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Rat liver mitochondrial dysfunction by addition of copper(II) or iron(III) ions

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ABSTRACT

Increased copper (Cu) and iron (Fe) levels in liver and brain are associated to oxidative stress and damage with increased phospholipid oxidation process. The aim of this work was to assess the toxic effects of Cu^{2+} and Fe^{3+} addition to rat liver mitochondria by determining mitochondrial respiration in states 3 (active respiration) and 4 (resting respiration), and phospholipid peroxidation. Both, Cu^{2+} and Fe^{3+} produced decreases in O_2 consumption in a concentration-dependent manner in active state 3: both ions by 42% with malate-glutamate as complex I substrate (concentration for half maximal response (C_{50}) 60 μM Cu^{2+} and 1.25 mM Fe^{3+}), and with succinate as complex II substrate: 64–69% with C_{50} of 50 μM Cu^{2+} and with C_{50} of 1.25 mM of Fe^{3+}. Respiratory control decreased with Cu^{2+} (C_{50} 50 μM) and Fe^{3+} (C_{50} 1.25-1-75 mM) with both substrates. Cu^{2+} produced a 2-fold increase and Fe^{3+} a 5-fold increase of thiobarbituric acid-reactive substances (TBARS) content from 25 μM Cu^{2+} (C_{50} 40 μM) and from 100 μM Fe^{3+} (C_{50} 1.75 mM). Supplementations with Cu^{2+} and Fe^{3+} ions induce mitochondrial dysfunction with phospholipid peroxidation in rat liver mitochondria. Although is proved that a Fenton/Haber Weiss mechanism of oxidative damage occurs in metal-ion induced mitochondrial toxicity, slightly different responses to the metal ions suggest some differences in the mechanism of intracellular toxicity. The decreased rates of mitochondrial respiration and the alteration of mitochondrial function by phospholipid and protein oxidations lead to mitochondrial dysfunction, cellular dyshomeostasis and cell death.

1. Introduction

The ions of the transition metals copper (Cu) and iron (Fe) are essential for life and clearly exhibit the hormesis phenomenon: they are needed at low levels for normal health in mammals (Recommended Daily Allowance for humans: 1–3 mg Cu/day and 10–15 mg Fe/day) but at higher levels (8–10 mg Cu kg^{-1} and 20–30 mg Fe kg^{-1}, both become toxic for humans [1–4] and animals [5–8]. The essential biological role of Cu and Fe is exerted by their ions Cu^{1+}, Cu^{2+}, Fe^{2+} and Fe^{3+}. The cellular and tisular levels of Cu and Fe ions are homeostatically determined by regulatory proteins and metallo chaperons that control metal ions absorption, transport and storage.

There is an increasing interest in the study of the toxic effects of Cu and Fe ions because they have been proposed as participants in the etiology of some pathology, especially of neurodegenerative diseases. Previous research has shown that increased Cu and Fe levels are associated to oxidative stress and oxidative damage in rat brain [9] and liver [6].

Regarding Cu, it is well known that increased Cu levels in the liver and brain, promote oxidative damage. The best known disorder associated to Cu dyshomeostasis and increased Cu levels, is Wilson's disease, an autosomal recesive disorder linked to the Cu-translocase expressed in hepatocytes. This enzyme is critical in the distribution and elimination of excess Cu from the organism [10]. Cu toxicity has been linked especially to neurological disorders and also to cancer progression, cardiovascular disease, atherosclerosis and diabetes [11].

Regarding Fe, hereditary hemochromatosis is a genetic disorder affecting Fe metabolism, producing a Fe overload in the body. There is also a secondary hemochromatosis, where the cause of Fe overload is not genetic, with the most common case of the iron overload from repeated blood transfusions [12,13]. The genetic form is due to a mutation in the human hemochromatosis protein (HFE gene) which is responsible for the regulation of Fe ions uptake. The common symptoms and pathology of Fe ions toxicity are: hepatomegaly, steatosis, insulin resistance, subclinical inflammation, skin pigmentation, joint diseases and lethargy [14,15].

The transition metals Fe and Cu are biochemically redox active: in the cell cytosol the stored cupric and ferric forms (Cu^{2+} and Fe^{3+}) are reduced by superoxide radical (O_2^{-}). The reduced forms (Cu^{1+} and Fe^{2+}) promote the homolysis of hydrogen peroxide (H_2O_2) and of organic hydroperoxides (ROOH) with formation of the powerful oxidant hydroxyl free radical ($HO^{\bullet}$) [6,16–20].

There are numerous reports in cells and organs in which Fe and Cu overloads lead to increased phospholipid peroxidation [6,7]. In a

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model system of phosphatidylcholine and phosphatidylserine (60:40) liposomes and H_2O_2, Cu^{2+} and Fe^{3+} promoted lipid peroxidation [20]. The results in cells and organs and in model systems were interpreted as the consequence of the homolytic scission of H_2O_2 and of endogenous ROOH with the generation of HO• and alkoxyl (RO•) radicals that start the free-radical mediated process of lipid peroxidation.

In eukariots, molecular oxygen (O_2) from the atmospheric air is reduced in mitochondria to water (H_2O) in an exergonic process coupled to adenosine triphosphate (ATP) synthesis. In aerobic and evolved organisms, oxidative phosphorylation occurs in mitochondria, where ATP synthesis is driven by the electron transfer in the respiratory chain from the reduced form of nicotinamide adenine dinucleotide (NADH) to O_2 [21].

