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Mitochondria are the main source and one of the targets of Pb (lead)-induced oxidative stress in the yeast *Saccharomyces cerevisiae*

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Abstract The yeast *Saccharomyces cerevisiae* is a useful model organism for studying lead (Pb) toxicity. Yeast cells of a laboratory *S. cerevisiae* strain (WT strain) were incubated with Pb concentrations up to 1,000 μmol/l for 3 h. Cells exposed to Pb lost proliferation capacity without damage to the cell membrane, and they accumulated intracellular superoxide anion ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2). The involvement of the mitochondrial electron transport chain (ETC) in the generation of reactive oxygen species (ROS) induced by Pb was evaluated. For this purpose, an isogenic derivative ρ^0 strain, lacking mitochondrial DNA, was used. The ρ^0 strain, without respiratory competence, displayed a lower intracellular ROS accumulation and a higher resistance to Pb compared to the WT strain. The kinetic study of ROS generation in yeast cells exposed to Pb showed that the production of $O_2^{\cdot -}$ precedes the accumulation of H_2O_2, which is compatible with the leakage of electrons from the mitochondrial ETC. Yeast cells exposed to Pb displayed mutations at the mitochondrial DNA level. This is most likely a consequence of oxidative stress. In conclusion, mitochondria are an important source of Pb-induced ROS and, simultaneously, one of the targets of its toxicity.

Keywords Lead (Pb) toxicity · Mitochondria · Reactive oxygen species (ROS) · Respiratory-deficient cells · Viability · Yeast

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Introduction

Lead (Pb) is considered a priority pollutant by the US Environmental Protection Agency (US EPA 2006) and is classified as a probable human carcinogen by the International Agency for Research on Cancer (ATSDR 2007). Lead can enter the environment as a result of various activities such as mining and smelting. It can also enter the environment through the wastewaters from industries that produce lead-acid batteries or lead wire and pipes (ATSDR 2007). Exposure to Pb can occur through different sources such tap water (as a result of Pb leaching from plumbing systems and fixtures containing Pb or with Pb solder), leaded gasoline or contact with household dust from Pb-based paints (Rossi 2008).

In the yeast *Saccharomyces cerevisiae*, Pb impairs the assimilation of nutrients (Chen and Wang 2007) and replication competence (Soares et al. 2002, 2003). Pb affected yeast metabolism because cells exposed to this metal displayed a decreased ability to process the dye FUN-1 [2-chloro-4-(2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene)-1-phenylquinolinium iodide] (Van der Heggen et al. 2010). The exposure of yeast cells to Pb induced a decrease in the intracellular level of reduced glutathione (Perez et al. 2013); nuclear morphological alterations, such as chromatin arranged in half rings (Bussche and Soares 2011) and nuclear DNA damage including mutations and telomere length alterations (Yuan and Tang 1999; Cui and Tang 2000; Yu et al. 2009). Pb-induced cell death is an active process, requiring the participation of cellular metabolism, as evidenced by the fact that the addition of cycloheximide, an inhibitor of protein synthesis, attenuated the Pb-induced loss of cell proliferation capacity. It was shown that exposure to Pb resulted in severe oxidative stress, which can be the trigger of programmed cell death by apoptosis (Bussche and Soares 2011).

The main role of the mitochondria is the production of ATP. However, mitochondria also participate in other functions

such as the biosynthesis of compounds, metabolite conversion and signal transduction (Pan 2011). Contrary to other organisms, yeasts are able to survive even after losing their mitochondrial DNA (mtDNA). Therefore, the yeast *S. cerevisiae* constitutes an appropriate model system to study the involvement of mitochondrial function in Pb-induced oxidative stress. In the present work, the contribution of the mitochondrial electron respiratory chain in the production of reactive oxygen species (ROS) in cells exposed to Pb will be investigated by using a wild-type strain of *S. cerevisiae* and an isogenic derivative ρ^0 strain lacking mtDNA. Additionally, to obtain further insight about Pb-induced ROS production, intracellular levels of superoxide anions ($O_2^{\cdot-}$) and oxygen peroxide (H_2O_2), the two main forms of ROS, will be measured. The impact of intracellular Pb-induced ROS accumulation on mitochondrial damage will also be evaluated.

