citrate:total iron ratio was 1:1, but not when this ratio was higher. It is concluded that the increase of lipid peroxidation by Fe(III) is observed only when low citrate:Fe(II) ratios were used. Similar results were obtained using ATP as a ligand of iron. Monitoring the rate of spontaneous Fe(II) oxidation by measuring oxygen uptake in buffered medium, in the absence of mitochondria, Fe(III)-stimulated oxygen consumption was observed only when a low citrate:Fe(II) ratio was used. This result suggests that Fe(III) may facilitate the initiation and/or propagation of lipid peroxidation by increasing the rate of Fe(II)citrate-generated reactive oxygen species. (Mol Cell Biochem **196**: 163–168, 1999)

Key words: Fe(II)citrate, free radicals, iron, lipid peroxidation, mitochondria, reactive oxygen species

Abbreviations: EGTA – Ethyleneglycolbis (β-aminoethyl-ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES – 2-hydroxyethyl-1-piperazineethanesulfonic acid; MES – morpholinoethanesulphonic acid; MOPS – morpholinopropanesulfonic acid; TBARS – thiobarbituric acid-reactive substances

Introduction

It is recognized that lipid peroxidation plays an important role in cell injury and death under oxidative stress conditions (for reviews, see refs. [1, 2]). Peroxidative destruction of internal cell membranes, such as the endo(sarco)plasmic reticulum and mitochondria, is linked to the disruption of both Ca^{2+} homeostasis and cellular energetics [2–7]. In order to induce lipid peroxidation in biomembranes, several oxidizing agents have been tested, including the hydroxyl radical in ionizing radiation studies [8], triplet acetone [9, 10], perhydroxyl radical [11], the peroxynitrite anion [12], and certain iron complexes [5, 6, 13–18].

It is proposed that iron complexes of low molecular weight are involved in important mechanisms of cell injury, such as post-ischemic oxidative damage and liver injuries associated with iron-overload diseases (for reviews, see refs. [19, 20]). We have previously reported that micromolar amounts of Fe(II)citrate [5, 6] or Fe(II)ATP [18] induce lipid peroxidation of mitochondrial membranes and alterations in

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membrane proteins. Although it has been studied extensively, the mechanism by which iron complexes of low molecular weight initiate lipid peroxidation is still controversial. The hydroxyl radical ($HO^{\cdot}$), an important radical in cellular oxidative stress, is often dismissed as an initiator of lipid peroxidation because $HO^{\cdot}$ scavengers do not usually inhibit peroxidation promoted by iron complexes [21–23]. A possible $HO^{\cdot}$ formation in specific hydrophobic sites, involving iron bound to membranes, is controversial [23]. Minotti and Aust [14, 22] have proposed that a ferrous-ferric-dioxygen (Fe(II)-Fe(III)-O_2) complex is the species that initiates lipid peroxidation. Others favor the participation of the perferryl (Fe(II)-O_2 or Fe(III)-$O_2^{\cdot-}$) [15, 24, 25] or ferryl ion (FeO^{2+} or $FeOH^{3+}$) (for review, see ref. [23]).

In the present study, we investigated the influence of Fe(III) on Fe(II)citrate-mediated lipid peroxidation of mitochondrial membranes, measured as antimycin A-insensitive oxygen uptake and formation of thiobarbituric acid-reactive substances (TBARS). Our results show that Fe(III) stimulates both spontaneous Fe(II) oxidation and Fe(II)citrate-induced lipid peroxidation only when low citrate:Fe(II) ratios are used.

Materials and methods

Reagents and solutions

Ferrous ions ($Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$) solutions were prepared in Milli-Q water and used immediately. Fe(III)citrate solutions were prepared daily by dissolving $FeCl_3$ (4 mM final concentration) in 4 mM citric acid and adjusting the pH to 7.2 by the addition of KOH. The citrate present in the Fe(III) solution was taken into account in calculating the total citrate content mentioned in figure legends. Antimycin A, citrate, EGTA, HEPES, rotenone and thiobarbituric acid were obtained from Sigma Chemical Co (St. Louis, MO, USA).

