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INORGANIC COMPOUNDS

The involvement of transition metal ions on iron-dependent lipid peroxidation

Marisa G. Repetto · Nidia F. Ferrarotti · Alberto Boveris

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Abstract The metals iron (Fe) and copper (Cu) are considered trace elements, and the metals cobalt (Co) and nickel (Ni) are known as ultra-trace elements, considering their presence in low to very low quantity in humans. The biologic activity of these transition metals is associated with the presence of unpaired electrons that favor their participation in redox reactions. They are part of important enzymes involved in vital biologic processes. However, these transition metals become toxic to cells when they reach elevated tissue concentrations and produce cellular oxidative damage. Phospholipid liposomes (0.5 mg/ml, phosphatidylcholine (PC)/phosphatidylserine (PS), 60/40) were incubated for 60 min at 37°C with 25 μM of Fe^{2+} in the absence and in the presence of Cu^{2+}, Co^{2+}, and Ni^{2+} (0–100 μM) with and without the addition of hydrogen peroxide (H_2O_2, 5–50 μM). Iron-dependent lipid peroxidation in PC/PS liposomes was assessed by thiobarbituric acid-reactive substances (TBARS) production. Metal transition ions promoted lipid peroxidation by H_2O_2 decomposition and direct homolysis of endogenous hydroperoxides. The Fe^{2+}-H_2O_2-mediated lipid peroxidation takes place by a pseudo-second order process, and the Cu^{2+}-mediated process by a pseudo-first order reaction. Co^{2+} and Ni^{2+} alone do not induce lipid peroxidation. Nevertheless, when they are combined with Fe^{2+}, Fe^{2+}-H_2O_2-mediated lipid peroxidation was stimulated in the presence of Ni^{2+} and was inhibited in the presence of Co^{2+}. The understanding of the effects of transition metal ions on phospholipids is relevant to the prevention of oxidative damage in biologic systems.

Keywords Iron · Copper · Cobalt · Nickel · Lipid peroxidation · Phospholipid oxidative damage

M. G. Repetto · N. F. Ferrarotti · A. Boveris
Laboratory of Free Radical Biology,
Department of Physical Chemistry,
School of Pharmacy and Biochemistry,
University of Buenos Aires,
C1113AAD Buenos Aires, Argentina

N. F. Ferrarotti
Laboratory of Clinical Immunology,
Department of Clinical Biochemistry,
School of Pharmacy and Biochemistry,
University of Buenos Aires,
C1113AAD Buenos Aires, Argentina

M. G. Repetto (✉)
Química General e Inorgánica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires,
Junin 954, C1113AAD Buenos Aires, Argentina
e-mail: mrepetto@ffyb.uba.ar; mrepetto.ffyb.uba@gmail.com

Introduction

The transition metals iron, copper and cobalt are integral part of important enzymes involved in vital biologic processes. However, these transition metals become toxic to cells at elevated tissue concentrations. Nickel is not an essential element, but it is present in the environment in small amounts and became bioavailable to humans as a consequence of environmental contamination. Tissue accumulation of transition metal ions is extremely toxic, leading to many pathologic conditions consistent with oxidative damage to biologic membranes and molecules (Sies 1991; Halliwell and Gutteridge 1999; Ozcelik and Uzun 2009). Although the toxicity of transition metals has been recognized, the mechanisms of their toxic action still remain poorly understood. A growing body of evidence indicates that transition metal cytotoxicity is likely exerted through oxidative stress and damage as precursors of

cellular damage leading to apoptosis or necrosis (Halliwell and Gutteridge 1999; Chance et al. 1979; Cadenas 1989; Carter 1995; Fang et al. 2002; Oteiza et al. 2004).

The concept of oxidative stress describes an imbalance between the physiologic rate of oxidant production and the velocity of antioxidant defense system for the maintenance of biologic oxidative damage at a minimum (Sies 1991; Boveris et al. 2008). The physiologic generation of the products of the partial reduction of oxygen, O_2^-, and H_2O_2, constitute the biologic basis of the process of lipid peroxidation in mammalian cells. The lipid peroxidation of membrane phospholipids induced by reactive oxygen species leads to membrane damage and is considered the main mechanism for the onset of several pathologies.

