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Electron transport chain dysfunction by H_2O_2 is linked to increased reactive oxygen species production and iron mobilization by lipoperoxidation: studies using *Saccharomyces cerevisiae* mitochondria

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Abstract The mitochondrial electron transport chain (ETC) contains thiol groups (−SH) which are reversibly oxidized to modulate ETC function during H_2O_2 overproduction. Since deleterious effects of H_2O_2 are not limited to –SH oxidation, due to the formation of other H_2O_2-derived species, some processes like lipoperoxidation could enhance the effects of H_2O_2 over ETC enzymes, disrupt their modulation by –SH oxidation and increase superoxide production. To verify this hypothesis, we tested the effects of H_2O_2 on ETC activities, superoxide production and iron mobilization in mitochondria from lipoperoxidation-resistant native yeast and lipoperoxidation-sensitized yeast. Only complex III activity from lipoperoxidation-sensitive mitochondria exhibited a higher susceptibility to H_2O_2 and increased superoxide production. The recovery of ETC activity by the thiol reductanct β-mercaptoethanol (BME) was also altered at complex III, and a role was attributed to lipoperoxidation, the latter being also responsible for iron release. A hypothetical model linking lipoperoxidation, increased complex III damage, superoxide production and iron release is given.

Keywords Yeast · Oxidative stress · Lipid peroxidation · Thiol oxidation · Respiratory chain · β-mercaptoethanol · Linolenic acid

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Introduction

The mitochondrial electron transport chain (ETC) transfers electrons from the oxidation of metabolic fuels to a final acceptor coupled with the translocation of H^+ to generate an electrochemical gradient whose energy is used for a variety of vital processes including ATP synthesis (Dimroth et al. 2000), ion homeostasis (Nicholls 2005), protein import (Martin et al. 1991), and programmed cell death (Ly et al. 2003), among others. Since these processes are important to cell survival, it is not surprising that mitochondrial dysfunction has been proposed to be involved in almost all pathological processes (Seppet et al. 2009). Thus, the study of the mechanisms underlying ETC dysfunction is an issue of constant growing interest, because it will help to

clarify the participation of mitochondria in disease, which, in turn, will allow use of this organelle as a promissory target for pharmacological and therapeutical interventions.

A key factor that affects ETC function is oxidative attack by reactive oxygen species (ROS) produced, primarily upon activation of NADPH oxidase (Nox) (Krause 2007), or by alterations in electron fluxes in the redox sites of complexes I and III of the ETC (Turrens 2003). The primary ROS produced from those sources is superoxide radical $\left(O_2^{\bullet-}\right)$. However, $O_2^{\bullet-}$ is less reactive than other ROS, and its deleterious effects are partially mediated by rapid dismutation to H_2O_2 (Halliwell and Gutteridge 1999), which is reflected by the several-fold higher, steady-state concentrations of H_2O_2 with respect to $O_2^{\bullet-}$ calculated for the mitochondrial matrix (Cadenas and Davies 2000). H_2O_2 reversibly oxidizes thiol groups (−SH) of cysteine residues and modifies the activity of proteins (Jones 2008). Indeed, the oxidation of reactive –SH groups has been demonstrated in the matrix surface of complexes I, II and IV (Lin et al. 2002) and in Rieske Fe-S protein, as well as core protein I of complex III (Hurd et al. 2007).

Functional studies have shown that –SH reversible modification can modulate the activity of ETC complexes. For example, in a previous study from our group, it was shown that the inhibition of complexes II and III by micromolar doses of H_2O_2 can be reverted by treatment with the thiol reductant β-mercaptoethanol (BME) (Cortés-Rojo et al. 2007). In an earlier report, it was described that –SH groups localized in distinct sites of complex II regulate its catalytic activity (Lê-Quôc et al. 1981). The ascorbate-iron system has been also proposed to inhibit ETC function through the oxidation of –SH groups, given that the pre-incubation of rat synaptosomes with glutathione prevented the inactivation of complexes II and III by the former oxidant system (Cardoso et al. 1999). However, because reversion of ETC inhibition by this antioxidant was not tested, the possibility that glutathione was acting merely as a ROS scavenger cannot be discarded, since it may instead be maintaining –SH groups in a reduced state. In addition, it has been observed that –SH groups of certain cysteine residues from complex I become gluthationylated in response to ROS generation, leading to enzyme inhibition without a further increase in $O_2^{\bullet-}$ production. Therefore, it was suggested that cysteine modification may protect complex I from oxidative damage through a cycle of –SH oxidation-regeneration (Hurd et al. 2008). Taking into account this suggestion and the data from the other works referred to above, it is also plausible that the physiological role of reversible –SH oxidation in the ETC could be to serve as a sensor of the redox state of mitochondria. In such a case, the purpose would be to decrease the electron fluxes towards respiratory complexes under oxidative conditions, thereby avoiding further ROS production and ETC damage by limiting electron leakage in the redox sites of ETC.

Despite the role that H_2O_2 can play during ETC inhibition by –SH oxidation, it must be expected that other ROS participate at the same time in such phenomena due to metal or enzyme-catalyzed conversion of H_2O_2 into other stronger oxidants like hydroxyl radicals ($OH^{\bullet}$) or sulfur-centered radicals ($S^{\bullet}$) (Chen et al. 1999), which, on the other hand, are highly reactive with fatty acyl chains (North et al. 1992; Schoneich et al. 1992). Mitochondria are also an important site of iron handling and storage, as this organelle actively participates in synthesis of both iron-sulfur (Fe-S) clusters and haem (Kwok and Kosman 2006), and ETC enzymes are rich in these prosthetics groups. On the other hand, both H_2O_2 and $O_2^{\bullet-}$ can raise the concentration of free iron available for $OH^{\bullet}$ production through degradation of haem groups and Fe-S clusters of several proteins (Longo et al. 1999; Jang and Imlay 2007). Thus, the abundance of iron in mitochondria, along with the formation of H_2O_2-derived radical species, may enhance the deleterious effects of H_2O_2 on ETC function through the damage in the phospholipids where the ETC enzymes are embedded.

In a previous report, we have shown that lipoperoxidation increases the sensitivity of complexes III and IV to an oxidative insult, impairs cytochrome *b* reduction and increases ROS production at complex III (Cortés-Rojo et al. 2009). Considering that H_2O_2 would indirectly promote lipoperoxidation and that this process can enhance the damage in ETC during ROS attack, we hypothesized that augmented sensitivity of ETC to H_2O_2 due to lipoperoxidation may disrupt the reversible modulation of ETC activity by –SH oxidation and increase both ROS production and iron release. To test this hypothesis, we evaluated the effects of H_2O_2 on ETC function, reversion of ETC damage by a thiol reductant, ROS production and mitochondrial labile iron levels in mitochondria from yeast with native (lipoperoxidation-insensitive) and manipulated (lipoperoxidation-sensitive) fatty acid composition.

Materials and methods

Reagents

α-Linolenic acid (*cis,cis,cis*-9,12,15-octadecatrienoic acid, purity ≥99%), Igepal CA-630, Mn-SOD from *E. coli*, catalase, H_2O_2 (30% v/v), butylated hydroxytoluene (BHT), and the reagents used for lipoperoxidation assays and ETC activity measurements were obtained from Sigma Chemical. Co. (St. Louis, MO, USA). Zymolyase 20 T was purchased from ICN Biomedicals, Inc. (Aurora, OH, USA). Calcein acetoxymethyl ester (calcein-AM) was acquired from Molecular Probes (Eugene, OR, USA). Chelex 100 resin was obtained from Bio-Rad Laboratories, Inc.

(Hercules, CA, USA). All other reagents were of the highest purity commercially available.