Isolation of rat liver mitochondria

Mitochondria were isolated by conventional differential centrifugation from the livers of adult Wistar strain rats fasted overnight. The homogenate was prepared in 250 mM sucrose, 1.0 mM EGTA and 5.0 mM HEPES buffer, 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. These mitochondria contained 8–10 nmol/mg endogenous calcium as determined by atomic absorption spectroscopy. The respiratory control (respiratory rate of state 3/state 4) of mitochondrial preparations were more than 4.0, measured with succinate (2 mM) as substrate.

Standard incubation procedure

The experiments were carried out at 30°C in a reaction medium containing 130 mM KCl, 10 mM HEPES buffer pH 7.2, 5.0 µM rotenone, 0.5 µM antimycin A and 10 µM Ca^{2+} as determined by atomic absorption spectroscopy. Other additions are indicated in the figure legends.

Oxygen uptake measurements

Oxygen uptake was measured using a Clark-type electrode (Yellow Springs Instruments Co.) in a glass chamber equipped with magnetic stirring. The lag phase of the oxygen uptake burst was defined as the time required to reach the maximal velocity of oxygen consumption after Fe(II)/Fe(III) addition (see example in Fig. 1).

Thiobarbituric acid-reactive substances (TBARS)

TBARS production by mitochondria was measured according to Buege and Aust [26]. Briefly, 0.4 ml samples were taken after 10 min incubation in the conditions described above and mixed with 0.4 ml of 1% thiobarbituric acid in 0.05 N NaOH, 0.2 ml of 20 % H_3PO_4 and 40 µl of 10 N NaOH. The

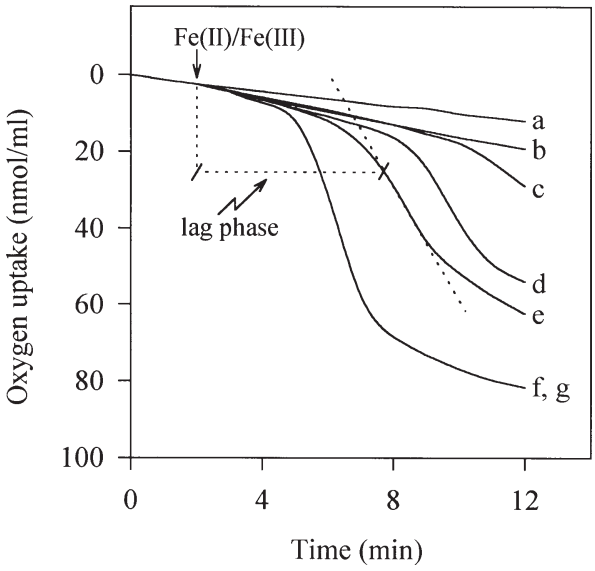

Fig. 1. Effect of Fe(III) on oxygen uptake due to lipid peroxidation induced by Fe(II)-citrate: Dependence on citrate:iron ratio. Rat liver mitochondria (RLM – 1 mg protein/ml) were incubated in standard reaction medium containing 0.05 mM (b, c), 0.1 mM (d, e) or 2 mM (a, f, g) citrate. At the arrow, 25 µM Fe(II) was added to all experiments (except in line a) and 25 µM Fe(III) was added to experiments represented by lines a, c, e and g. Data presented are representative of 3 experiments reproducible within 10%.

mixture was heated at 90–100°C for 15 min in the presence of 1 mM butylated hydroxytoluene. After cooling, 1.5 ml butanol was added to the solution. The mixture was shaken and centrifuged at 3000 rpm during 5 min. The optical density of the organic layer was determined at 535 nm. The molar extinction coefficient used to calculate TBARS concentrations was 1.56×10^5 $M^{-1}cm^{-1}$ [26].