From a molecular point of view hydroxyl radical ($HO^{\cdot}$) generation, formed from H_2O_2 and Fe^{2+} by the Fenton reaction, has been considered for a long time as the likely rate-limiting step for physiologic lipid peroxidation (Chance et al. 1979; Halliwell and Gutteridge 1999; Cadenas 1989). The Fenton–Haber–Weiss reaction (Haber and Weiss 1934) and Fenton-like reactions are frequently used to explain the toxic effects of redox-active metals where $M^{(n)+}$ is usually a transition metal ion (Eq. 1):

$$M^{(n)+} + H_2O_2 \rightarrow M^{(n+1)+} + HO^{\cdot} + HO^- \quad (1)$$

The in vivo production of $HO^{\cdot}$ in the Fenton reaction, with $M^{(n)+}$ as an ionic form of Fe, Co, Cr, Co and other metals, is the current hypothesis in the field (Oteiza et al. 2004; Valko et al. 2006). Interestingly, the concentration of intracellular redox active transition metals is either low or negligible: free Fe^{2+} is 0.2–0.5 μM and the pool of free Cu^{2+} is about a single ion per cell (Valko et al. 2006). However, trace (nM) levels of cellular and circulating active transition metal ions seem enough for the catalysis of a slow Fenton reaction in vivo at the physiologic levels of H_2O_2 (0.1–1.0 μM) (Chance et al. 1979).

Metal ions in the intracellular compartment and in plasma are mainly bound to proteins, membranes, amino-acids, nucleic acids and other cell constituents, so that the concentration of "free" aqueous metal ions are very low. However, this does not mean that only the "free" forms are active in the Fenton reaction. The transition metals Fe, Cu, Co and Ni have been recognized as redox-active metals in biologic systems and they act as pro-oxidants both in vitro and in vivo (Oteiza et al. 2004; Valko et al. 2006). Although the mechanisms of the pro-oxidant effect of transition metals are still under study, a vast evidence supports the occurrence of reactions of metal ions with H_2O_2, hydroperoxides and biologic membranes, the latter ones, the main target of oxidative damage. In other words, by one mechanism, transition metals produce lipid peroxidation by stimulation of H_2O_2 promoting, by HO generation, of a free-radical mediated processes (Chance et al. 1979; Carter 1995; Halliwell and Gutteridge 1999), and by another mechanism, they bind to negatively charged phospholipids that alters the physical properties of the bilayer and favors the initiation and propagation reactions of lipid peroxidation (Oteiza et al. 2004).

Regardless of the radical generating system used or of the nature of the lipid substrate, several studies involving Fe ions have shown that, without exception, oxidation of Fe^{2+} or reduction of Fe^{3+} is necessary for the initiation of lipid peroxidation (Halliwell and Gutteridge 1999). The extent of lipid peroxidation depends on the simultaneous availability of Fe^{2+} and Fe^{3+} and the critical 1:1 ratio of Fe^{2+} to Fe^{3+} provides the maximal rate of Fe-dependent lipid peroxidation (Ohyashiki et al. 2002).

The aim of this study is to evaluate the involvement of the transition metal ions Cu^{2+}, Co^{2+}, and Ni^{2+} in the mechanism of Fe^{2+}-mediated lipid peroxidation.

Materials and methods

Reagents and solutions

Brain phospholipids, phosphatidylcholine (PC) and phosphatidylserine (PS) were purchased from Avanti Polar Lipids Chem. Inc. (Birmingham, UK). $FeSO_4$, $CuSO_4$, $CoCl_2$, and $NiCl_2$ and 2-thiobarbituric acid, butylhydroxytoluene, butanol, Tris, and phosphates were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Liposome preparation

Chloroform solution of the brain phospholipids PC and PS were brought to dryness blowing N_2 for 5–10 min. Dried phospholipids, at the molar ratio of 60/40 for PC/PS, a mixture that yields stable liposomes, were suspended in 20 mM Tris–HCl, 140 mM NaCl solution (pH 7.40) at 0.5 mg of phospholipid/ml (2.6 mM phospholipid). Small vesicles (liposomes) were obtained by three cycles of 45 s sonication in a Branson 250 Sonifier (Branson Ultrasonic, Danbury, CT, USA) at 80 W (Verstraeten and Oteiza 2002). Milli Q water was used in liposome preparation and for the reaction media.

Measurement of lipid peroxidation

Liposomes were suspended in 140 mM NaCl, 20 mM Tris–HCl (pH 7.40) at 0.5 mg of phospholipids/ml and incubated at 37°C for 60 min with gentle agitation in the absence or in the presence of Fe^{2+}, Cu^{2+}, Co^{2+}, and Ni^{2+} (5–100 μM) with or without the addition of H_2O_2 (5–50 μM). The stock solution of $FeSO_4$ (2.5 mM) was

considered unstable and prepared immediately before use. Other transition metal stock solutions were considered stable and kept refrigerated for one week. The extent of lipid peroxidation in the samples was determined by the 2-thiobarbituric acid-reactive substances (TBARS) assay (Fraga et al. 1988). Lipid peroxidation is expressed as nmol of TBARS/mg of phospholipid.