Mitochondria isolation and fatty acid manipulations

Mitochondria were prepared from an industrial wild-type diploid strain of *Saccharomyces cerevisiae* (Yeast Foam, a kind gift from Professor Michel Rigoulet, IBGC, University of Bordeaux-2, France). Cells were grown in Ylac medium (2% DL-lactate, 1% yeast extract, 0.12% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, pH 5.0 with NaOH) and harvested as described previously (Cortés-Rojo et al. 2009). Mitochondria were isolated by differential centrifugation from spheroplasts (Guérin et al. 1979; Avéret et al. 1998), and protein concentration was assayed by the Biuret method (Gornall et al. 1949). For enrichment of yeast membranes with linolenic acid (C18:3), 1 mM of this fatty acid solubilized with 5% (v/v) Igepal CA-630 was added to growth medium immediately prior to inoculation with cells from starter cultures. We previously reported that the fatty acid composition of yeast mitochondria grown in the absence of C18:3 (−C18:3) consisted of 4.63±0.01% palmitic acid (C16:0), 50.73±0.05% palmitoleic acid (C16:1), 2.73±0.02% stearic acid (C18:0), and 41.9±0.02% oleic acid (C18:1). Supplementation of yeast cultures with C18:3 produces in mitochondria an enrichment with this fatty acid of 79.15±4.63%, while percentages of native fatty acids are: 9.3±0.02% C16:0, 2.08±0.003% C16:1, 7.01±0.02% C18:0, and 2.43±0.01% C18:1 (Cortés-Rojo et al. 2009).

Treatments with H_2O_2 and antioxidants

Mitochondria (at proper protein concentrations for each assay as indicated below) were incubated in 50 mM KH_2PO_4 buffer (pH 7.6 with NaOH) for 30 min. at 4 °C with H_2O_2 at the concentrations indicated in the legend for each figure. Stock solutions of H_2O_2 were prepared immediately before each experiment. In experiments of protection against lipoperoxidation, mitochondria were incubated with 5 μM butylated hydroxytoluene (BHT) for 15 min at 4 °C before treatment with H_2O_2. To test the reversion of the effects of H_2O_2 on ETC activities, mitochondria were incubated with BME for 15 min at 4 °C after treatment with H_2O_2. For measurement of complex II activity, BME was removed by washing mitochondria twice with fresh KH_2PO_4 buffer to avoid the unspecific reduction of succinate-2,6-dichlorophenolindophenol (DCIP) by BME. Controls were treated in the same way, except that H_2O_2 addition was omitted.

Evaluation of lipid peroxidation

Levels of lipoperoxidation were evaluated with the thiobarbituric acid (TBA) assay (Buege and Aust 1978). Mitochondria (0.1 mg/ml) were treated as described above, and the experimental procedures were carried out as described elsewhere (Cortés-Rojo et al. 2007, 2009).

Measurement of ETC activities

Partial reactions of ETC were evaluated according to the procedures described previously (Uribe et al. 1985; Cortés-Rojo et al. 2007, 2009). Briefly, intact mitochondria were permeabilized by detergent treatment with Triton X-100 (Hallberg et al. 1993), re-suspended in KH_2PO_4 buffer and treated with H_2O_2 and antioxidants as described above. Enzymatic activities were recorded at room temperature in a Perkin Elmer Lambda 18 UV/vis spectrophotometer. Succinate-DCIP oxidoreductase activity (complex II) was assayed in 0.3 mg/ml protein, following the succinate-stimulated (10 mM) secondary reduction of 80 μM DCIP at 600 nm in the presence of 1 μg antimycin A and 0.75 mM KCN. The antimycin A-sensitive succinate-cytochrome *c* oxidoreductase activity [representative of the complex III activity due to the use of endogenous ubiquinone-6 pool as a secondary electron donor (Matsuno-Yagi and Hatefi 1996)] was evaluated in 0.1 mg/ml protein, following at 550 nm the succinate-stimulated (10 mM) reduction of 1.5 mg cytochrome *c* in the presence of 0.75 mM KCN. The reaction was stopped by the addition of 1 μg antimycin A. Cytochrome *c* oxidase activity (complex IV) was determined by measuring at 550 nm the cyanide-sensitive oxidation of 250 mg dithionite-reduced cytochrome *c* in the presence of 1 μg antimycin A. This reaction was stopped by the addition of 0.75 mM KCN. The activities of the complexes were calculated from the slopes of the absorbance. For complex II, the background reduction of DCIP in the absence of succinate was subtracted from the reduction of DCIP stimulated with succinate. For complex III, the reduction of cytochrome *c* in the presence of antimycin A plus succinate was subtracted from the cytochrome *c* reduction only with succinate. For complex IV activity, the oxidation of cytochrome *c* in the presence of cyanide was subtracted from the oxidation of cytochrome *c* in the absence of cyanide. Molar extinction coefficients of 21 mM^{-1} cm^{-1} and 19.1 mM^{-1} cm^{-1} were used for DCIP and cytochrome *c*, respectively.

Determination of superoxide formation

The production of $O_2^{\bullet-}$ was estimated by measuring the superoxide dismutase (SOD)-sensitive, succinate-stimulated reduction of cytochrome *c* (Boveris and Cadenas 1975; Muller et al. 2002). Mitochondria (0.1 mg/ml) were treated with 1.0 mM H_2O_2 as described above, and cytochrome *c* reduction was evaluated under the procedures described for

the measurement of succinate-cytochrome *c* oxidoreductase activity, except that mitochondria were incubated with 100 U/ml MnSOD for 5 min before each determination. The inhibition of cytochrome *c* reduction by MnSOD was attributed to $O_2^{\bullet-}$ production in the Q_O site of complex III, given that in our hands, 100 U/ml MnSOD inhibited the antimycin A-resistant reduction of cytochrome *c* by $O_2^{\bullet-}$ at levels comparable with the inhibition of cytochrome *c* reduction achieved with stigmatellin (Cortés-Rojo et al. 2009).

Estimation of iron release

Iron release was estimated with calcein-AM, a lipophilic ester that penetrates membranes and is cleaved by unspecific esterases to yield calcein, whose fluorescence is quenched upon binding to labile iron. To remove contaminating iron that could interfere with the experimental conditions, the buffers used for this and the other determinations were treated with Chelex 100 resin by following the manufacturer's instructions. Intact mitochondria (0.1 mg/ml) were incubated 30 min in isotonic buffer (0.6 M mannitol, 10 mM tris maleic acid (pH 6.8)) with 1.0 mM H_2O_2 alone or, where indicated, with 5 μM BHT or 100 U/ml catalase 15 min previous to H_2O_2 treatment. Incubation with H_2O_2 was stopped by adding 100 U/ml catalase to remove remaining H_2O_2 and avoid calcein bleaching. Mitochondria were then loaded with 2 μM calcein-AM for 30 min and washed twice to remove the remaining extramitochondrial calcein-AM. Final resuspensions were carried out with hypotonic 50 mM KH_2PO_4 buffer to disrupt mitochondrial integrity and allow the accessibility of the non-membrane-permeant iron chelator EDTA to calcein-iron complexes. Changes in fluorescence were followed with a Shimadzu RF5000U spectrofluorometer with constant stirring (excitation 494 nm; emission 517 nm). The dequenching of calcein fluorescence after the addition of 1 mM EDTA was attributed to the release of iron from calcein-iron complexes, as this probe binds to both Fe^{2+} and Fe^{3+} with high affinity constants and is insensitive to calcium and magnesium ions even at 1000-fold excess (Breuer et al. 1995); moreover, the intracellular concentration of other calcein-binding species is very low (Chen et al. 2006).

Data analysis

Results are expressed as the mean ± standard error of at least 4 independent experiments. Statistical significances ($P<0.05$, $P<0.01$) were determined with Student's *t*-test using Sigma Plot software v10.0.