Results

Figure 1 shows that addition of 25 µM Fe(II) to a mitochondrial suspension in the presence of a high concentration of citrate (2 mM) caused a transitory burst of antimycin A-insensitive oxygen uptake (line f). Previous data from our group [18] showed that this oxygen burst is inhibited by butylated hydroxytoluene, demonstrating that it is due to lipid peroxidation of the mitochondrial membrane. Co-addition of 25 µM Fe(III)and 25 µM Fe(II), in the presence of 2 mM citrate (line g), induced the same oxygen uptake burst profile obtained in line f. When lower concentrations of citrate were employed (0.05 mM, lines b–c and 0.1 mM, lines d–e), the rate of iron-dependent lipid peroxidation decreased. Lipid peroxidation mediated by iron has been reported to be dependent on the citrate concentration [14]. Under these conditions of low citrate concentrations, the oxygen uptake burst obtained in the presence of Fe(II) plus Fe(III) (lines c and e) was significantly greater than in the presence of Fe(II) alone (lines b and d). Moreover, the lag phase that accompanies the oxygen uptake burst was reduced from 7 min in the presence of 0.1 mM citrate plus Fe(II) (line d) to 5.5 min by Fe(III) addition (line e). These results indicate that, at low citrate levels, Fe(III) stimulates Fe(II)-induced lipid peroxidation. In the experimental conditions of Fig. 1, spontaneous Fe(II) oxidation is responsible for up to 5 nmols of O_2 uptake (result not shown). The appropriated corrections due to spontaneous Fe(II) oxidation do not result in significant alterations in the data presented in Fig. 1. A detailed study of spontaneous Fe(II) oxidation will be presented in Fig. 5.

Minotti and Aust [14] obtained peroxidation of microsomal phospholipid liposomes by Fe(II)citrate with optimum activity occurring at a Fe(II):Fe(III) ratio of 1:1. The effect of different Fe(II):Fe(III) ratios on mitochondrial lipid peroxidation was assessed by measuring total oxygen uptake (Fig. 2A) or TBARS formation (Fig. 2B). In the presence of a citrate:total iron ratio of 1:1 (●) (citrate = 0.05 mM; iron being either Fe(II), Fe(III) or a mixture of Fe(II) plus Fe(III)) maximal stimulation of lipid peroxidation occurred when the Fe(II):Fe(III) ratio was in the range of 1:1 to 1:2. In the presence of a high citrate concentration (2 mM) (■), this optimal range of Fe(II):Fe(III) ratio was not observed, rather lipid peroxidation diminished in proportion to the decrease in the initial Fe(II) concentration.

Figure 3 shows the influence of 25 µM Fe(III) on total oxygen uptake (panel A) and on the lag phase that accompanies the oxygen uptake burst (panel B) when different citrate:Fe(II) ratios (from 2:1–32:1, with Fe(II) at 25 µM) were used. As expected, an increase in citrate concentration induced an increase in total oxygen uptake (panel A), both in the