The stimulation of Fe^{2+}-promoted TBARS production by transition metal ions (Cu^{2+}, Co^{2+}, and Ni^{2+}) was calculated as: ΔTBARS (Fe^{2+} + Me^{2+})/ΔTBARS (Fe^{2+}), where ΔTBARS (Fe^{2+} + Me^{2+}) is the increase in TBARS by the addition of Fe^{2+} and Me^{2+} over the control (no additions) values and ΔTBARS (Fe^{2+}) is the increase in TBARS by the addition of Fe^{2+} over the control values.

Data analysis

The experimental data was analyzed by one way ANOVA followed by the Tukey test for statistical significance. The values given in tables and figures are means ± standard error of the mean of at least three independent experiments with different liposome preparations. Statistical significance was considered with $P < 0.05$.

Results

Effects of transition metal ions on phospholipid auto-oxidation

Incubation of phospholipid unilamellar liposomes with Fe^{2+} or Cu^{2+} at 37°C for 60 min induced a marked process of phospholipid peroxidation. The initial TBARS content, at 0 time of incubation, due to previous and endogenous lipid peroxidation, was increased about 10-times by 100 μM Fe^{2+} and 4-times by 100 μM Cu^{2+} (Table 1; Fig. 1). The transition metal ion concentration that produced a fifty percent of maximal TBARS production ($C_{50\%}$) was 16 μM for Fe^{2+} and 21 μM for Cu^{2+}. At variance, Co^{2+} and Ni^{2+} showed no significant effects on phospholipid auto-oxidation, except for a slight effect of Co^{2+} (Table 1; Fig. 1).

Considering the effect of a second ion, Cu^{2+}, Co^{2+}, and Ni^{2+} (assayed at 10 μM) in the Fe^{2+}-mediated phospholipid peroxidation, Cu^{2+} showed a synergistic pro-oxidative effect, Ni^{2+} exhibited an additive effect, and Co^{2+} showed a protective effect (Table 1). The effects of Cu^{2+}, Ni^{2+} and Co^{2+} on the pro-oxidant action of Fe^{2+}, calculated by the ratio ΔTBARS (Fe^{2+} + Me^{2+})/ΔTBARS (Fe^{2+}), showed a synergistic pro-oxidant effect of 92% for 10 μM Cu^{2+}, an additive oxidant effect of 48% for 10 μM Ni^{2+}, and an inhibitory effect of 70% for 10 μM Co^{2+} (Table 1).

Table 1 Effect of the transition metals Fe^{2+}, Cu^{2+}, Co^{2+} and Ni^{2+} in phospholipid autooxidation and peroxidation

System (Phospholipids + Me^{2+})	TBARS (nmol/mg phospholipid)	Δ TBARS (Fe^{2+} + Me^{2+})/Δ TBARS (Fe^{2+})
Without metals	0.8 ± 0.1	–
Fe^{2+} (10 μM)	2.6 ± 0.1	1.0
Fe^{2+} (100 μM)	8.0 ± 0.2	
Cu^{2+} (10 μM)	1.0 ± 0.1	
Cu^{2+} (100 μM)	3.2 ± 0.1	
Co^{2+} (10 μM)	0.8 ± 0.1	
Co^{2+} (100 μM)	0.9 ± 0.1	
Ni^{2+} (10 μM)	1.0 ± 0.2	
Ni^{2+} (100 μM)	1.1 ± 0.1	
Fe^{2+} (10 μM) + Cu^{2+} (10 μM)	5.02 ± 0.03	1.92
Fe^{2+} (10 μM) + Cu^{2+} (100 μM)	1.91 ± 0.06	0.73
Fe^{2+} (10 μM) + Co^{2+} (10 μM)	1.85 ± 0.05	0.70
Fe^{2+} (10 μM) + Co^{2+} (100 μM)	0.63 ± 0.07	0.24
Fe^{2+} (10 μM) + Ni^{2+} (10 μM)	3.85 ± 0.08	1.48
Fe^{2+} (10 μM) + Ni^{2+} (100 μM)	3.50 ± 0.06	1.34