Mitochondria are formed by a double membrane. The inner membrane has convolutions called cristae which provide a very large surface area. It contains the respiratory chain electron carriers (complexes I-IV), ATP synthase (complex V), adenosine diphosphate (ADP)-ATP translocases and other membrane transporters. The enzymatic complexes I-IV constitutes the electron transfer chain. Electron transfer through these complexes is coupled to proton (H^+) pumping from the mitochondrial matrix to the intermembrane space (the space between the inner and the outer mitochondrial membranes), being the inner membrane impermeable to H^+. Thus, an electrochemical potential of H^+ is produced from electron flow and it is the proton motive force for ATP synthesis by the F_0F_1-ATP synthase (complex V) [21].

Because of a small electron leak in the inner membrane, $O_2^{\cdot-}$ is a by-product of mitochondrial electron transfer. It is mainly produced in complex III by semi-ubiquinone ($UQH^{\cdot}$) autooxidation (75%) and in complex I by the NADH-dehydrogenase flavin mononucleotide ($FMNH^{\cdot}$) autooxidation (25%) [22]. Superoxide radical ($O_2^{\cdot-}$) is the mitochondrial stoichiometric precursor of H_2O_2. The antioxidant enzyme superoxide dismutase (SOD) (Mn-SOD in the matrix and Cu,Zn-SOD in the intermembrane space) catalyzes its dismutation into H_2O_2 and O_2. Mitochondria are the main intracellular source for $O_2^{\cdot-}$, H_2O_2 and $HO^{\cdot}$ productions and the cellular generation of $O_2^{\cdot-}$ and of H_2O_2 is quantitatively and by far a mitochondrial phenomenon. There are other sources for these two chemical species but they are less important in quantitative terms. About 1–2% of the O_2 consumed in tissue normal respiration is converted to $O_2^{\cdot-}$. In this way, the chemical species $O_2^{\cdot-}$ is the precursor of most of the reactive oxygen species (ROS). Early experimental reports recognized successively the mitochondrial production of H_2O_2 and of $O_2^{\cdot-}$. The determined mitochondrial rate of H_2O_2 production accounts for 0.26–0.96% of the rate of organ O_2 uptake. For perfused liver and heart, the ratios were 0.96 and 0.67%, showing a very significant H_2O_2 production [23,24]. $O_2^{\cdot-}$ (and H_2O_2) production is larger in metabolic state 4 (inactive, absence of ADP) and minimal in state 3 (active, with ADP). The mitochondrial O_2 uptake in states 4 and 3 accounts for 1.32% and 0.22% of the respiration in rat liver, for 0.93% and 0.13% in rat heart and for 0.18% and 0.01% in rat brain. Thus, $O_2^{\cdot-}$ and H_2O_2 are produced as a physiological phenomenon of mitochondrial respiration and of oxidative metabolism.

Fe and Cu induced oxidative damage has been extensively report in different experimental models. However, the actual molecular events underlying the oxidative damage remain elusive. While the Fenton/Haber-Weiss reaction serves as an explanation to the oxidative modifications of macromolecules observed, the availability of the intracellular metals to participate in Fenton chemistry is uncertain. Moreover, additional concomitant reactions between Fe and Cu with biological targets may have been overlooking. The simultaneous study of both Fe and Cu, while stressing the differences between the toxic mechanisms of both metals will help outline the main events that are critical for the impairment of cell function.

Therefore, the aim of this work is to determine the toxic effect of Fe and Cu ions by assessing mitochondrial respiration and phospholipid peroxidation in Cu^{2+} and Fe^{3+} added rat liver mitochondria, to analyze the participation of H_2O_2 and $O_2^{\cdot-}$ in the observed toxic effect and finally to compare the toxicity of the two metals.

2. Materials and methods

2.1. Chemicals

Chemicals were from Sigma-Aldrich Chemical Co. (St. Louis, MO). Other reagents were of analytical grade.

2.2. Animals

Sprague-Dawley male rats (200 g) were from the Central Animal House, School of Pharmacy and Biochemistry, University of Buenos Aires, and were acclimatized under laboratory conditions 7 days before experiments. Rats were provided with standard diet and water *ad libitum* and were maintained under controlled temperature (23–25 °C) and humidity (50%) with an alternating 12 h light–dark cycle. Rats were anesthetized with 15% urethane at 1.5 g × kg^{-1} (ip) and the liver was excised and suspended in 250 mM sucrose and 10 mM HEPES buffer solution (pH 7.40) as isolation medium at 0 °C. Animal care was givenin compliance with Argentine regulations (ANMAT) and with the Guidelines for Ethical Treatment in Animal Experimentation of the American Physiological Society (Bethesda, MD).

2.3. Isolation of mitochondria

Liver was cut and suspended in EDTA-free isolation medium at a ratio of 5 mL/g of tissue and the tissue was finely cut with scissors and washed 3–5 times. The preparation, always kept at 0 °C, was passed through a Potter-Elvehjem homogenizer and centrifuged at 700*g* (Sorvall-Du Pont Instruments, Model RC5S) during 10 min at 0–2 °C. The pellet (nuclei, unbroken cells and other tissue components) was discarded and the supernatant was centrifuged at 8000*g* during 10 min at 0–2 °C. Supernatant was discarded and this new pellet, containing mitochondria able to perform oxidative phosphorylation, was washed and re-suspended in isolation medium [25–27].