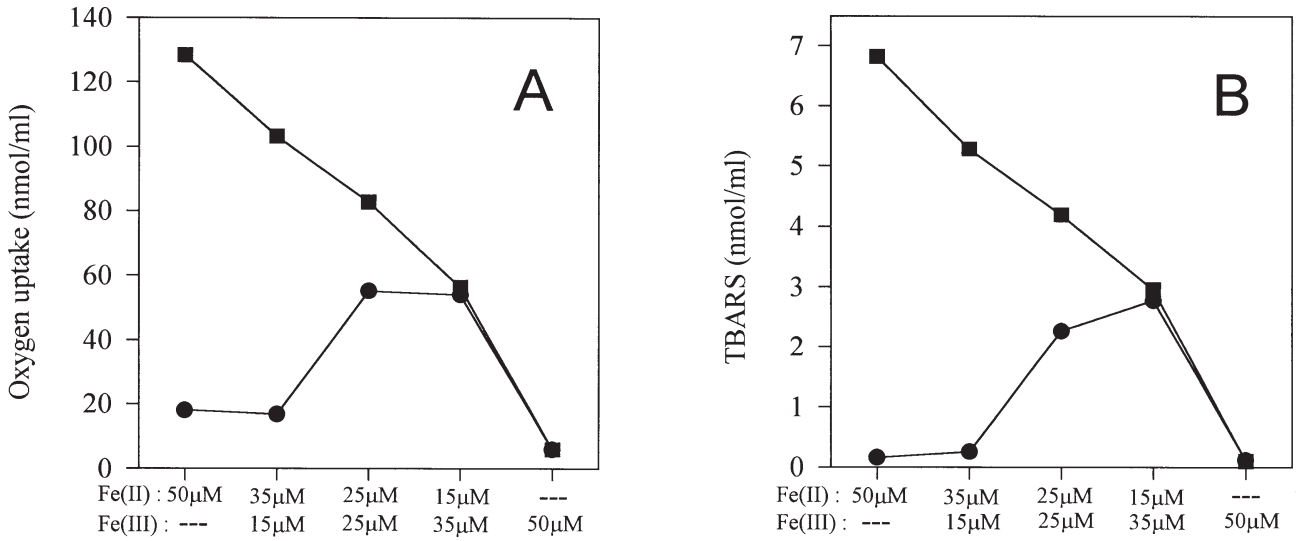

Fig. 2. Effect of the Fe(II):Fe(III) ratio on oxygen uptake (A) or TBARS formation (B) due to lipid peroxidation induced by Fe(II)citrate: Dependence on citrate concentration. RLM (1 mg protein/ml) were incubated in standard reaction medium containing 0.05 mM (●) or 2 mM citrate (■). Fe(II), Fe(III) or both were added at 2 min to obtain the concentrations indicated on the abscissa. Total oxygen uptake and TBARS formation were determined 12 min after Fe(II)/Fe(III) addition. Data are presented as means of 3 experiments reproducible within 10%.

presence (■) and in the absence (●) of Fe(III). However, only at low citrate:Fe(II) ratios (2:1 and 4:1, which corresponds to 50 and 100 µM citrate, respectively) Fe(III) significantly stimulated the total oxygen uptake (Fig. 3A). At low citrate concentrations, Fe(III) (■) also shortens significantly the lag phase that accompanies the oxygen uptake burst induced by Fe(II)citrate (●) (Fig. 3B). These results strongly suggest that Fe(III) stimulates the Fe(II)-mediated lipid peroxidation reaction mainly in its initial phase. At high citrate levels, Fe(III) lacks the ability to stimulate lipid peroxidation of the mitochondrial membrane.

Figure 4 shows that when citrate and Fe(III) concentrations were increased together (■) (so that the ratio between citrate and total Fe(II) plus Fe(III) remained at 1:1, with a fixed concentration of Fe(II)) the lag phase was reduced more rapidly than in the absence of Fe(III) (●). Stimulation of Fe(II)citrate-mediated lipid peroxidation by Fe(III) did not occur at high citrate levels (2 mM, ▲). In the presence of 5 µM Fe(II) and 2 mM citrate (result not shown) a lag phase of 6–7 min preceding the oxygen consumption was observed. In this situation, the addition of Fe(III) (5–50 µM) did not alter whatsoever the lag period.

The initial rate of Fe(II) spontaneous oxidation was measured by monitoring the oxygen consumption in buffered medium in the absence of mitochondria (Fig. 5). A Fe(II) concentration of 200 µM was used in order to obtain more significant results. The presence of Fe(III) (40–200 µM) stimulates the initial oxygen consumption rate when a low citrate:Fe(II) ratio (0.4 mM citrate) (■) was used. In the presence of 8 mM citrate (●), the presence of Fe(III) did not increase oxygen consumption.