Materials and methods

Yeast strains and growth conditions

In this work, the strain of *S. cerevisiae* BY4741 was used. The strain was purchased from the EUROSCARF collection (Frankfurt, Germany). In addition, the isogenic derivative ρ^0 strain (BY4741-ρ^0), which lacks mtDNA, was used. The strains were routinely maintained at 4 °C on YPD agar slants [10 g/l yeast extract (Difco-BD, Sparks, MD, USA), 20 g/l peptone (Difco-BD), 20 g/l glucose (Merck, Darmstadt, Germany) and 20 g/l agar (Merck)].

The mitochondrial respiratory-deficient (RD) (ρ^0) mutant was obtained as described in the literature with minor modifications (Rickwood et al. 1988). Briefly, BY4741 wild type cells were two-step grown in YPD containing 40 μg/ml ethidium bromide (Merck) at 25 °C in the dark at 150 rpm for 24 h. The verification of respiratory deficiency was carried out by the complete lack of growth on YPGly, media that contains a non-fermentable carbon source (2 % (v/v) glycerol, Merck). The lack of mtDNA was subsequently confirmed by staining with 4,6-diamidino-2-phenylindole (DAPI) (see below).

Pre-cultures were prepared in 10 ml of YPD broth in 100-ml Erlenmeyer flasks. Cells were incubated at 25 ºC on an orbital shaker at 150 rpm for 8–10 h. Cultures in the exponential growth phase were obtained by inoculating 100 ml of YPD broth in 250-ml Erlenmeyer flasks with pre-cultures and then grown overnight (OD_{600}~1.0) under the same conditions as the pre-culture.

Treatment of yeast cells with Pb

Cells in the exponential growth phase were harvested by centrifugation (2,000×*g*, 5 min), washed twice with deionised water and re-suspended in 10 mmol/l 2-(*N*-morpholino)ethanesulfonic acid (MES) pH buffer (Sigma-Aldrich, St. Louis, MO, USA) at pH 6.0, containing 2 % (w/v) glucose, to approximately 1×10^7 cells/ml. MES is a suitable pH buffer for heavy metal toxicity studies because it does not complex lead (Soares et al. 1999) and yeast cells maintain viability when incubated in this buffer for 48 h (Soares et al. 2000).

Cell suspensions (40 ml) that contained 1×10^7 cells/ml in 10 mmol/l MES buffer (pH 6.0) with 2 % (w/v) glucose and the appropriate volume of lead solution ($Pb(NO_3)_2$) from a stock standard solution of 2,000 mg/l (Merck) were shaken in 100-ml Erlenmeyer flasks at 150 rpm at 25 °C.

Cell viability assays

Two methodologies were used to assess yeast cell viability. The clonogenic assay consisted of removing samples (two replicates) of Pb-treated and non-treated cells at different intervals of time. Samples were serially diluted with sterile deionised water and plated on YPD agar (two replicates of the convenient dilutions). The colonies were counted after 3–5 days of incubation at 25 °C. The % viability was calculated using the number of colony-forming units (cfu)/ml at zero time as reference (100 %).

Viability was also determined by image-based cytometry using propidium iodide (PI). At the defined times reported in the figures, samples of Pb-treated and non-treated cells were placed in a final concentration of 15 μmol/l PI (Sigma, Steinheim, Germany) for 5 min at 25 °C. After incubation, samples were diluted with MES buffer (pH 6.0, with 2 % [w/v] glucose) at ~2×10^6 cells/ml. Image-based cytometric analysis was performed in a Tali (Invitrogen-Life Technologies, Carlsbad, CA, USA) image-based cytometer. In each sample, ~10,000 cells were analysed (excitation filter: 543/22 nm; emission filter: long pass 585 nm). As a positive control (PI positive cells), the yeast was heated to 65 °C for 60 min. Then, the cells were stained and analysed as described above.

Quantification of respiratory-deficient mutants

The formation of RD mutants was monitored through their inability to grow on non-fermentable media (YPGly) (Gomes et al. 2008). Convenient dilutions of Pb-treated and non-treated samples were plated on YPD and YPGly as described above. The % of RD cells (RD%) was calculated using the following equation:

$$\mathrm{RD}\ (\%) = [(\text{number of cfu/ml on YPD} - \text{number of cfu/ml on YPGly})/(\text{number of cfu/ml on YPD})] \times 100$$