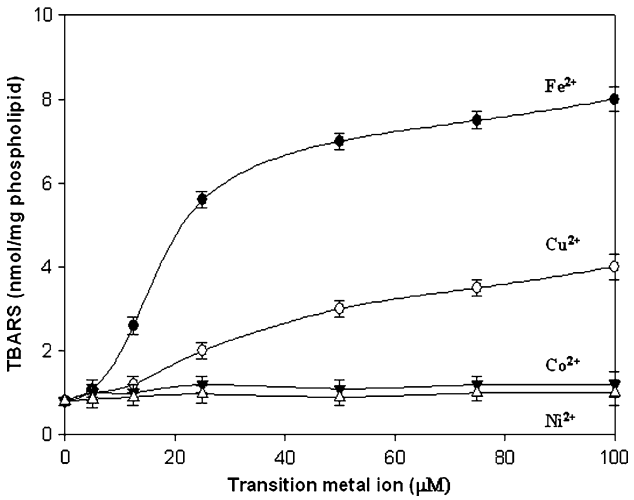

Fig. 1 Effect of the transition metal ions iron, copper, cobalt and nickel on phospholipid autoxidation process (expressed as TBARS) in liposomes (0.5 mg/ml, PC/PS) incubated at 37°C during 60 min with 0–100 μM of transition metal ions

Effect of transition metals ions in H_2O_2- and Me^{2+}-mediated phospholipid peroxidation

The joint effect of Me^{2+} and H_2O_2 in phospholipid peroxidation was assayed in the presence of Fe^{2+} and Cu^{2+}, (the two transition metal ions that were effective in promoting phospholipid auto-oxidation and peroxidation). The optimal concentration of H_2O_2 to promote the process was 10 μM for Fe $^{2+}$ and Cu^{2+} (Fig. 2). To note, H_2O_2 itself without the addition of Me^{2+} did not sustain phospholipid

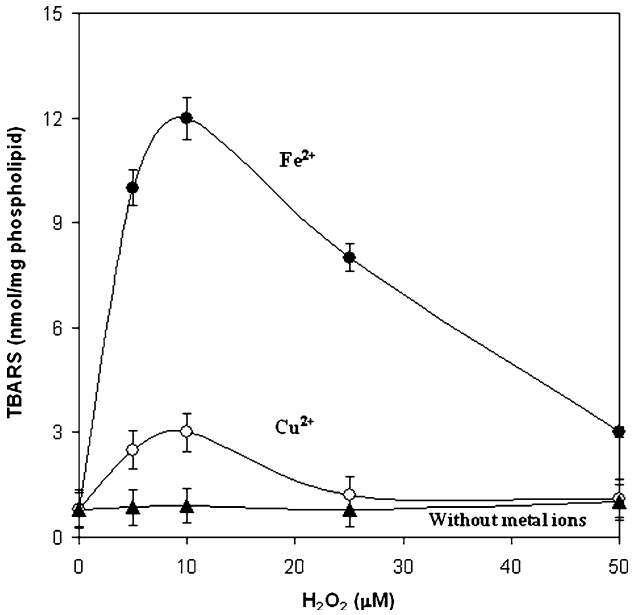

Fig. 2 Dependence of the Fe^{2+}- and Cu^{2+}-promoted TBARS production (lipid peroxidation) on H_2O_2 concentration (Fe^{2+} and Cu^{2+} = 25 μM, H_2O_2 = 0–50 μM)

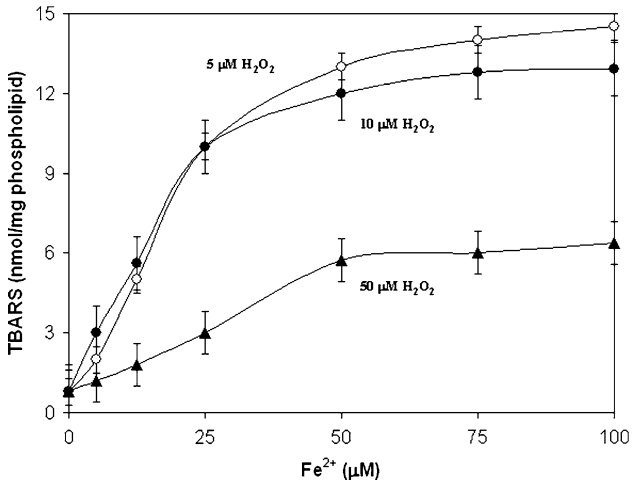

Fig. 3 Lipid peroperoxidation (expressed as TBARS) in the Fe^{2+}- and H_2O_2-promoted process at different Fe^{2+} (0–100 μM) and H_2O_2 concentrations (5, 10, 50 μM)