Discussion

It has been reported that Fe(III) is able to stimulate Fe(II)-dependent lipid peroxidation [14, 15, 22, 24, 27]. This phenomenon has been linked to different hypothetical mechanisms for initiation of lipid peroxidation: direct interaction of Fe(III) with membrane lipids [27], increased concentration of iron-derived radicals [15] or formation of a Fe(II)-Fe(III)-O_2 complex [14, 22]. The results presented in this paper show that Fe(III) stimulates lipid peroxidation of rat liver mitochondrial membrane only at low citrate:Fe(II) ratios. At low citrate concentrations, Fe(III) induces a significant decrease in the initiation phase of the lipid peroxidation analyzed as the lag period that accompanies the oxygen uptake burst (Figs 1, 3B and 4). Similar results were obtained using ATP as a ligand of iron (results not shown). Although the mechanism by which Fe(III) stimulates lipid peroxidation remains unknown, we propose that Fe(II)citrate complex could have its redox potential decreased by a co-complexation of Fe(III) in the presence of low citrate levels. This would promote increased rates of both Fe(II) oxidation (Fig. 5) and oxyradical production, thus stimulating mitochondrial lipid peroxidation. The presence of high levels

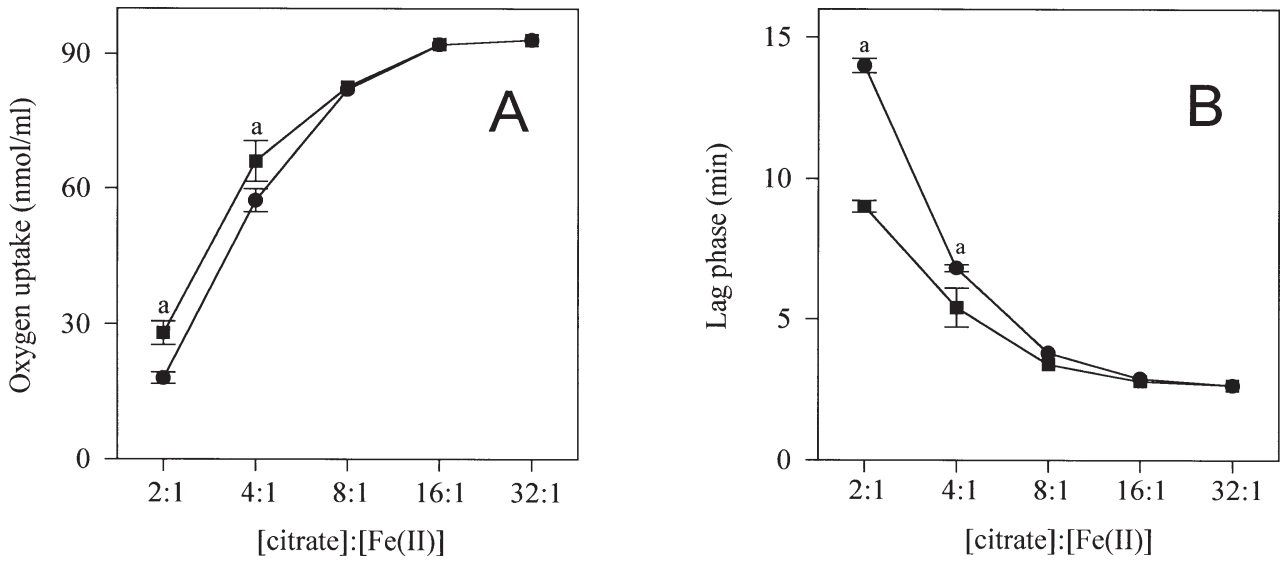

Fig. 3. Stimulation of Fe(II)citrate-induced lipid peroxidation by Fe(III). RLM (1 mg protein/ml) were incubated in standard reaction medium containing concentrations of citrate necessary to obtain the citrate:Fe(II) ratios indicated on the abscissa. Either 25 µM Fe(II) (●) or 25 µM Fe(II) plus 25 µM Fe(III) (■) were added at 2 min. In (A), total oxygen uptake was determined 10 min after Fe(II)/Fe(III) addition. In (B), the lag phase that accompanies oxygen uptake burst was determined as the time required to reach the maximal velocity of oxygen consumption after Fe(II)/Fe(III) addition (as shown in Fig. 1). Data are presented as means of 3 experiments ± S.D. *Note:* (a) $p < 0.05$ for unpaired Student's two-tailed *t*-test.