Assessment of intracellular ROS accumulation

Intracellular ROS production, namely, superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), were monitored with dihydroethidium (DHE; Sigma) and 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA; Sigma), respectively. Cells were suspended at 1×10^7 cells/ml in MES buffer (10 mmol/l, pH 6.0) with 2 % (w/v) glucose and incubated at 25 °C for 10 min with DHE (in a final concentration of 8 μmol/l) or 20 min with H_2DCFDA (in a final concentration of 20 μmol/l) before Pb treatment. Yeast cells were subsequently exposed to Pb and placed in quadruplicate in a 96-well flat microplate (Orange Scientific, Braine-l'Alleud, Belgium), 200 μl per well, and the fluorescence intensity was measured (relative fluorescence units [RFU]) at defined intervals of time reported in the figures in a microplate reader at a fluorescence excitation wavelength of 485/14 nm and an emission of 620/8 nm (DHE) or at a fluorescence excitation of 485/14 nm and an emission of 535/25 nm (H_2DCFDA).

Fluorescence microscopy

For epifluorescence microscopy analysis, cells were treated in the same conditions described above for DHE and H_2DCFDA. In the double staining protocol (H_2DCFDA/PI), Pb-treated and non-treated (control) cells were incubated with 20 μmol/l H_2DCFDA for 30 min and with 15 μmol/l PI for 5 min at 25 °C in the dark. DNA visualisation was carried out by incubating yeast cells with DAPI (Sigma) at a final concentration of 3 μmol/l for 15 min at room temperature in the dark. Subsequently, cells were washed and suspended in 50 mmol/l PBS buffer (pH 7.4).

Cells were observed using an epifluorescence microscope (Leica Microsystems, Wetzlar GmbH, Germany) equipped with an HBO 100 mercury lamp and the following filter sets from Leica: N2.1 (excitation filter [band pass filter, BP] BP 515–560, dichromatic mirror 580 and suppression filter [long pass filter, LP] LP 590) for DHE; I3 (excitation filter BP 450–490, dichromatic mirror 510 and suppression filter LP 515) for H_2DCFDA/PI; and A (excitation filter BP 340–380, dichromatic mirror 400 and suppression LP 425) for DAPI. The images were acquired with a Leica DC 300 F camera (Leica Microsystems, Heerbrugg, Switzerland) using a 100× oil immersion N plan objective and processed using Leica IM 50-Image manager software.

Reproducibility of the results and statistical analysis

All experiments were repeated, independently, three to seven times. In fluorescence measurements, the observed trends were fully consistent among the independent experiments and a typical example is shown. Fluorescence data were expressed as the mean ± standard deviation (SD) of quadruplicate measurements. The data reported for viability are the mean ± SD, presented with 95 % confidence; the means values were subjected to unpaired *t* test or one-way ANOVA followed by Tukey–Kramer multiple comparison method.

Results

Pb induces loss of cell proliferation capacity without disruption of membrane integrity

The toxic effect of lead was evaluated using the laboratory strain *S. cerevisiae* BY4741 (WT strain) as the model organism. Viability was assessed using two complementary methodologies: a clonogenic assay based on the ability of a single cell to grow into a colony on YPD agar plates, and a membrane integrity test based on the exclusion of propidium iodide dye. The exposure of yeast cells to Pb resulted in a progressive loss of cell proliferation capacity. After a 3-h incubation with 1,000 μmol/l Pb, cell viability fell to ~17 % (Fig. 1a). The ability to reproduce remains a "gold standard" in determining viability. However, cell membranes can be damaged as a consequence of a stress imposed on the cell. As a selective barrier between intracellular content and its surrounding environment, the integrity of the cell membrane constitutes an essential characteristic of a viable cell. The WT cells that were exposed to 1,000 mmol/l Pb for 3 h retained their membrane integrity (Fig. 1b) because they remained impermeable to propidium iodide (Fig. 1c and d). The comparative analyses of the viability, assessed by the two methods, confirm that in the yeast *S. cerevisiae*, the loss of proliferation capacity in cells exposed to Pb was not associated with and was not a consequence of the disruption of membrane integrity.

Mitochondrial function is implicated in Pb-induced oxidative stress

ROS can have different origins, and the mitochondrial respiratory chain is one of the main sources (Guidot et al. 1993; Barros et al. 2004). In the yeast *S. cerevisiae*, the mtDNA encodes several subunits of the electron respiratory chain (Evans 1983). Thus, respiration can be completely abolished by inducing the loss of mtDNA. To further investigate the requirement of mitochondrial respiratory activity on Pb-induced oxidative stress, a WT isogenic derivative ρ^0 strain was used. This ρ^0 strain was unable to grow on media with a respiratory carbon source (glycerol) (data not shown), and the strain lacks mtDNA, as confirmed by DAPI staining (Fig. 2a). To compare ROS production by the WT and the isogenic derivative ρ^0 strain, cells of both strains were pre-incubated with H_2DCFDA and subsequently exposed to 250 and 1,000 μmol/l Pb for 3 h. As shown in Fig. 2b, the WT strain displayed an ROS level ~4 times higher than the ρ^0 strain.