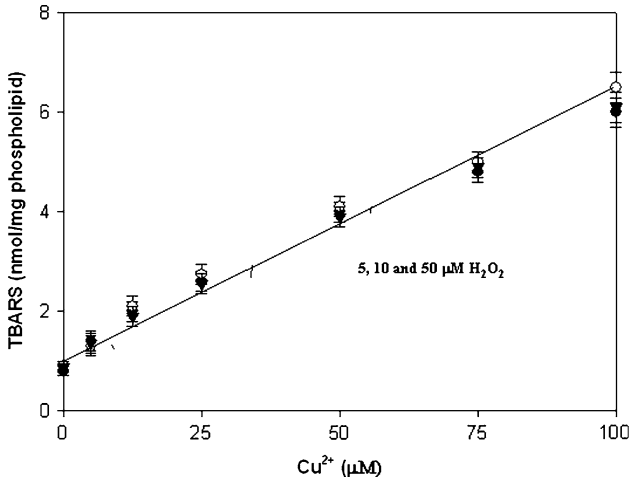

Fig. 4 Dependence of Cu^{2+}-promoted TBARS production (lipid peroxidation) in the presence of different H_2O_2 concentrations (Cu^{2+} = 0–100 μM;H_2O_2 = 5, 10, 50 μM)

peroxidation and showed that the reaction medium was transition metal free (Fig. 2). The two ions, Fe^{2+} and Cu^{2+} made with H_2O_2 an effective chemical system to promote lipid peroxidation in phospholipid liposomes (Fig. 2). In terms of TBARS production, Fe^{2+} and Cu^{2+} were about twice more effective with H_2O_2 than in its absence (Figs. 1, 2). For both ions, Fe^{2+} and Cu^{2+}, the H_2O_2 that produced fifty percent of maximal phospholipid peroxidation ($C_{50\%}$) was similar and was 2.5 μM H_2O_2. Nevertheless, the maximal level of TBARS observed was 4-times higher for Fe^{2+} than for Cu^{2+} (Fig. 2).

TBARS were determined after liposome incubations with 5, 10 and 50 μM H_2O_2 and Fe^{2+} concentrations in the range 0–100 μM (Fig. 3). Maximal phospholipid peroxidation was observed at 5–10 μM H_2O_2 and at 25 μM Fe^{2+}, with $[H_2O_2]/[Fe^{2+}]$ ratios of 0.2–0.4 (Fig. 3). The phospholipid peroxidation $C_{50\%}$ was 29 μM Fe^{2+}, about twice the level required in the absence of H_2O_2 ($P < 0.05$).

The effect of Cu^{2+} on Me^{2+}- and H_2O_2-mediated phospholipid peroxidation was assayed in the range of 0–100 μM Cu^{2+} (Fig. 4). The whole process was independent of H_2O_2 concentrations and linearly dependent on Cu^{2+} concentration in a pseudo-first order process (Fig. 4). In this system of Me^{2+}- and H_2O_2-mediated lipid peroxidation, 10 μM Cu^{2+} showed an additive effect with 10 Fe^{2+}, and Co^{2+} and Ni^{2+}, both at 10 μM exhibited a marked inhibitory effect (Table 2). The effects of Cu^{2+}, Ni^{2+} and Co^{2+} on the pro-oxidant effect of Fe^{2+} were calculated by the ratio ΔTBARS $(Fe^{2+} + Me^{2+})$/Δ TBARS (Fe^{2+}) and were: pro-oxidant of 15–25% for 10–50 μM for Cu^{2+}; inhibitory of 57–79% for 10–50 μM Co^{2+}; and inhibitory of 59% for 10 μM Ni^{2+} and prooxidant for 50 μM Ni^{2+} (Table 2).

Time course of the Me^{2+}- catalyzed Fenton-type process of lipid peroxidation

The time course of TBARS formation in PC/PS (60/40) liposomes was followed for 90 min in the presence of 10 μM H_2O_2 and 10 μM Fe^{2+}, in the absence or added with 50 μM Me^{2+}. The process was linear during 60 min in Fe^{2+}-dependent lipid peroxidation and during 90 min in the presence of $Fe^{2+}$and of a second Me^{2+} ion (Fig. 5). Considering the time point of 60 min, the inclusion of Cu^{2+} in the reaction medium increased the process by

Table 2 Effect of the transition metal ions Cu^{2+}, $Co^{2+}$and Ni^{2+} in the Fe^{2+}- and H_2O_2-mediated lipid peroxidation process (H_2O_2 = 10 μM)