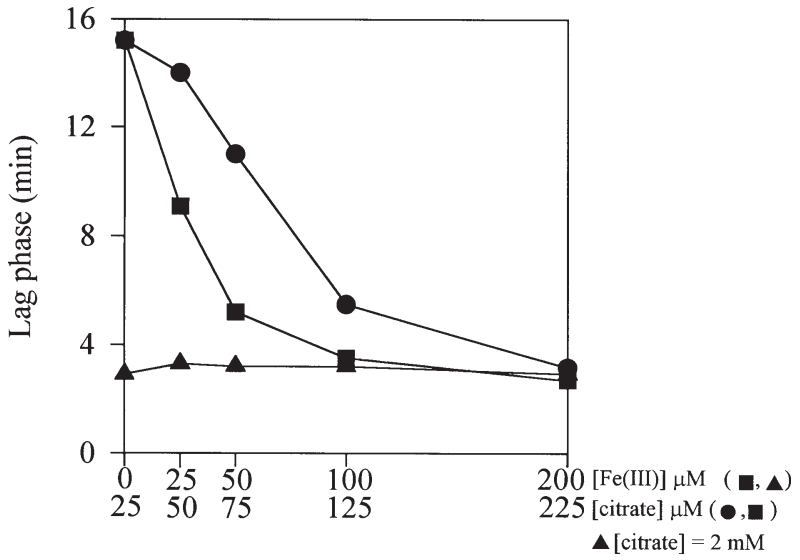

Fig. 4. Effect of Fe(III) on the lag phase that accompanies oxygen uptake due to lipid peroxidation induced by Fe(II)citrate: Dependence on citrate concentration. RLM (1 mg/ml) were incubated in standard reaction medium containing either 2 mM citrate (▲) or concentrations indicated on the abscissa (●, ■). At 2 min, either 25 μM Fe(II) (●) or 25 μM Fe(II) plus the concentrations of Fe(III) indicated on the abscissa (■, ▲) were added. Data are presented as means of 3 experiments reproducible within 10%.

of citrate would drive Fe(III) out of the putative Fe(II)-citrate-Fe(III) complex to form an unreactive Fe(III)citrate complex. Accordingly, Tadolini and Hakin [28] have observed that Fe(III) promotes an increase in deoxyribose degradation,

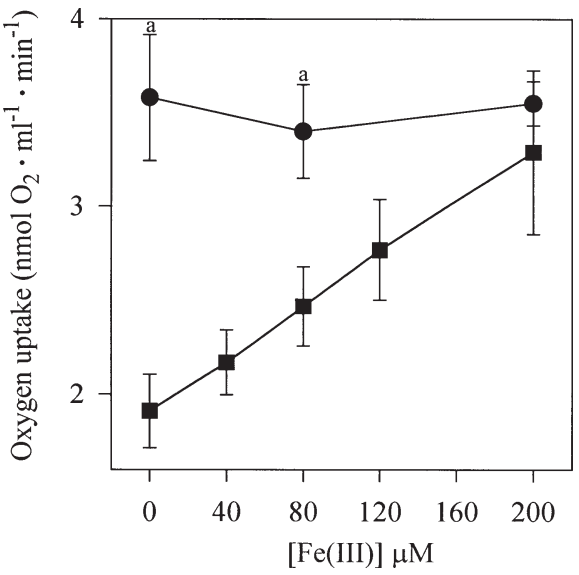

Fig. 5. Effect of Fe(III) on oxygen uptake due to spontaneous Fe(II) oxidation: Dependence on citrate concentration. The experiments were conducted in standard reaction medium (without the addition of RLM) in the presence of 0.4 mM (■) or 8 mM (●) citrate. Oxygen uptake was determined during the first 30 sec after the addition of 200 μM Fe(II). Data are presented as means of 5 experiments ± S.D. *Note:* (a) $p < 0.05$ for unpaired Student's two-tailed *t*-test.

as an index of oxyradical formation, caused by Fe(II) autoxidation in MES or MOPS buffered media (in the absence of citrate). In this last case the buffer could act as a low affinity ligand of iron.