2.4. Mitochondrial oxygen consumption

Mitochondrial O_2 consumption depends on the mitochondrial metabolic state and on the type of tissue. In Table 1, the metabolic states that define the mitochondrial respiratory rates are given.

Mitochondrial respiration was determined in states 3 (active respiration) and 4 (resting respiration) that fully describe mitochondrial function and the quality of the mitochondrial preparation, which is mainly evaluated by the respiratory control, the ratio of the rates of O_2 uptake in state 3 and state 4.

Oxygen uptake was determined polarographically using a Clark-type electrode (Hansatech Oxygraph System DW1) at 25 °C. The O_2 concentration of air-equilibrated reaction medium at 25 °C is 0.25 mM O_2. Mitochondrial O_2 uptake was determined in the following reaction medium: 120 mM KCl, 5 mM KH_2PO_4, 3 mM 4-(2-Hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) and 1 mg/mL bovine serum albumin (BSA), pH 7.20 (adjusted with 1 M KOH). Since Cu^{2+} and Fe^{3+} effects were determined, the chelators ethylenediaminetetraacetic acid (EDTA) and ethyleneglycol-aminoethyl-tetraacetic acid (EGTA) were avoided, and the omission did not affect mitochondrial respiratory rates. For the assay, 1 mL of respiration medium was placed in the

Table 1
Mitochondrial metabolic states [28,29].

State	Oxygen	ADP	Substrate	O_2 upake	Limiting factor
1	Available	Low	Low	Slow	ADP
2	Available	High	Very low	Slow	Substrate
3	Available	High	High	Fast	Respiratory chain
4	Available	Low	High	Slow	ADP
5	Unavailable	High	High	None	Oxygen

electrode chamber, that was closed and the substrates were added with a Hamilton syringe. State 4 respiratory rate was determined with either 2 mM glutamate and 5 mM malate as substrate for complex I or 10 mM succinate as substrate for complex II. After the addition of the mitochondrial suspension the respiration in state 4 and in state 3 were successively recorded.

For copper and iron ions exposure, the corresponding salts were added to the chamber before the mitochondrial suspension from a 10 mM Cu^{2+} stock solution ($CuSO_4 \cdot 5H_2O$) or a 100 mM Fe^{3+} stock solution ($FeCl_3 \cdot 6H_2O$) to obtain final Cu^{2+} concentrations of 10–200 μM and final Fe^{3+} concentrations of 0.25–4 mM.

2.5. Mitochondrial phospholipid peroxidation

Mitochondrial phospholipid peroxidation was determined by the thiobarbituric acid-reactive substances (TBARS) assay, as described by Fraga et al. (1988) [30]. The sample, a rat liver mitochondrial suspension of about 1.5 mg protein/mL, was 10 min incubated with Cu^{2+} or Fe^{3+} in the reaction medium for O_2 consumption in absence or presence of substrate (2 mM glutamate and 5 mM malate). After that, samples were twice washed by centrifugation at 8200*g* and re-suspended in isotonic phosphate buffer solution (PBS) for TBARS determination. The sample was added to 4% butylhydroxytoluene in ethanol, 20% trichloroacetic acid, and 0.7% thiobarbituric acid. The deproteinized supernatant was heated at 100 °C for 20 min, and the absorption of the pink solution was determined at 535 nm ($\varepsilon = 156\ mM^{-1}\ cm^{-1}$) and expressed as nmol TBARS × mg of $protein^{-1}$.

2.6. Protein content

Protein contents were measured using the Folin reagent with BSA as standard [31].

2.7. Data analysis

Values in text, figures and tables are expressed as mean ± standard error of the mean (SEM) from at least 3 independent determinations. The software GraphPad InStat3 was used and one-way analysis of variance (ANOVA) and Dunnett's tests as *post hoc* tests were performed. Significance is indicated by the p value, where $p < 0.05$ was considered significant, $p < 0.01$ was considered very significant and $p < 0.001$ was considered extremely significant.

3. Results

3.1. Oxygen consumption of rat liver mitochondria added with Cu^{2+} or Fe^{3+} ions

Both Cu^{2+} and Fe^{3+} produced a decrease in the O_2 consumption of active state 3. The rate of O_2 uptake was determined immediately after loading the metals to the chamber. The metal concentration for half maximal response was determined by the C_{50} [6]. With malate-glutamate as complex I substrate, both Cu^{2+} and Fe^{3+}, produced a significant decrease of about 42% in O_2 consumption in state 3, with C_{50} of 60 μM Cu^{2+} (Fig. 1A), and of 1.25 mM Fe^{3+} (Fig. 1B). The coupling between respiration and phosphorylation was assessed by the determination of the respiratory control (RC) ratio. This index decreased in a concentration-dependent manner from 50 μM Cu^{2+} (37% with a C_{50} of 60 μM, Fig. 1C) and 1.5 mM Fe^{3+} (35% with a C_{50} of 1.25 mM, Fig. 1D).