System (Phospholipids + Me^{2+} + H_2O_2)	TBARS (nmol/mg phospholipid)	Δ TBARS (H_2O_2 + Fe^{2+} + Me^{2+})/Δ TBARS (H_2O_2 + Fe^{2+})
Without metals	0.8 ± 0.1	–
Fe^{2+} (10 μM)	5.6 ± 0.1	1.0
Fe^{2+} (50 μM)	12.0 ± 0.1	
Cu^{2+} (10 μM)	2.0 ± 0.1	–
Cu^{2+} (50 μM)	4.0 ± 0.1	
Co^{2+} (10 μM)	0.8 ± 0.1	–
Co^{2+} (50 μM)	0.9 ± 0.1	
Ni^{2+} (10 μM)	1.0 ± 0.2	–
Ni^{2+} (50 μM)	1.1 ± 0.1	
Fe^{2+} (10 μM) + Cu^{2+} (10 μM)	6.4 ± 0.1	1.14
Fe^{2+} (10 μM) + Cu^{2+} (50 μM)	6.8 ± 0.1	1.21
Fe^{2+} (10 μM) + Co^{2+} (10 μM)	3.2 ± 0.1	0.57
Fe^{2+} (10 μM) + Co^{2+} (50 μM)	3.8 ± 0.2	0.68
Fe^{2+} (10 μM) + Ni^{2+} (10 μM)	3.3 ± 0.1	0.59
Fe^{2+} (10 μM) + Ni^{2+} (50 μM)	6.3 ± 0.3	1.13

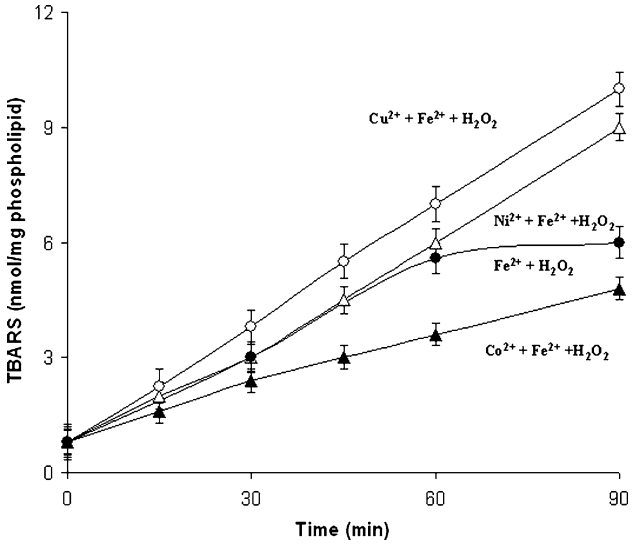

Fig. 5 Time course of the production of TBARS in lipid peroxidation processes in which the Fenton reaction, catalyzed by Fe^{2+} and other transition metal ions is the kinetically rate-limiting step (H_2O_2 = 10 μM, Fe^{2+} = 10 μM, and Cu^{2+}, Co^{2+} and Ni^{2+} = 50 μM)

22%, whereas Ni^{2+} has no significant effect and Co^{2+} partially protected liposomes, by 38%, from phospholipid peroxidation (Fig. 5).

Discussion

The toxicity of transition metal ions in biologic systems is a complex process that involves reactions that reach equilibrium, as the binding to cellular structures and the redox reactions with cellular components, and free-radical mediated reactions that are transient and out-of-equilibrium. These latter reactions involve the Me^{2+}- and free radical-mediated initiation reactions of lipid peroxidation that leads to irreversible oxidation in which O_2 is added to carbon free radicals ($R^{\cdot}$) to form oxyradicals ($ROO^{\cdot}$) and stable oxidation products (TBARS).

The transition metals Fe and Cu are part of the active centers of enzymes and as such they undergo cyclic reduction and oxidation. The free ions of these transition metals, Fe^{2+}, Fe^{3+}, Cu^{+} and Cu^{2+}, in plasma and cells, react with physiologically relevant metabolites, as O_2, O_2^{-}, H_2O_2 and ascorbic acid, as described by the thermodynamic oxidation potentials (Kohen and Nyska 2002) and results in a redox state of the $Me^{(n)+}/Me^{(n+1)+}$ couple that is nearly at equilibrium with the steady state of reductants and oxidants.

The free radical-mediated oxidative damage to lipids, proteins and DNA promoted by transition metal ions is properly summarized by stating that it seems to occur in aerobic cells by the generation of the hydroxyl radical, $HO^{\cdot}$ or a similar oxidizing species, by the Fenton reaction (Giulivi et al. 1995).

Transition metal ions strongly bind to phospholipids and alter the liposome bilayer and increase their susceptibility to lipid peroxidation (Oteiza et al. 2004). It is also known that these metal ions form complexes with endogenous polyacids (as citric acid) that keep the chemical property of the ion for catalyzing free-radical mediated reactions but prevent the structural effects in biomembranes (Chance et al. 1979; Carter 1995; Oteiza et al. 2004).

Hydrogen peroxide does not induce phospholipid peroxidation in a cell-free system and in the absence of transition metal ions (Fig. 2), indicating that Me^{n+} ions are necessary for the initiation reaction of lipid peroxidation.