Another interpretation for the increase in lipid peroxidation promoted by Fe(III) has been presented by Minotti and Aust [14, 22], who proposed that the Fe(II)-Fe(III)-O_2 complex initiates lipid peroxidation. This conclusion was supported by the observation that maximal lipid peroxidation occurred when a critical 1:1 ratio of Fe(II):Fe(III) was present. This observation was based only on experiments conducted without organic ligands of iron [22] or in the presence of low citrate concentrations [14]. However, as shown by the present results, the 1:1 ratio of Fe(II):Fe(III) does not produce maximal levels of lipid peroxidation when high citrate concentrations are used.

In conclusion, we propose that: (i) Fe(III) stimulates the autoxidation of Fe(II)citrate by forming a putative co-complex, in the presence of low citrate levels, leading to increased formation of oxyradicals and lipid peroxidation; and (ii) Fe(III) ions could also interact with the mitochondrial phospholipids [27] when low citrate levels are present, somehow facilitating the initiation or propagation process of lipid peroxidation, as proposed for others trivalent metal ions [29]. High levels of citrate would remove Fe(III) from the membranes. Both mechanisms (i) and (ii) could take place at the same time, thus explaining the role of Fe(III) in lipid peroxidation. In any case, the modulation of Fe(II)-mediated lipid peroxidation caused by Fe(III) and citrate is possibly of *in vivo* significance during processes of hepatic oxidative stress in iron-overload conditions.