Similar results were obtained when a complex II substrate (succinate) and state 3 mitochondria were used (Fig. 2). Significant decreases in O_2 consumption (64–69%) with a C_{50} of 50 μM Cu^{2+} (Fig. 2A) and with a C_{50} of 1.25 mM Fe^{3+} (Fig. 2B) were observed. The RC index was decreased by 40% in a concentration-dependent manner from 25 μM Cu^{2+} (C_{50} 50 μM, Fig. 2C) and from 3 mM Fe^{3+} (C_{50} 1.75 mM,

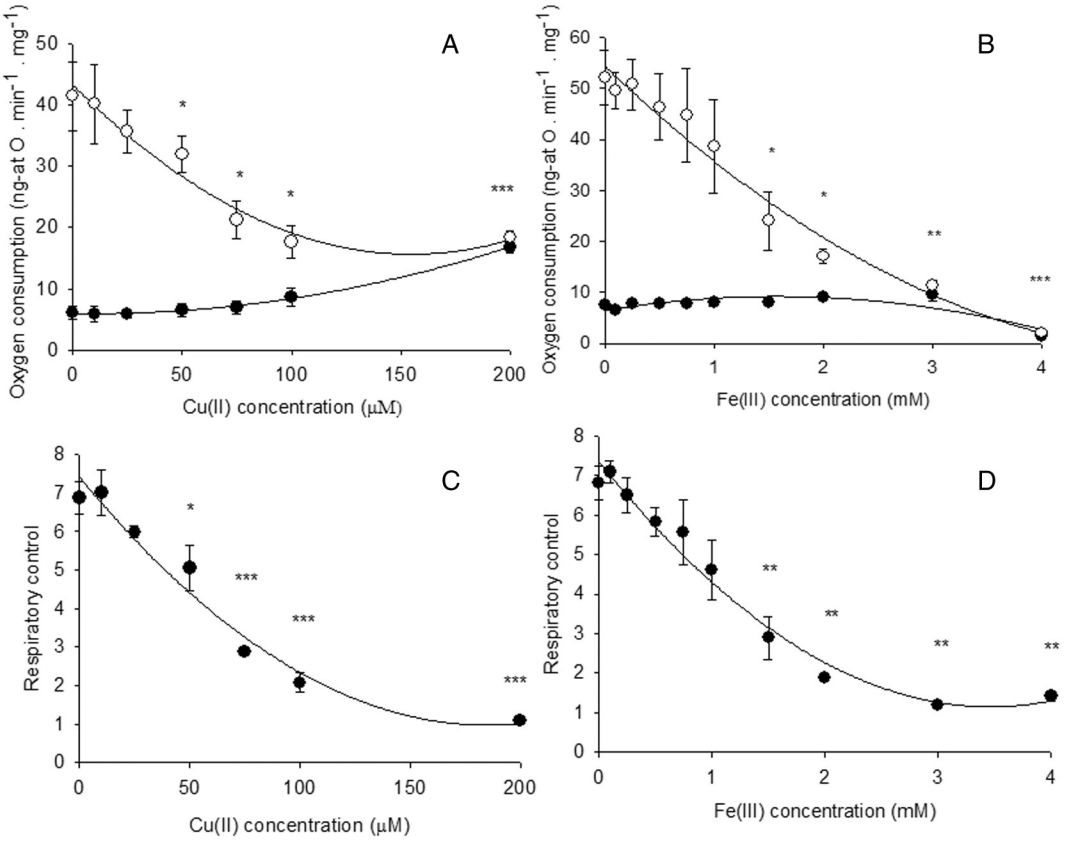

Fig. 1. Oxygen consumption (A and B) (○, state 3; ●, state 4) and respiratory control (C and D) of rat liver mitochondria exposed to Cu^{2+} (A and C) and Fe^{3+} supplementation with complex I substrate (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