Results

Effect of C18:3 on mitochondrial lipoperoxidation by H_2O_2

Lipoperoxidation levels were measured in mitochondria exposed to increasing millimolar concentrations of H_2O_2 to test its ability to damage mitochondrial membranes and verify if the incorporation of C18:3 can enhance the damage in membrane lipids (Fig. 1). –C18:3 mitochondria were insensitive to all of the concentrations of H_2O_2 tested (white bars, panel a). In contrast, gradual increments in lipoperoxidation up to 6.3 fold were reached in +C18:3

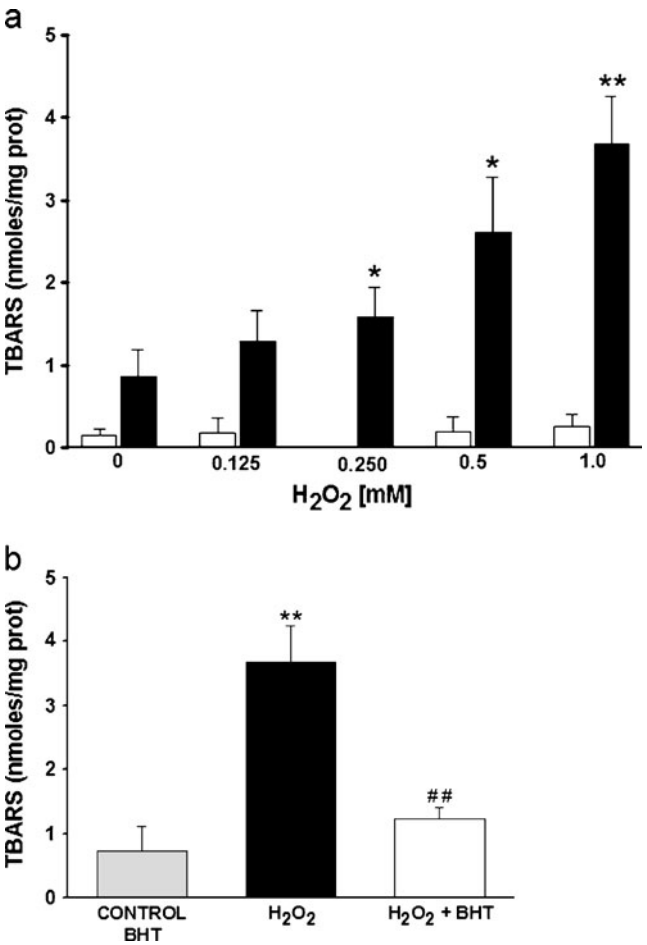

Fig. 1 Effect of H_2O_2 on lipoperoxidation levels in -C18:3 mitochondria (*white bars*) and +C18:3 mitochondria (*black bars*) (**a**) and protection by BHT (**b**). Mitochondria were treated as described in "Materials and methods" with indicated concentrations of H_2O_2 during 30 min (**a**). Mitochondria were pre-incubated during 15 min with BHT previous to H_2O_2 challenge (**b**). Data are presented as mean ± S.E. of at least four independent experiments. Panel a: significantly different when compared to –C18:3 mitochondria at the same H_2O_2 concentration (*$P<0.05$, **$P<0.01$, Student's test); panel b: significantly different when compared to control BHT (**$P<0.01$, Student's test); significantly different when compared to H_2O_2 1.0 mM ($^{\#\#}P<0.01$, Student's test)

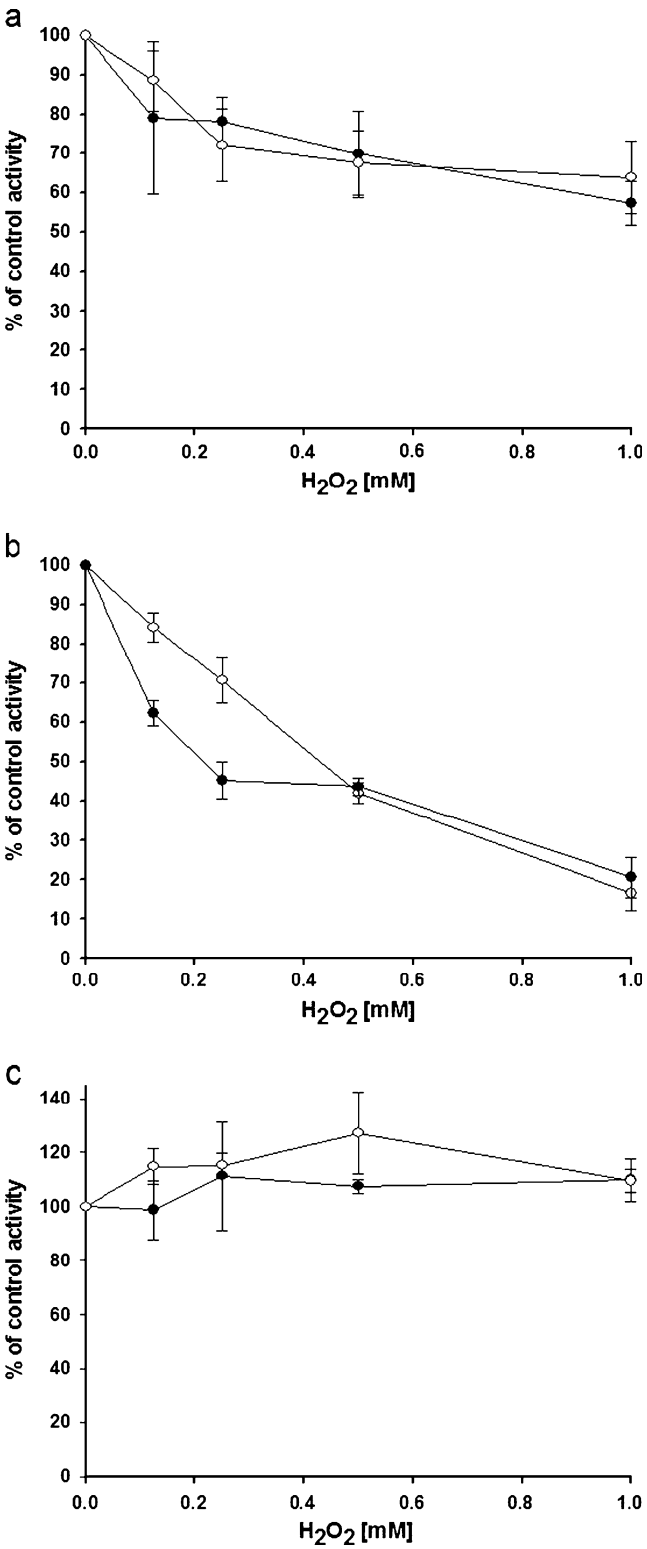

Fig. 2 Effect of H_2O_2 on partial reactions of ETC in −C18:3 (*white circles*) and +C18:3 (*black circles*) mitochondria. Mitochondria were incubated for 30 min at 4 °C with the indicated concentrations of H_2O_2. Succinate-DCIP oxidoreductase (complex II) (**a**), antimycin A-sensitive succinate-cytochrome *c* oxidoreductase (complex III) (**b**) or cyanide-sensitive cytochrome *c* oxidase (complex IV) activities (**c**) were determined as described in "Materials and methods." Data are expressed as the percentage of the remaining activities with respect to control activities (0 mM H_2O). The values of control activities (100%) were as previously reported (Cortés-Rojo et al. 2009). Data are presented as mean ± S.E. of at least four independent experiments

mitochondria (black bars, panel a). These results indicate that H_2O_2 can induce damage in mitochondrial membranes when constituted by unsaturated fatty acyl chains with more than two unsaturations. In another set of experiments, +C18:3 mitochondria were pre-incubated with the lipophilic antioxidant BHT before challenge with 1.0 mM H_2O_2 to corroborate that the effects observed in +C18:3 mitochondria are attributable to toxic effects in lipids. As observed in panel b (Fig. 1), BHT treatment protected from lipoperoxidation to an extent similar to the values observed in mitochondria incubated only with BHT. Therefore, this finding corroborates the ability of H_2O_2 to damage membrane phospholipids.