Acknowledgements

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References

1. Kappus A: Lipid peroxidation: mechanisms, analysis, enzymology and biological relevance. In: H Sies (ed). Oxidative Stress. Academic Press Inc., London, 1985, pp 273–310
2. Halliwell B, Chirico S: Lipid peroxidation: Its mechanism, measurement, and significance. Am J Clin Nutr 57: 715S–725S, 1993
3. Bindoli A: Lipid peroxidation in mitochondria. Free Rad Biol Med 5: 247–261, 1988
4. Dinis TCP, Almeida LM, Madeira VMC: Lipid peroxidation in sarcoplasmic reticulum membranes: Effect on functional and biophysical properties. Arch Biochem Biophys 301: 256–264, 1993
5. Castilho RF, Meinicke AR, Almeida AM, Hermes-Lima M, Vercesi AE: Oxidative damage of mitochondria induced by Fe(II)citrate is potentiated by Ca^{2+} and includes lipid peroxidation and alterations in membrane proteins. Arch Biochem Biophys 308: 158–163, 1994
6. Castilho RF, Kowaltowski AJ, Meinicke AR, Vercesi AE: Oxidative damage of mitochondria induced by Fe(II)citrate or *t*-butyl hydroperoxide in the presence of Ca^{2+}: Effect of coenzyme Q redox state. Free Rad Biol Med 18: 55–59, 1995
7. Formark-Andrée P, Lee C-P, Dallner G, Ernster L: Lipid peroxidation and changes in the ubiquinone content and the respiratory chain enzymes of submitochondrial particles. Free Rad Biol Med 22: 391–400, 1997
8. Stark G: The effect of ionizing radiation on lipid membranes. Biochim Biophys Acta 1071: 103–122, 1991
9. Nantes IL, Cilento G, Bechara EJH, Vercesi AE: Chemiluminescent diphenyllacetaldehyde oxidation by mitochondria is promoted by cytochromes and leads to oxidative injury of the organelle. Photochem Photobiol 62: 522–527, 1995
10. Kowaltowski AJ, Castilho RF, Grijalba MT, Bechara EJH, Vercesi AE: Effect of inorganic phosphate concentration on the nature of inner mitochondrial membrane alterations mediated by Ca^{2+} ions. A proposed model for phosphate-stimulated lipid peroxidation. J Biol Chem 271: 2929–2934, 1996
11. Aikens J, Dix TA: Perhydroxyl radical ($HO_2^{\cdot}$) initiated lipid peroxidation. The role of fatty acid hydroperoxides. J Biol Chem 266: 15091–15098, 1991
12. Gadelha FR, Thomson L, Fagian MM, Costa AD, Radi R, Vercesi AE: Ca^{2+}-independent permeabilization of the inner mitochondrial membrane by peroxynitrite is mediated by membrane protein thiol cross-linking and lipid peroxidation. Arch Biochem Biophys 345: 243–250, 1997
13. Gutteridge JMC: Ferrous ion-EDTA-stimulated phospholipid peroxidation. A reaction changing from alkoxyl-radical- to hydroxyl-radical-dependent initiation. Biochem J 224: 697–701, 1984
14. Minotti G, Aust SD: An investigation into the mechanism of citrate-Fe(II)-dependent lipid peroxidation. Free Rad Biol Med 3: 379–387, 1987
15. Ursini F, Maiorino M, Hochstein P, Ernster L: Microsomal lipid peroxidation: Mechanisms of initiation. The role of iron and iron chelators. Free Rad Biol Med 6: 31–36, 1989
16. Shivaswamy V, Ramakrishna Kurup CK, Ramasarma T: Ferrous-iron induces lipid peroxidation with little damage to energy transduction in mitochondria. Mol Cell Biochem 120: 141–149, 1993
17. Carini R, Parola M, Dianzani MU, Albano E: Mitochondrial damage and its role in causing hepatocyte injury during stimulation of lipid peroxidation by iron nitriloacetate. Arch Biochem Biophys 297: 110–118, 1992
18. Hermes-Lima M, Castilho RF, Meinicke AR, Vercesi AE: Characteristics of Fe(II)ATP complex-induced damage to the rat liver mitochondrial membrane. Mol Cell Biochem 145: 53–60, 1995
19. Bacon BR, Britton RS: The pathology of hepatic iron overload: A free radical-mediated process? Hepatology 11: 127–137, 1990
20. Bacon BR, Tavill AS, Britton RS: In: JH Brock, JW Halliday, MJ Pippard, LW Powell (eds). Iron Metabolism in Health and Disease. W.B. Sauders Company Ltd, London, 1994, pp 311–351
21. Gutteridge JMC: The role of superoxide and hydroxyl radicals in phospholipid peroxidation catalysed by iron salts. FEBS Lett 150: 454–458, 1982
22. Minotti G, Aust SD: The requirement for iron(III) in the initiation of lipid peroxidation by iron(II) and hydrogen peroxide. J Biol Chem 262: 1098–1104, 1987
23. Halliwell B, Gutteridge JMC: Role of free radicals and catalytic metal ions in human disease: An overview. Meth Enzymol 186: 1–85, 1990
24. Hochstein P, Ernster L: ADP-activated lipid peroxidation coupled to the TPNH oxidase system of microsomes. Biochem Biophys Res Commun 12: 388–394, 1963
25. Sassa H, Takaishi Y, Terada H: The triterpene celastrol as a very potent inhibitor of lipid peroxidation in mitochondria. Biochem Biophys Res Commun 172: 890–897, 1990
26. Buege JA, Aust SD: Microsomal lipid peroxidation. Meth Enzymol 52: 302–310, 1978
27. Aruoma OI, Halliwell B, Laughton MJ, Quinlan GJ, Gutteridge JMC: The mechanism of initiation of lipid peroxidation. Evidence against a requirement for an iron(II)-iron(III) complex. Biochem J 258: 617–620, 1989
28. Tadolini B, Hakim G: The mechanism of iron (III) stimulation of lipid peroxidation. Free Rad Res 25: 221–227, 1996.
29. Verstraeten SV, Nogueira LV, Schreier S, Oteiza PI: Effect of trivalent metal ions on phase separation and membrane lipid packing: role in lipid peroxidation. Arch Biochem Biophys 338: 121–127, 1997