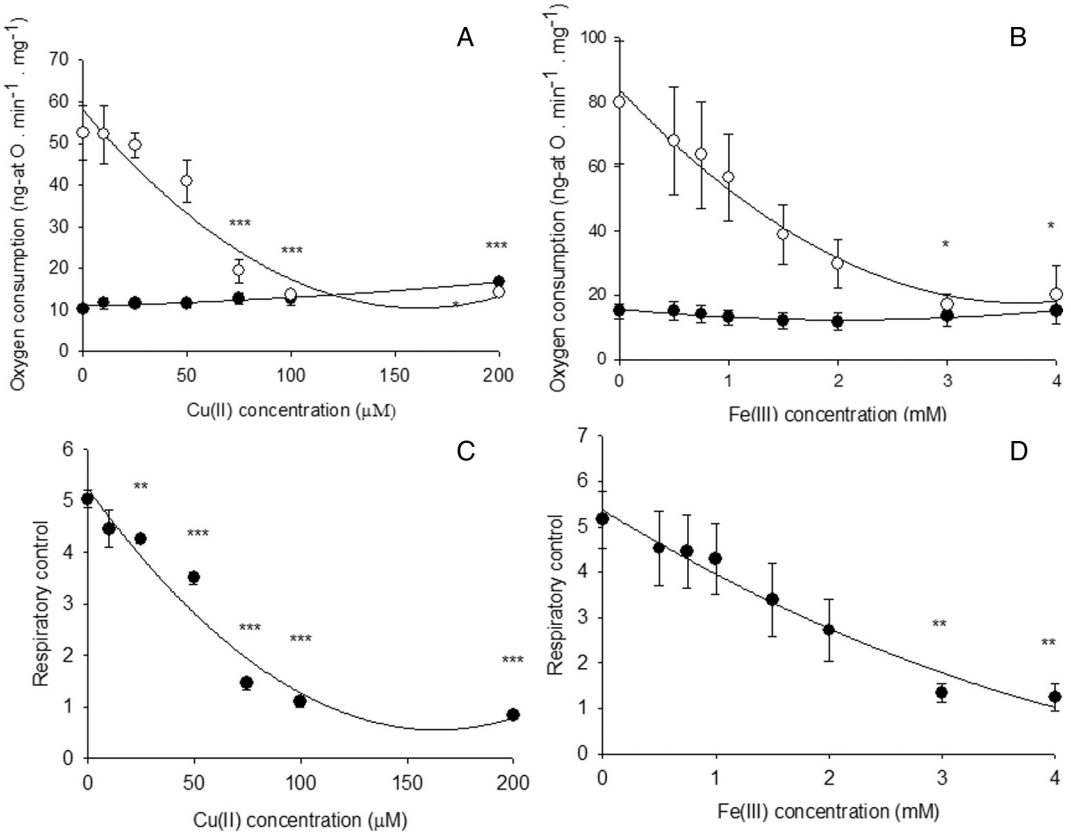

Fig. 2. Oxygen consumption (A and B) (○, state 3; ●, state 4) and respiratory control (C and D) of rat liver mitochondria exposed to Cu^{2+} (A and C) and Fe^{3+} (B and D) supplementations with complex II substrates (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

Fig. 2D). It is clear that the observed respiration inhibitions are independent of the site of substrate entry to the respiratory chain. From here on, only malate-glutamate was used as respiratory substrate.

To test the ability of the metal ions to interact with the O_2^- and H_2O_2 produced in state 4, O_2 consumption in state 3 and respiratory control were determined after different metal ion exposure times in state 4 (Fig. 3). It was found that the longer the time of the metal ion exposure in state 4 (state of larger O_2^- and H_2O_2 production), the higher the decrease in state 3 oxygen consumption (Fig. 3). This is observed in the slope of O_2 consumption decrease, which is 2-fold higher when mitochondria are exposed for longer times to Cu^{2+} (Fig. 3A) and to Fe^{3+} in state 4 (Fig. 3B).

The same toxicity enhancing effect by metal incubation in state 4 was observed in respiratory control (Figs. 3C and D): the slope for the decrease in respiratory control is almost twice when mitochondria are longer incubated in state 4 with the transition metal ions.

3.2. Phospholipid peroxidation in rat liver mitochondria added with Cu^{2+} or Fe^{3+} ions

The oxidative structural damage in liver mitochondria exposed to high concentrations of Cu^{2+} or Fe^{3+} was determined through phospholipid peroxidation that was measured as TBARS production. For that, mitochondria were exposed to the metal ions in metabolic state 4 for 10 min, so that the respiration by-products O_2^- and H_2O_2 were formed inside mitochondria. Here, Cu^{2+} produced a 2-fold increase in TBARS content from 25 μM (C_{50} 40 μM, Fig. 4A) and Fe^{3+} produced a 5-fold increase in TBARS at 100 μM Fe^{3+} with a C_{50} of 1.75 mM (Fig. 4B).

TBARS were also determined in metabolic state 1, where substrate and ADP levels are negligible, so there is neither significant O_2 consumption nor O_2^- and H_2O_2 productions (Table 1). High Cu^{2+} concentrations (for instance, 100 μM) produce a significant increase (4-fold) in phospholipid peroxidation only in mitochondria respiring in the presence of substrate. This indicates that the metal ions themselves are not enough to start lipid peroxidation if respiratory substrates are absent (state 1, without substrate) (Fig. 5).

The same effect was observed in iron supplementation, with a 2-fold increase in phospholipid peroxidation at 100 μM Fe^{3+}. A 4-fold increase in phospholipid peroxidation was found in non-respiring mitochondria (state 1) when Fe^{3+} concentration was raised to 500 μM (Fig. 5).

4. Discussion

Liver mitochondria have been recently used to assess the oxidative damage in the organelles produced by tert-butyl hydroperoxide [32–33] and by drugs, as the insecticide fipronil [34], the anticonvulsivant valproic acid [35] and pioglitazone, used in the treatment of type II diabetes [36].

There are reasons to use liver mitochondria to test Cu^{2+} and Fe^{3+} toxicities. First, mitochondria are the subcellular organelles responsible for cell bioenergetics and oxygen metabolism, what is of great importance when studying oxidative stress and damage. Mitochondria have the master role in O_2 metabolism and mitochondrial function is an indicator of full cell metabolism. Second, mitochondrial O_2 consumption reports about organelle integrity and oxidative damage by the phospholipid peroxidation of mitochondrial membranes. Third, mitochondria are an O_2^- and H_2O_2 producing system whose production rates are easily modulated by switching among different metabolic states (Table 1) or by addition of respiratory substrates or inhibitors to the reaction medium. Thus, it is possible to study the effect of Cu^{2+} and Fe^{3+} on mitochondria by determining the rates of respiration and of lipid peroxidation.