Influence of C18:3 incorporation on ETC inhibition by H_2O_2

Partial reactions of ETC were evaluated after H_2O_2 exposure to explore whether incorporation of C18:3 into membranes alter the sensitivity of respiratory complexes to this oxidant. In both –C18:3 and +C18:3 mitochondria, less than 35% of complex II activity was lost with concentrations up to 0.5 mM H_2O_2 (Fig. 2, panel A). Additionally, more than 50% of the activity was preserved even after exposure to 1.0 mM H_2O_2. No significant differences in the sensitivity of complex II to H_2O_2 were detected at any concentration of H_2O_2. In contrast with complex II, the sensitivity of complex III to H_2O_2 was higher, irrespective of C18:3 incorporation (Fig. 2, panel B). Moreover, +C18:3 mitochondria were 22% and 25.3% more prone to H_2O_2 inhibition at 0.125 and 0.25 mM H_2O_2, respectively. However, sensitivity to H_2O_2 was similar at concentrations above 0.5 mM, reaching a drop in enzymatic activity of about 60% and 80% with 0.5 and 1.0 mM H_2O_2, respectively. Complex IV showed full resistance to H_2O_2, as no concentration of this oxidant exerted an inhibitory effect in either –C18:3 or +C18:3 mitochondria (Fig. 2 panel C). These results indicate that the succinate-cytochrome *c* oxidoreductase segment is the most sensitive component of the yeast ETC to the deleterious effects of H_2O_2 and may indicate that in +C18:3 mitochondria, other factors different than that of –SH oxidation are involved in the augmented sensitivity of complex III to H_2O_2.

Protection from damaging effects of H_2O_2 on ETC by BHT and BME

The effects of BHT and BME were evaluated in mitochondria challenged with H_2O_2 to explore the involvement of –

SH reversible oxidation and lipoperoxidation in the inhibition of ETC activities. A null protective effect in complex II was obtained by pre-incubation with 5 μM BHT for 15 min prior to exposure to 1.0 mM H_2O_2 in both –C18:3 and +C18:3 mitochondria (Fig. 3, panel A). Conversely, the treatment for 15 min with 2.0 mM BME after H_2O_2 challenge restored in ~84–88% the activity of complex II, independently of C18:3 incorporation. These observations may indicate that complex II impairment by H_2O_2 proceeds mainly through reversible –SH oxidation in an independent way of lipoperoxidation.

The inhibition in complex III activity by treatment with 0.5 mM H_2O_2 was not prevented by BHT pre-treatment in either –C18:3 or +C18:3 mitochondria (Fig. 3, panel B). BME treatment restored this activity by 82% in –C18:3 mitochondria. Remarkably, BME exerted in +C18:3 mitochondria a lower recovering effect, as the same treatment restored this enzymatic activity by only 60%. These results lead us to suggest that complex III activity from +C18:3 mitochondria were impaired by another factor in addition to that of reversible –SH oxidation. To elucidate whether lipoperoxidation detected in +C18:3 mitochondria (Panel A, Fig. 1) is such a factor contributing to enhanced complex III impairment, mitochondria were sequentially incubated with BHT-H_2O_2-BME at the times and concentrations indicated in the legend to Fig. 3. In –C18:3 mitochondria, the combined use of BHT and BME did not enhance the restoring effect produced by BME alone. In +C18:3 mitochondria, the same treatment significantly enhanced the restoring effect of BME in 8%. These observations may mean that the lower restoring effect of BME in +C18:3 mitochondria are due in part to the partial contribution of lipoperoxidation to enhanced sensitivity of complex III to H_2O_2–damaging effects. Moreover, the null effect of BHT alone in +C18:3 mitochondria may indicate that –SH oxidation is the factor determining complex III inhibition at concentrations starting from 0.5 mM H_2O_2 in such a way that –SH oxidation may overlap the contribution of lipoperoxidation to complex III impairment.

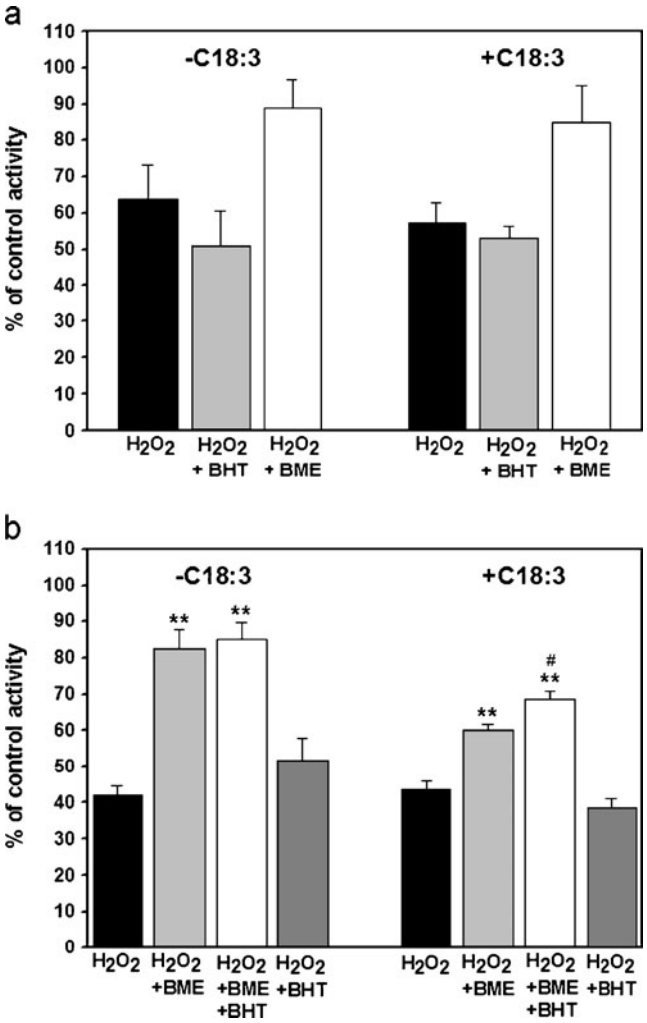

Fig. 3 Effects of BHT and BME over the damage induced by H_2O_2 on ETC partial reactions. To test protective effects of BHT alone, mitochondria were pre-incubated for 15 min with 5 μM BHT prior to incubation for 30 min with 1.0 mM (**a**) or 0.5 mM (**b**) H_2O_2. To test the restoring effect of BME alone, mitochondria were treated for 15 min with 2.0 mM (**a**) or 1.0 mM (**b**) BME after treatment for 30 min with 1.0 mM (**a**) or 0.5 mM (**b**) H_2O_2. Sequential treatment with BHT-H_2O_2-BME (**b**) was carried out by first pre-incubating mitochondria with 5 μM BHT. After 15 min, 0.5 mM H_2O_2 was added and incubated for 30 min. Then, a final incubation was done with 1.0 mM BME. All incubations were carried out under the conditions described in "Materials and methods" After incubation procedures, succinate-DCIP oxidoreductase (**a**) or antimycin A-sensitive succinate-cytochrome *c* oxidoreductase (**b**) activities were measured as described in "Materials and methods". No significant differences in enzymatic activities were observed with respect to controls when BME, BHT or BME plus BHT were added in the absence of H_2O_2. Data are presented as mean ± S.E. of at least four independent experiments. Significantly different when compared to H_2O_2 (**$P<0.01$); significantly different when compared to +C18:3 mitochondria treated with H_2O_2 + BME ($^{\#}P<0.05$, Student's test)