Iron is the most abundant transition metal in the body (about 4 g in adult humans), almost all in the form of hemoproteins and iron-sulfur centers. Free iron concentrations are particularly low (0.2–0.5 μM; Valko et al. 2006), mainly as Fe^{2+} due to the biologic reductants O_2^- and ascorbic acid (Fang et al. 2002). Superoxide radicals are able to reduce the Fe^{3+} of ferritin and some iron-sulfur centers to Fe^{2+}, making Fe^{2+} readily released (Gutteridge 1984).

As mentioned in Introduction, it has been considered for a relatively long time that the main source of $HO^{\cdot}$ formation in aerobic cells is the Fenton reaction ($\Delta E^{\circ} = +0.307$ V), with H_2O_2 homolyzed and reduced (Eq. 2):

$$Fe^{2+} + H_2O_2 \rightarrow [Fe(II)H_2O_2] \rightarrow Fe^{3+} + HO^- + HO^{\cdot} \quad (2)$$

An alternative explanation for the Fenton reaction postulates the formation of an oxidizing Fe(IV) species, as FeO^{2+} or Fe(IV)=O (Lloyd et al. 1997). These perferryl intermediates are thought as powerful oxidants, similar to HO· but distinguishable from it. In the Fenton reaction, $HO^{\cdot}$ derives exclusively from H_2O_2 and not from H_2O (Lloyd et al. 1997). Yamazaki and Piette 1990, after a EPR spin-trapping study of the reaction of Fe^{2+} with H_2O_2, proposed two reaction pathways from the $[Fe(II)H_2O_2]$ intermediate of reaction 2, one that generates $HO^{\cdot}$ and the other one that produces a perferryl species.

Transition metal ions also stimulate Fe^{2+}-mediated lipid peroxidation by the reductive cleavage of endogenous lipid hydroperoxides (ROOH) of membrane phospholipids to the corresponding alkoxyl ($RO^{\cdot}$) and peroxyl ($ROO^{\cdot}$) radicals in a process that is known as ROOH-dependent lipid peroxidation (Eqs. 3, 4):

$$Fe^{2+} + ROOH \rightarrow RO^{\cdot} + OH^- + Fe^{3+} \quad (3)$$

$$Fe^{3+} + ROOH \rightarrow RO_2^{\cdot} + H^+ + Fe^{2+} \quad (4)$$

The mechanisms of these two reactions appear to involve the formation of Fe(II)-Fe(III) or Fe(II)-O_2-Fe(III) complexes with maximal rates of $HO^{\cdot}$ radical formation at a ratio Fe(II)/Fe(III) of 1 (Gutteridge 1984; Tadolini and Hakim 1996).

In the conditions of this study, at low level of H_2O_2, Fe^{2+} induces lipid peroxide decomposition, generating peroxyl and alkoxyl radicals and favoring lipid peroxidation. Theses results agree with the evidence given by Ohyashiki et al. (2002), showing that the onset of the Fe^{3+} stimulatory effect on Fe^{2+}-dependent lipid peroxidation is mainly due to the reactive oxygen species production via Fe^{2+} oxidation and that endogenous ROOH is not directly involved in the Fe^{3+} stimulatory effect.

Copper is an essential element, as it is part of the active center of cytochrome oxidase, the enzyme responsible for O_2 uptake in mammalian cells (Chance et al. 1979), with a content of about 80 mg in adult humans. Increased contents of Cu in the human body, especially in the central nervous system exert toxic effects with metabolic disturbances (Carter 1995; Ozcelik and Uzun 2009). The Cu^+ ion is considered an effective catalyst for the Fenton reaction (Eq. 5), and Cu^{2+} and Cu^+ are known for their capacity to decompose organic hydroperoxides (ROOH) to form $RO^{\cdot}$ and $ROO^{\cdot}$ (Eqs. 6, 7) (Halliwell and Gutteridge 1984; Klotz et al. 2003).

$$Cu^+ + H_2O_2 \rightarrow [Cu(I) - H_2O_2] \rightarrow Cu^{2+} + HO^- + HO^{\cdot} \quad (5)$$

$$Cu^+ + ROOH \rightarrow RO^{\cdot} + OH^- + Cu^{2+} \quad (6)$$

$$Cu^{2+} + ROOH \rightarrow RO_2^{\cdot} + H^+ + Cu^+ \quad (7)$$

As a summary, this study indicates the likelihood of Fe^{2+}-dependent initiation reactions for lipid peroxidation in biologic systems. The process of lipid peroxidation has been recognized as free radical-mediated and physiologically occurring (Halliwell and Gutteridge 1999; Chance et al. 1979) with the supporting evidence of in situ organ chemiluminescence (Boveris et al. 1980). The main initiation reaction is understood to be mediated by $HO^{\cdot}$ (Eq. 2) or by a ferryl intermediate, both with the equivalent potential for hydrogen abstraction from an unsaturated fatty acid, with formation of an alkyl radical ($R^{\cdot}$) (Eq. 8):