In this work, the addition of Cu^{2+} or Fe^{3+} at different concentrations in the respiration medium was first tested to find the concentration

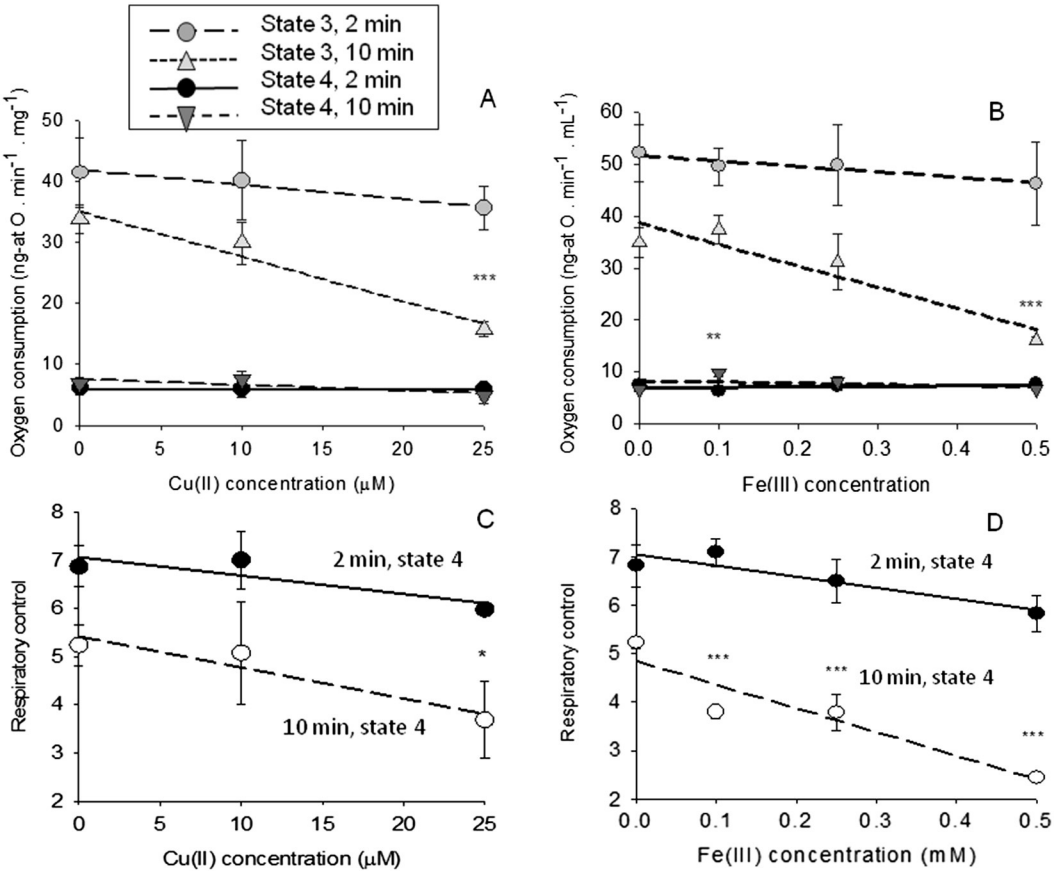

Fig. 3. Oxygen consumption of rat liver mitochondria exposed to Cu^{2+} (A) and Fe^{3+} (B) added to the reaction medium with 2 or 10 min of exposure of the metal ions in state 4. Malate-glutamate as substrate (**$p < 0.01$; ***$p < 0.001$).

range where changes in mitochondrial respiration occur. Cu^{2+} was found to significantly decrease O_2 consumption from 50 to 75 μM but the trend to the decrease of this parameter was observed from 10 μM Cu^{2+}, with both malate-glutamate or succinate as substrates. Fe^{3+} addition did not show any decrease in O_2 consumption up to 250 μM (results not shown) and statistically significant changes were only observed at concentrations higher than 1.5 mM. In these determinations, the O_2 uptake corresponds to the first minute after addition of the metal ions, in a direct effect of metal ions on mitochondrial respiration (Figs. 1 and 2). Nevertheless, when mitochondria were incubated with the metal ions (Cu^{2+} or Fe^{3+}) for 10 min in resting state 4, to allow the interaction of the metal ions with the O_2^- and the H_2O_2 produced in this metabolic state, quite lower metal concentrations where needed to achieve a significant decrease in mitochondrial respiration (Fig. 3). A concentration of 25 μM Cu^{2+} was enough to produce a significant decrease in O_2 consumption in active state 3 as well as in RC. However, 100 μM Fe^{3+} was only able to increase O_2 consumption in state 4 and to decrease the RC without changes in active respiration. A 500 μM Fe^{3+} concentration was needed to produce a significant decrease in O_2 consumption in active state 3.

An evident but not minor observation was that Cu^{2+} turns out to be much more toxic than Fe^{3+}, since 5- to 10-fold higher concentrations were needed for Fe^{3+} to produce effects of the same magnitude than Cu^{2+}. This difference between metal concentration and magnitude of