Effect of H_2O_2 and C18:3 incorporation on succinate-dependent $O_2^{\bullet-}$ production

The effect of MnSOD in succinate-stimulated cytochrome *c* reduction was evaluated to determinate if enhanced complex III damage in +C18:3 mitochondria leads to ROS production due to increased electron leakage. It can be observed that residual cytochrome *c* reduction after 30 min incubation with 1.0 mM H_2O_2 proceeds solely in the presence of succinate under any experimental conditions (Fig. 4), which suggests that no other source of superoxide production than the ETC was active in mitochondrial preparations. The addition of 100 U MnSOD 5 min previous to the start of the spectrophotometric register did not alter the rate of cytochrome *c* reduction in –C18:3 mitochondria (panel a), possibly meaning that complex III inhibition by 1.0 mM H_2O_2 does not promote $O_2^{\bullet-}$

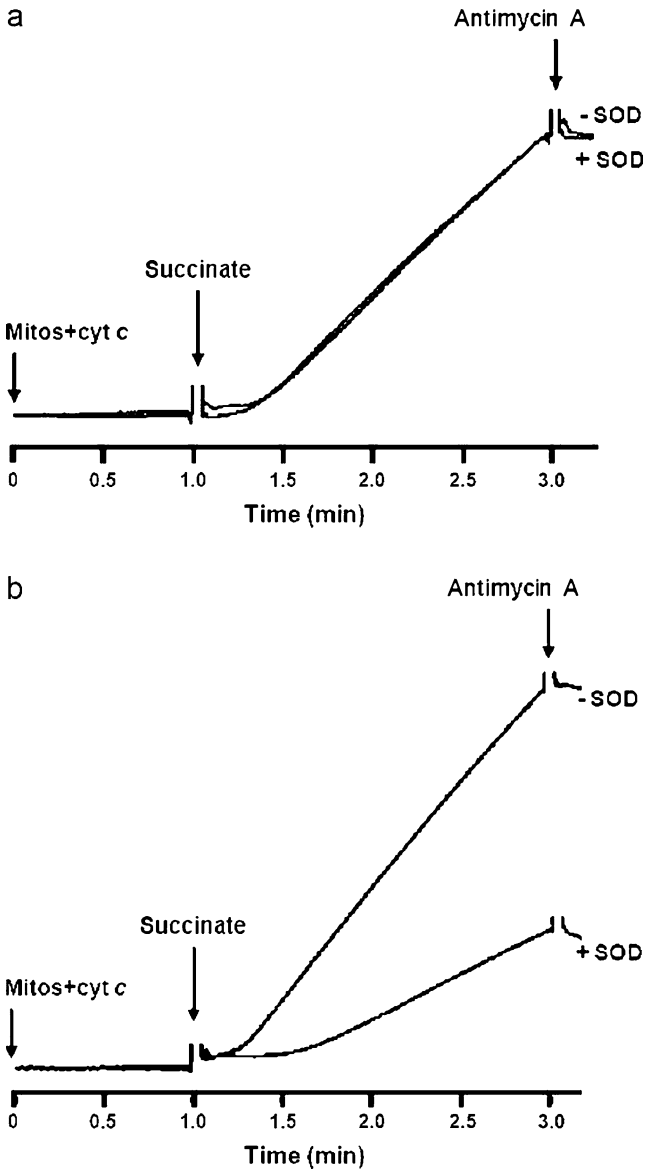

Fig. 4 Effect of MnSOD on time traces of cytochrome *c* reduction by succinate after H_2O_2 treatment. -C18:3 (**a**) or +C18:3 (**b**) mitochondria were incubated for 30 min with 1.0 mM H_2O_2 as described in "Materials and methods." Succinate-cytochrome *c* oxidoreductase activity was evaluated under the procedures described for the measurement of succinate-cytochrome *c* oxidoreductase activity, except that mitochondria were incubated with 100 U/ml MnSOD for 5 min before each determination. Succinate and antimycin A were added at the times indicated by *arrows*. Data are representative of $n \geq 4$

production. In +C18:3 mitochondria (panel b), the addition of MnSOD decreased significantly the reduction of cytochrome *c* in average by 62% ($n \geq 4$, $P < 0.05$), indicating that only 38% of this activity is attributable to complex III enzymatic activity, as supported by the observation that the addition of antimycin A fully inhibited cytochrome c reduction. Thus, these results may imply that increased sensitivity of complex III to H_2O_2 due to +C18:3 incorporation is associated with a rise in electron leakage at complex III to generate enzymatically measurable concentrations of $O_2^{\bullet -}$.

Alterations in mitochondrial labile iron by H_2O_2 and its relation with lipoperoxidation

Mitochondrial labile iron (defined as the fraction of iron accessible to a chelating agent) was estimated to investigate the role of lipoperoxidation in the ability of H_2O_2 to release iron from mitochondrial pools. This was achieved through the use of the fluorescent probe calcein. Figure 5 shows representative time plots of iron detection in mitochondria

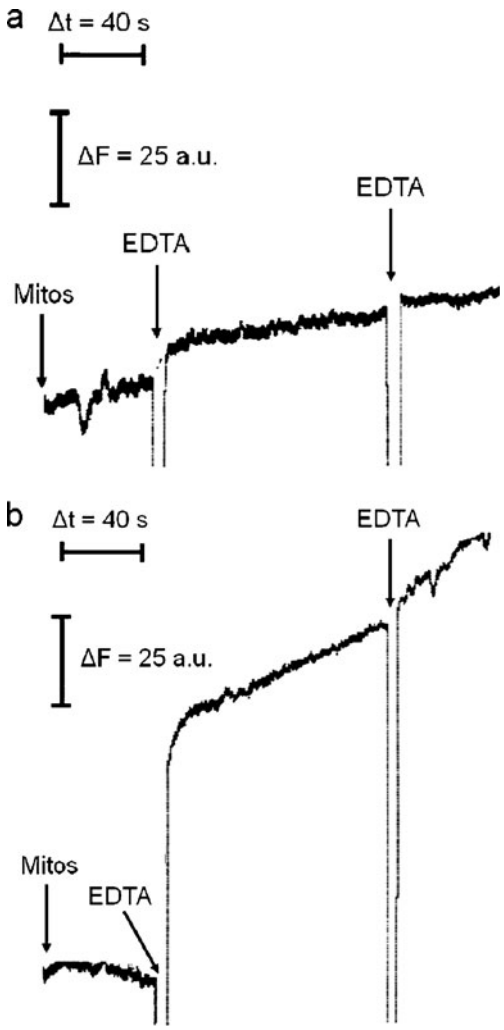

Fig. 5 Measurement, using calcein assay, of mitochondrial iron mobilization induced by H_2O_2. Intact –C18:3 (**a**) or +C18:3 (**b**) mitochondria were incubated for 30 min with 1.0 mM H_2O_2 as described in Fig. 4, except that 0.6 M mannitol buffer was used instead of phosphate buffer and 100 U/ml catalase was added at the end of the incubations to ensure H_2O_2 removal and avoid calcein bleaching. Loading of mitochondria with calcein-AM and spectrofluorometric determinations in hypotonic buffer to allow EDTA accessibility were done as described in "Materials and methods." Mitochondria and EDTA were added at the times indicated by *arrows*. Data are representative of $n \geq 3$

treated with 1.0 mM H_2O_2. In –C18:3 mitochondria (panel A), the addition of 1 mM EDTA induced a slight increase in calcein fluorescence, and no further changes were observed after the addition of another similar amount of the chelant. In contrast, in +C18:3 mitochondria (panel B); EDTA produced a notable increment in both fluorescent signal and the slope of the trace. Further addition of EDTA caused only a moderate augmentation in the slope of the trace. These effects could be interpreted as a biphasic behavior of the dissociation of calcein-iron complexes, where the instantaneous rise in calcein fluorescence may represent a rapid phase of dissociation of calcein-iron complexes, while the posterior change in the slope may correspond to a slow phase of such dissociation.