$$HO^{\cdot} + RH \rightarrow H_2O + R^{\cdot} \quad (8)$$

In the lipid peroxidation of the brain PC-PS liposomes, hydrogen abstraction occurred at the allilic carbons 9 and 10 of the oleic acid chain. Secondary initiation reactions are provided by hydrogen abstraction by $RO^{\cdot}$ and $ROO^{\cdot}$ (Eqs. 9, 10) at the mentioned tertiary carbons:

$$RO^{\cdot} + RH \rightarrow ROH + R^{\cdot} \quad (9)$$

$$RO_2^{\cdot} + RH \rightarrow ROOH + R^{\cdot} \quad (10)$$

$$R^{\cdot} + O_2 \rightarrow RO_2^{\cdot} \quad (11)$$

The $R^{\cdot}$ and $ROO^{\cdot}$ radicals (Eqs. 8–11) are central to the free radical-mediated process of lipid peroxidation. The addition reaction of R with O_2 to yield $ROO^{\cdot}$ (Eq. 11) yield a product that is able to abstract hydrogen atoms and to regenerate $R^{\cdot}$ for a new cycle (Eq. 10). The whole process, by repetition of reaction 11, consumes O_2 and produces malondialdehyde (O=HC–CH_2–CH=O), 4-hydroxynonenal and other dialdehydes as secondary and end products of lipid peroxidation. The process produces TBARS at an

approximate ratio of 0.12 TBARS/O_2 and normally utilized as measurement of the rate and extent of lipid peroxidation (Hochstein et al. 1964).

From the observed effects of Fe^{2+} and Cu^{2+}, it can be stated that Fe^{2+} and Cu^{2+} promote lipid peroxidation in phospholipid liposomes by homolysis of H_2O_2 with $HO^{\cdot}$ generation and by scission of ROOH with $RO^{\cdot}$ and $ROO^{\cdot}$ production and by altering liposome surface structure.

Nickel and cobalt may participate, similarly to iron and copper, in the metal-catalyzed peroxidation of cellular phospholipids. Although not clear yet, it is generally considered that Fenton-type reactions may be involved in the toxic effects of these metals in vivo.

Nickel is a long term recognized human carcinogen that forms compounds with oxidation states −1 to +4, with the prevalent form of +2. The main recognized biochemical effects of Ni^{2+} are the interference in DNA repair (Hartwig et al. 2002) and the promotion of lipid peroxidation and protein carbonyl formation (Athar et al. 1987; Huang et al. 1994, Chen et al. 1998; Hfaiedh et al. 2008). The oxidative effects were ascribed to the Ni(III)-Ni(II) complex and to the oxene species $(NiO)^{2+}$ that are formed at neutral pH (Nieboer et al. 1989; Kasprzak et al. 2003). Ni^{2+} does not promote lipid peroxidation in the presence of hydroperoxides but it does in the presence of Fe^{2+}/H_2O_2 mixtures, in such conditions is oxidized to Ni^{3+} and reduced back to Ni^{2+} by cellular reductant as O_2^{-}, in a Haber/Weiss-type reaction (Krezel and Bal 2004; Salnikow and Kasprzak 2005).

Cobalt ions, Co^{2+}, did not promote phospholipids peroxidation directly and showed an almost negligible pro-oxidant effect in the presence of Fe^{2+}/H_2O_2. Co^{2+} could act as pro-oxidant through its capacity to replace redox-active metals from their binding sites in hemoproteins. The possibility that Co^{2+}-mediated free radical generation contributes to cobalt toxicity was studied by EPR spin trapping and it was found that O_2^{-} was formed in the reaction of H_2O_2 with Co^{2+}, a reaction inhibited when Co^{2+} was chelated with ADP, EDTA or citrate (Carter 1995).

The observed and reported effects of Co^{2+} and Ni^{2+} in vivo are better explained by their structural effects after binding to the liposome and cell surfaces. The current hypothesis is that ions with redox capacity stimulate Fe^{2+}-initiated lipid peroxidation, but ions without redox capacity increase Fe^{2+}-initiated lipid peroxidation by increasing lipid packing, bringing phospholipid acyl chains closer together, thus favoring the propagation step of lipid peroxidation (Verstraeten et al. 1997). It is well known that the densely packed phopholipids in liposomes and biomembranes are less sensitive to lipid peroxidation than phospholipids in solution (Verstraeten and Oteiza 1995). Further studies are needed to better understand the cellular effects of this essential, but also toxic trace elements and their functional interaction with nutrients and biologic systems.

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