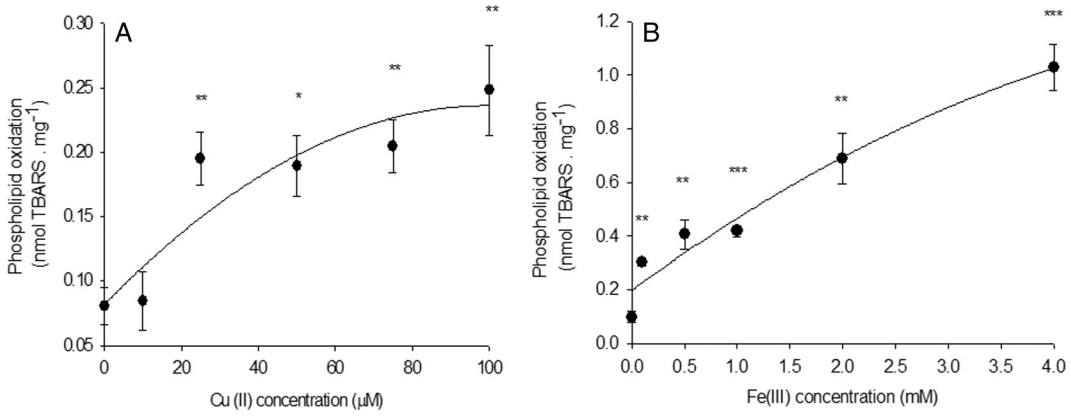

Fig. 4. Respiratory control in rat liver mitochondria exposed to Cu^{2+} (A) and Fe^{3+} (B) in the reaction medium for 2 and 10 min of exposure in state 4. Malate-glutamate was used as substrate (*$p < 0.05$; ***$p < 0.001$).

toxic effect, in favour of Cu, was also observed when determining phospholipid peroxidation.

Both Cu^{2+} and Fe^{3+} were able to increase TBARS content in mitochondria after 10 min of incubation with the metal ions in a dose-dependent manner. Statistically significant changes were observed at 25 μM Cu^{2+} and 100 μM Fe^{3+}. For Cu^{2+}, this coincides with the decrease of mitochondrial active respiration in the same conditions. For 100 μM Fe^{3+}, despite of not producing changes in O_2 consumption in state 3, mitochondrial function showed in this condition a slight but significant increase in respiration rate in state 4 (typically understood as a sign of uncoupling) and, in consequence, a decrease in the respiratory control index. Interestingly, the metal-ion induced oxidative damage to phospholipids to start the process of lipid peroxidation depended on mitochondrial respiration in state 4. Despite of the supplementation with the metal ions at 100 μM, Cu^{2+} or Fe^{3+}, no changes in TBARS productions were observed when a respiratory substrate was not added. Nevertheless, Fe^{3+} at 500 μM was able to produce mitochondrial phospholipid peroxidation independent on respiration (500 μM Fe^{3+} was the lowest concentration where a decrease in active O_2 consumption was observed). Moreover, 100 μM Fe^{3+} that significantly increased phospholipid peroxidation without affecting active respiration, depending on respiring mitochondria to produce the observed oxidative damage. These results indicate that, beyond the widely described Fenton-Haber Weiss mechanism to explain the toxicity of redox active metals, these metal ions present slightly different toxicity mechanisms.

Since the metal-ions induced phospholipid peroxidation depends on state 4 respiration and the presence of respiratory substrates, toxicity seems to take place through the reaction of these metal ions with H_2O_2 (from O_2^- dismutation) yielding $HO^\bullet$ through the Fenton/Haber-Weiss reaction; HO• starts the free-radical mediated phospholipid peroxidation with a structural alteration of the mitochondrial membrane. The linkage between Cu and Fe ions supplementation and HO• generation was observed in two experiments. First, the metal ion toxicities in liver mitochondria were greatly enhanced when the metal ions were incubated with mitochondria in state 4, the metabolic state of highest steady state concentrations of H_2O_2 and O_2^-. The longer the time that mitochondria were respiring in state 4 (with the higher production of O_2^- and H_2O_2) the more marked the inhibition of state 3 respiration. Moreover, we assessed the dependence of the oxidative damage to phospholipids on the availability of substrate for mitochondrial respiration in state 4. Here, we found that Cu^{2+} itself is not able to induce phospholipid peroxidation while Fe^{3+} does, depending on its concentration (Fig. 5).

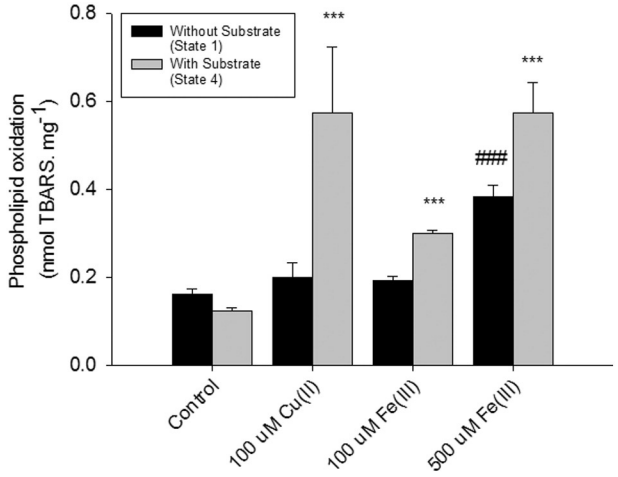

Fig. 5. Phospholipid peroxidation determined as TBARS in rat liver mitochondria exposed to Cu^{2+} (A) and Fe^{3+} (B) supplementation at different concentrations. Metal ion exposure was performed in state 4 for 10 min before sampling for the TBARS assay (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

Concerning the molecular mechanism of Cu^{2+} and Fe^{3+} toxicities it seems reasonable to consider that the metal ions are adsorbed at the outer mitochondrial surface and reduced to Cu^{1+} and Fe^{2+} by transmembrane reductases. Hydrogen peroxide, especially in state 4 conditions, diffuses from inside mitochondria to the reaction medium and encounters Cu^{1+} and Fe^{2+} at the interface, both ready to catalyze H_2O_2 homolysis and $HO^\bullet$ formation.