The averages of the effects produced by EDTA in calcein fluorescence are shown in Fig. 6. In –C18:3 mitochondria (panel A), none of the treatments significantly altered calcein fluorescence when the results were compared with those in controls, although catalase treatment before incubation with H_2O_2 caused, in some cases, an increment in the fluorescent signal which was not statistically significant. As shown in Fig. 5, H_2O_2 treatment produced a huge rise in calcein fluorescence in +C18:3 mitochondria (panel B). Remarkably, no signal was detected in control mitochondria. BHT pre-treatment significantly inhibited the increment caused by H_2O_2, although a residual signal was detected. In the same way, catalase pre-treatment counteracted significantly the effect of H_2O_2 at a level comparable with that observed in –C18:3 mitochondria. In this respect, it is important to point out that the addition of catalase or BHT did not disturb calcein fluorescence in the absence of mitochondria (data not shown). In addition, BHT and catalase were removed through washing with isotonic buffer before calcein loading. Hence, these facts supports the interpretation that catalase decreases calcein fluorescence due to removal of H_2O_2 from the incubation medium rather than catalase affects calcein fluorescence by an unspecific interaction. Together these results indicate that lipoperoxidation elicited by H_2O_2 in +C18:3 mitochondria increases iron mobilization.

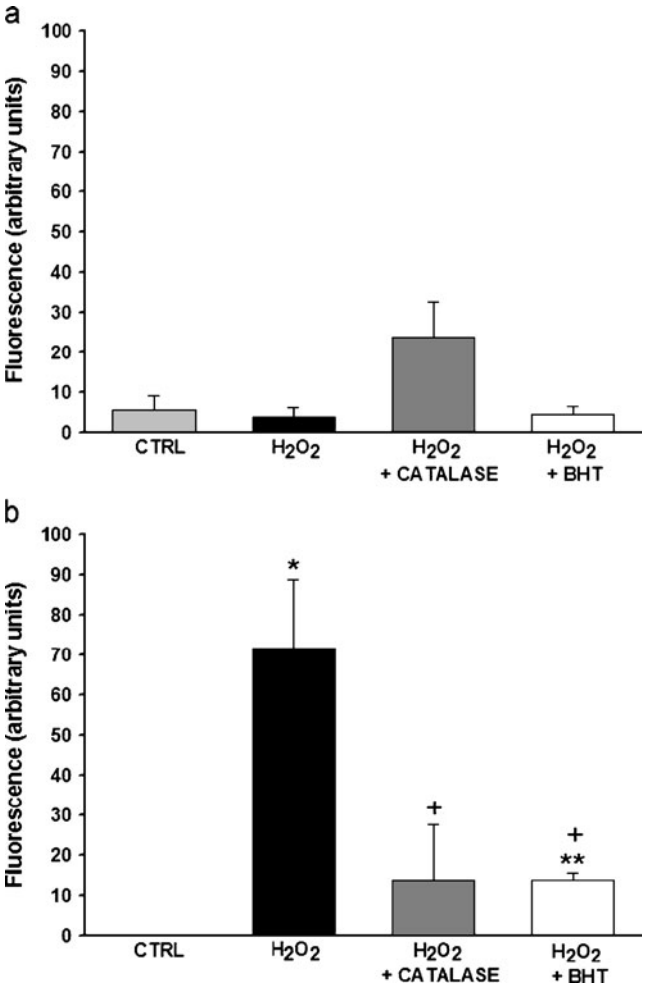

Fig. 6 Quantification of the changes in calcein fluorescence upon EDTA addition in -C18:3 (**a**) and +C18:3 (**b**) mitochondria. Incubations with H_2O_2 and fluorescence determinations were done as described in Fig. 5, except when indicated, 100 U/ml catalase or 5 μM BHT were added 15 min previous to H_2O_2 treatment. Significantly different when compared to control (*$P<0.05$, **$P<0.01$, Student's test); significantly different when compared to H_2O_2 ($^{+}P<0.05$)

Discussion

Reversible oxidation of reactive –SH from cysteine residues modulates the biological activity of several proteins including transcription factors (Zheng et al. 1998), chaperones (Jakob et al. 2000), and enzymes (Kim et al. 2000). In the last example, it has been demonstrated that -SH oxidation and further reaction with low-molecular weight thiols like glutathione, serve as a mechanism to protect enzyme activity during oxidative stress (Hondorp and Matthews 2004). In the case of mitochondrial ETC, there are several proteomic and functional studies that support the role of –SH-reversible modifications in the regulation of ETC activity (Lê-Quôc et al. 1981; Cardoso et al. 1999; Lin et al. 2002; Cortés-Rojo et al. 2007; Hurd et al. 2007). Furthermore, it has been observed that –SH modifications in ETC during oxidative stress may serve as a mechanism to decrease deleterious ROS generation, as the inhibition of complex I activity by glutathionylation of a cysteine residue also decreases $O_2^{\bullet-}$ production (Hurd et al. 2008). However, the reactions of –SH with ROS and thiol reductants depend on some factors like conversion of -SH into thiolate ion ($-S^-$), –SH oxidation state, pH of the surrounding environment, the binding of cysteines with metals and the pKa values of $-S^-$ (Forman et al. 2004; Jacob et al. 2004; Lin et

al. 2002; Kiley and Storz 2004). Therefore, the regulatory effect of –SH reversible oxidation on ETC function may strongly depend on these factors due to the H^+ gradient established across the inner membrane by the ETC. In this paper, we report that lipoperoxidation can also alter the regulatory effect of reversible –SH oxidation on ETC function during damage by H_2O_2, which was associated with increased ROS production and iron release from mitochondrial pools.

S. cerevisiae represent a proper model to study the influence of lipoperoxidation on cell physiology because native yeast membranes contain only saturated and mono-unsaturated acyl chains, which are resistant to lipoperoxidation (Holman 1954). However, the sensitivity of yeast to lipoperoxidation can be augmented through the supplementation of growth medium with lipoperoxidation-prone, polyunsaturated fatty acids (PUFA). It has been demonstrated that PUFA supplementation represses the synthesis of mono-unsaturated fatty acids in yeast through down-regulation of the Δ^9-fatty acid desaturase gene *OLE1*, leading to substitution of the native mono-unsaturated fatty acids with the supplemented PUFA (Martin et al. 2007). We have shown that under our culture conditions, enrichment up to 79% with C18:3 is achieved in the mitochondrial fraction when this PUFA is added in lactate-based medium (Cortés-Rojo et al. 2009). Thus, the comparison among the effects produced by H_2O_2 in –C18:3 and +C18:3 mitochondria allow discrimination of the role of lipoperoxidation on ETC dysfunction from the effects produced by H_2O_2 and related ROS on other components different from membrane lipids.

No increase in lipoperoxidation levels was observed when –C18:3 mitochondria were treated with H_2O_2. This is in agreement with our previous report about the insensitivity of yeast to H_2O_2-induced lipoperoxidation (Cortés-Rojo et al. 2007). Conversely, a significant augmentation in this parameter was observed in +C18:3 mitochondria, reaching a maximum of 3.7 nmoles TBARS per mg protein (Fig. 1, panel A). These values are similar to those observed in rat liver and brain mitochondria in the presence of 0.5 mM H_2O_2 (Cortés-Rojo et al. 2007). Nevertheless, these levels are far from those obtained during the treatment of +C18:3 mitochondria with Fe^{2+} (15–60 nmoles TBARS per mg protein) (Cortés-Rojo et al. 2009). This difference can be related to H_2O_2's weakness as an oxidant and because it does not react directly with PUFA to initiate lipoperoxidation, but promotes this process through formation of $OH^{\bullet}$ radicals catalyzed by transition metals. In contrast, Fe^{2+} initiates and propagates lipoperoxidation through the formation of a highly oxidant species with $OH^{\bullet}$ characteristics (North et al. 1992). Therefore, it could be inferred that the amount of iron released from mitochondrial pools by the treatment of +C18:3 mitochondria with H_2O_2 may be far lower than the amount used during in vitro incubations, which would explain the differences observed between the extent of lipoperoxidation induced by H_2O_2 and Fe^{2+}.

In agreement with the results obtained in +C18:3 mitochondria, it has been demonstrated that the exogenous addition of H_2O_2 to renal mitochondria can promote iron release (Ueda et al. 1993). Taking into account that H_2O_2 did not promote an increase in iron levels in –C18:3 mitochondria and that rat mitochondria are susceptible to lipoperoxidation due to the presence of PUFA in its membranes (Malis et al. 1999), it is feasible to propose that lipoperoxidation is a primary event needed to initiate iron release by H_2O_2, which is line with the fact that BHT pre-treatment blocked the rise in labile iron observed with 1.0 mM H_2O_2 (Fig. 6). Our data also indicate that iron was not available in control+C18:3 mitochondria (Fig. 6, panel B) to start lipoperoxidation through the reaction with H_2O_2 to yield $OH^{\bullet}$. However, released iron could not be necessary to induce lipoperoxidation by H_2O_2, as it has been reported that the reaction of H_2O_2 with complex IV leads to the formation of sulfur-centered radicals (Chen et al. 1999) capable of initiating lipoperoxidation (Schoneich et al. 1992).

With regard to the effects of H_2O_2 on ETC activities, only complex III showed a higher sensitivity to H_2O_2 when C18:3 was incorporated into mitochondrial membranes, while complex II was inhibited in the same magnitude in both –C18:3 and +C18:3 mitochondria. This result confirms the notion that lipoperoxidation is not involved in the inhibition of complex II activity by ROS attack, as it has been demonstrated that the presence of C18:3 did not increase the sensitivity of the yeast complex II to Fe^{2+} exposure (Cortés-Rojo et al. 2009) and the pretreatment of yeast mitochondria or rat synaptosomes with lipophilic antioxidants did not prevent complex II inhibition by iron (Cortés-Rojo et al. 2009; Cardoso et al. 1999). Likewise, pre-treatment with BHT did not prevent damage by H_2O_2, irrespective of the presence of C18:3, further confirming the null role of lipoperoxidation in the deleterious effects of ROS in complex II. Thus, the similar recovery in complex II activity (~85%) obtained with BME in both types of mitochondria, indicates that –SH oxidation is the main factor affecting complex II activity, which is in agreement with previous reports about the existence of two classes of –SH groups with a different sensitivity to alkylating agents that regulates the catalytic activity of this enzyme (Lê-Quôc et al. 1981). At this point, it is important that the insensitivity of complex II to lipoperoxidation and the similar recovery of enzymatic activity with BME among –C18:3 and +C18:3 mitochondria, lead us to suggest that the regulation of ETC activity by –SH oxidation is maintained unaltered in the absence of lipoperoxidation, thereby supporting our view that reversible modulation of ETC

activity by –SH oxidation is disrupted only in ETC complexes that are sensitive to lipoperoxidation.

Complex III activity from +C18:3 mitochondria was 20% more sensitive to H_2O_2 at concentrations below 0.5 mM H_2O_2, while at concentrations between 0.5 and 1.0 mM H_2O_2 no apparent differences were observed with respect to the presence of –C18:3 (panel A, Fig. 2). These results could be erroneously interpreted, as lipoperoxidation does not play a role in complex III inhibition at concentrations above 0.5 mM. However, when compared with –C18:3 mitochondria, a loss (22%) in the ability of BME to recover complex III activity in +C18:3 mitochondria was observed, which may mean that other events different from –SH oxidation participate in complex III inhibition at those concentrations. Pre-treatment with BHT did not exert a protective effect in either –C18:3 or +C18:3 mitochondria, but the combination of BHT plus BME significantly improved in 8% the effect of BME only in +C18:3 mitochondria, a finding that may indicate lipoperoxidation partially alters the modulation of complex III activity by reversible –SH oxidation. Again, the latter finding corroborates the idea that lipoperoxidation affects the modulation of the ETC activity by changes in the oxidation state of cysteine.

Evidence for increased damage in complex III by H_2O_2-induced lipoperoxidation is the detection of a higher production of ROS in +C18:3 mitochondria by measuring SOD-sensitive cytochrome *c* reduction (Fig. 4). In this case, it was found that 62% of cytochrome *c* reduction does not proceed through normal complex III turnover, but rather by the production of $O_2^{\bullet-}$ radical due to electron leakage. Therefore, this finding confirms that the similar sensitivity of complex III to 0.5 mM H_2O_2 observed in both –C18:3 mitochondria and +C18:3 mitochondria was only apparent and that only 38% of electrons may be enzymatically transferred to complex IV through cytochrome *c* reduction, which in turn would affect other processes depending on overall ETC activity (e.g. membrane potential and ATP production). This fact exemplifies the importance of using SOD to measure complex III activity under oxidative stress conditions, as cytochrome *c* reduction by $O_2^{\bullet-}$ can unmask the decrease in the catalytic transfer of electrons to cytochrome *c*. Also, these results agree with our previous findings in which we reported that only 5% of cytochrome *c* reduction is attributable to complex III catalytic activity under strong lipoperoxidative conditions induced by Fe^{2+} (Cortés-Rojo et al. 2009). This finding may indicate that the amount of $O_2^{\bullet-}$ generated by the lipoperoxidative damage in complex III depends on the extent of lipoperoxidation in a non-linear relationship.

On the other hand, it has been demonstrated that the $O_2^{\bullet-}$ radical can promote iron release in yeast through the disassembly of the Fe-S clusters from mitochondrial enzymes (Longo et al. 1999). Although identification of the sources of the iron mobilized in +C18:3 mitochondria is beyond the scope of this work, it can be hypothesized that iron mobilization in +C18:3 mitochondria was stimulated by the damage in mitochondrial Fe-S clusters due to $O_2^{\bullet-}$ generated by the lipoperoxidative damage in complex III activity, a possibility which is supported by the finding of protective effects of BHT against both lipoperoxidation (Fig. 1) and iron release (Fig. 6), as well as the BHT-enhanced recovery of complex III activity in the presence of BME (Fig. 3). This idea is also reinforced by the null effects of H_2O_2 on $O_2^{\bullet-}$ production and iron mobilization in –C18:3 mitochondria. Therefore, $O_2^{\bullet-}$ production in complex III could be the link between lipoperoxidation and iron release in +C18:3 mitochondria due to H_2O_2 attack.

In contrast with the other complexes, complex IV was fully insensitive to H_2O_2 even in +C18:3 mitochondria. This observation can be explained by H_2O_2's ability to be a substrate for the enzyme rather than a deleterious agent, as it has been demonstrated that complex IV can function as a peroxidase using reduced cytochrome *c* as an electron donor (Vygodina and Konstantinov 2007). With respect to the participation of -SH oxidation in the modulation of complex IV activity, another study had shown that the modification of –SH groups with N-ethylmaleimide did not affect its catalytic activity (Chen et al. 1999), which is in agreement with our results. We have reported that complex IV activity from yeast mitochondria is sensitive to lipoperoxidation; for example, 30% of the enzymatic activity was lost at 18 nmoles TBARS per mg protein in the presence of 12.5 μM Fe^{2+} (Cortés-Rojo et al. 2009), but since the maximum level of lipoperoxidation reached with H_2O_2 was four times lower than the above mentioned value, it is possible the level of lipoperoxidation achieved with H_2O_2 is not enough to exert an effect in this complex.