To further explain the linkage between mitochondrial phospholipid peroxidation and the inhibition of respiration, it should be taken into account the nature of the mitochondrial inner membrane, where electron transfer and oxidative phosphorylation take place. About 25% of the mitochondrial inner membrane is composed of phospholipids (no other lipids are present) with 30% of them being cardiolipin, which consists in two phosphatidic acids bound to a glycerol molecule by the phosphate groups. Cardiolipin is tightly bound to mitochondrial membrane proteins and seems essential for proper protein function. The presence of conjugated double bonds in the fatty acids of phosphatidic acid makes cardiolipin highly susceptible of reacting with electrophiles and to undergo phospholipid peroxidation, altering in this way the phospholipid bilayer of the mitochondrial inner membrane and its ability to couple electron transfer with ATP production [37]. Therefore, metal-induced phospholipid peroxidation (Fig. 5) would alter the bilayer structure of the inner mitochondrial membrane and thus, respiratory chain function.

It is frequent to consider or suppose equivalence between O_2^- and H_2O_2 and ROS. It is clear in this study that metal ion toxicity is related to and dependent on O_2^- and H_2O_2 productions. On the other hand, the concept of ROS has been changing with time. Originally, ROS meant extracellular O_2^-, H_2O_2 and $HO^\bullet$ [38] and was proposed as the mechanism for luminal chemiluminescence after monocyte activation by lymphokines. The concept was later extended to the intracellular medium and the list of ROS grew with time. Today, it is accepted that includes O_2^-, H_2O_2 and $HO^\bullet$ and intermediates and products of the free radical-mediated lipid peroxidation, such as $ROO^\bullet$ (peroxyl radical), ROOH (organic hydroperoxide), $^1O_2$ (singlet oxygen) and peroxynitrite ($ONOO^-$), this latter the product of the reaction between and NO and O_2^-. As said before, the chemical species involved in the toxicity of Cu^{2+} and Fe^{3+} are the products of the partial reduction of O_2: O_2^-, H_2O_2 and $HO^\bullet$.

Finally, we have observed in this work the major ability of Cu to induce mitochondrial dysfunction. The mitochondrial porins, which are responsible for the extremely high permeability of the mitochondrial outer membrane to hydrophilic low molecular weight molecules [39], allow Cu^{2+} and Fe^{3+} ions to cross the outer membrane and enter the mitochondrial intermembrane space. Once within the organelle both Cu^{2+} and Fe^{3+} may be reduced. These reductive reactions are thermodynamically favored due to their positive redox potential ($E^\circ_{Cu^{2+}/Cu^{+}} = +0.159$ and $E^\circ_{Fe^{3+}/Fe^{2+}} = +0.770$ V) and likely coupled to the oxidation of GSH molecules ($E^\circ_{GSSG/GSH} = -0.240$ V) as well as protein –SH groups. An uncontrolled redox state of proteins would be reflected in impaired enzyme and cell functions [40]. Nevertheless, the kinetic hindrances faced by these reactions ultimately determine their occurrence, as observed in the contrasting lack of reactivity of Fe^{3+} to Cu^{2+}, despite the higher E° of the former. It is worth mentioning that without disregarding the importance of the –SH pool in the handling of the intracellular redox homeostasis, oxidative modifications of other protein groups such as –OH might have an impact on the outcome of the metal exposure. The way both ions, either in a physiological or an overload, context reach the mitochondrial matrix requires further clarification".

5. Conclusion

Both supplementations with copper and iron ions induce mitochondrial dysfunction related to phospholipid peroxidation in rat liver mitochondria. Although it is proved that a Fenton/Haber-Weiss reaction mechanism of oxidative damage occurs in metal-ion induced

mitochondrial toxicity, slightly different responses to the metal ions suggest some differences in the mechanism of intracellular toxicity. The decreased rates of mitochondrial respiration and the alteration of mitochondrial normal function by phospholipid peroxidation and protein oxidation lead to mitochondrial dysfunction, cellular dyshomeostasis and ultimately to cell death.

Both Fe and Cu induced phospholipid peroxidation is dependent on the mitochondrial production of H_2O_2 and $O_2^{\cdot-}$. However, the direct impact on the organelle functionality of far lower concentrations of Cu than Fe suggests a dissociating event in the mechanism of toxicity of both metals, which should been further studied.

Abbreviations

ADP	adenosine diphosphate
ATP	adenosine triphosphate
ANOVA	analysis of variance
BSA	bovine serum albumine
C_{50}	concentration of half maximal response
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-aminoethyl-tetraacetic acid
HEPES	4-(2-Hydroxyethyl)-1-piperazine ethanesulfonic acid
H_2O_2	hydrogen peroxide
ip	intraperitoneal
NADH	reduced form of nicotinamide adenine dinucleotide
$O_2^{\cdot-}$	superoxide anion
$HO^{\bullet}$	hydroxyl radical
RC	respiratory control
$RO^{\bullet}$	alkoxyl radical
$ROO^{\bullet}$	lipid peroxyl radical
ROOH	lipid hydroperoxide
ROS	reactive oxygen species
rpm	revolutions per minute
SEM	standard error of the mean
TBARS	thiobarbituric acid-reactive substances

Conflicts of interest

The authors declare that they have no conflict of interest.

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