Despite this paper and others which describe the inhibition of ETC complexes through –SH oxidation, other authors have reported that H_2O_2 had a null effect on ETC enzymes, although it does affect respiration through the inhibition of enzymes from the Krebs cycle, such as α-ketoglutarate dehydrogenase or aconitase (Nulton-Persson and Szweda 2001; Zini et al. 2007), and in some cases it is due to enzyme gluthationylation instead of a direct interaction of H_2O_2 with cysteines (Nulton-Persson and Szweda 2001). The discrepancies between these reports and our results could be partially attributed to the short times of incubation (3 to 7.5 min) and lower H_2O_2 concentrations (50 μM) used in those studies. In this regard, it must be pointed out that the concentrations of H_2O_2 used in the present study are in the range of the concentrations found in biological samples, e.g., lens cataracts (above 600 μM) (Spector et al. 1993), liver mitochondria from rats intoxicated with an organophosphate insecticide (up to 150 μmol)

(Łukaszewicz-Hussain and Moniuszko-Jakoniuk 2004), and the concentrations used to trigger apoptosis (600 μM) (Kim et al. 2003) or to study the in situ effects of H_2O_2 in mitochondrial energy transduction (up to 10 mM) (Tatsumi and Kako 1993).

Even when a total recovery in ETC activities was not observed through the use of antioxidants, the partial protective effects obtained could have a beneficial effect on overall cell function and survival during oxidative stress conditions. This is exemplified by a study in which the antioxidant silybin protected from hepatic fibrosis caused by iron overload, even when ETC activities, which were inhibited below 45%, were partially protected by silybin up to 71% (e.g. succinate dehydrogenase) (Masini et al. 2000). Thus, it would be interesting to investigate if increased protection exerted by accumulative effects of thiol reduction and lipoperoxidation inhibition (as observed in this study in complex III from +C18:3 mitochondria) may be beneficial in model diseases in which ETC function is compromised by both lipoperoxidation and –SH oxidation like Parkinson's disease (Jha et al. 2000; Beal 2003) or hepatitis C infection (Korenaga et al. 2005; Okuda et al. 2002). In this regard, it would be important to know the threshold activities of the ETC complexes that allow cell survival after oxidative insults in pathologies like ischemia-reperfusion, stroke, etc., to elucidate if the magnitude of the potentiation exerted by BHT on the recovering effects of BME could be enough to improve tissue function during such maladies.

The inefficacy of some antioxidants during certain pathologies that involve ETC dysfunction by ROS could be related, in part, to the impossibility of these molecules to provide protection from all the main events that disrupt ETC function. For example, the inefficacy of the mitochondria-targeted antioxidant MitoQ observed during the progression of Parkinson's disease (Snow et al. 2010) may be partially related to the fact that ETC impairment during this pathology proceeds mainly through thiol oxidation (Jha et al. 2000), and MitoQ does not scavenge H_2O_2 nor prevent –SH oxidation by peroxides (James et al. 2005). These antecedents and the results from this work support the idea from other authors that the antioxidants that operate through more than one mechanism of action could be more effective against diseases involving more than one ROS or distinct ROS targets, as exemplified by honokiol, a molecule capable of scavenging peroxyl and superoxide radicals (Dikalov et al. 2008). Therefore, an antioxidant protecting from lipoperoxidation and –SH oxidation may be more effective in protecting ETC function than a molecule acting only in one of these processes.

Based on our results and the antecedents described above about iron release by $O_2^{\bullet-}$ (Longo et al. 1999) and the effects of H_2O_2 in complex IV (Schoneich et al. 1992; Chen et al. 1999), we propose the following hypothetical model (Fig. 7). H_2O_2 inhibits the activities of complexes II and III by the oxidation of regulatory –SH groups without further increases in $O_2^{\bullet-}$ production (1). H_2O_2 does not

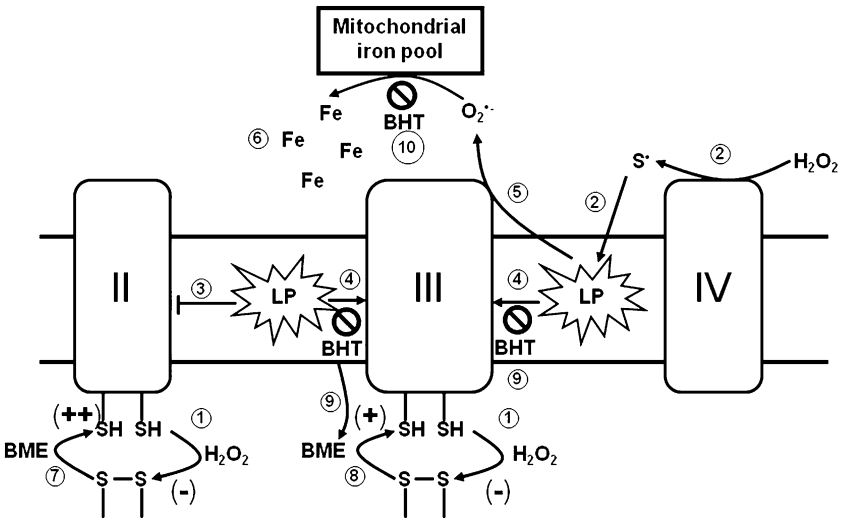

Fig. 7 Hypothetical model linking lipoperoxidation with increased ETC damage and iron release by H_2O_2. Complexes II and III are reversibly inhibited by H_2O_2 due to –SH oxidation (1). H_2O_2 does not inhibit complex IV activity but may stimulate the formation of sulfur-centered free radicals (S•) that induce lipoperoxidation (LP) as reported previously (Schoneich et al. 1992; Chen et al. 1999). Lipoperoxidation does not increase susceptibility of complex II to H_2O_2 (3). Conversely, lipoperoxidation increases the susceptibility of complex III to the inhibitory effects of H_2O_2 (4), causing increased $O_2^{\bullet-}$ production (5), which, in turn, stimulates iron release from mitochondria (6). –SH reduction by BME restores complex II activity (++) independently of lipoperoxidation induction (7); however, the recovery of complex III activity by H_2O_2 is partially impaired by lipoperoxidation (+) (8), as evidenced by the improvement in activity recovery by the combined use of BHT plus BME (9). The inhibition of lipoperoxidation also blocks iron mobilization (10) probably due to diminished $O_2^{\bullet-}$ production, as evidenced by null $O_2^{\bullet-}$ detection in –C18:3 after H_2O_2 insult (Fig. 4a)

decrease complex IV activity due to its peroxidase activity, and/or the oxidation of –SH groups are not involved in its catalytic activity. However, H_2O_2 reaction with complex IV leads to the formation of sulfur- centered radicals that stimulate lipoperoxidation (2), which does not affect complex II activity (3) but enhances the damage on complex III (4). Lipoperoxidative damage in complex III stimulates an electron leak and $O_2^{\bullet-}$ production (5), which in turn, lead to iron release from the mitochondrial pool to enhance oxidative conditions (6). BME can reduce oxidized thiols from complex II to restore its catalytic activity in a lipoperoxidation-independent way (7). In contrast, recovery of complex III activity by thiol reduction is affected when lipoperoxidation is stimulated (8), while thiol reduction simultaneous to lipoperoxidation inhibition improves the recovery in complex III activity (9). At the same time, the absence of lipoperoxidation avoids $O_2^{\bullet-}$ and iron mobilization (10).

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