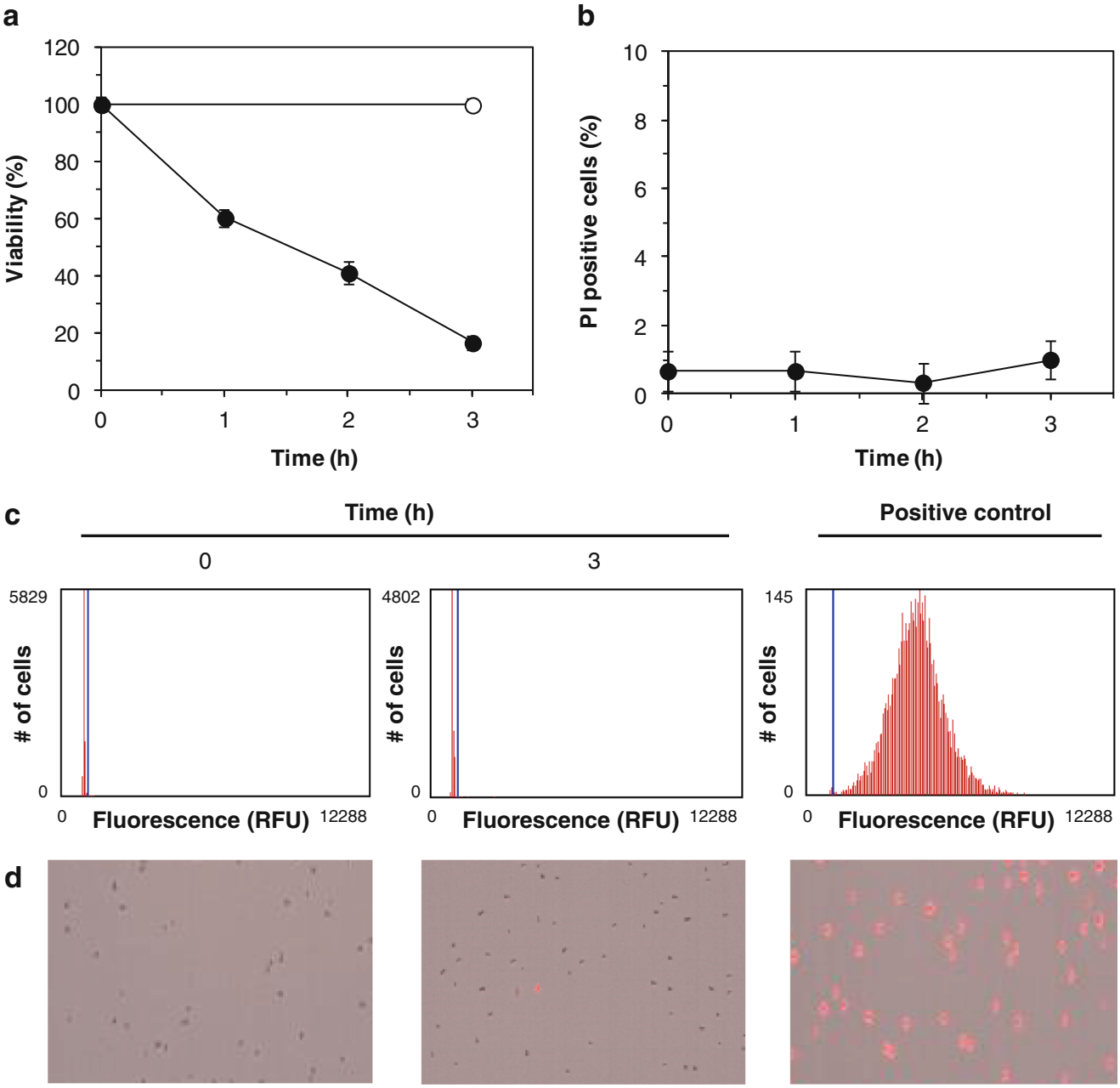

Fig. 1 Effect of Pb on the viability of *S. cerevisiae* BY4741. Yeast cells were suspended in MES buffer (10 mmol/l, pH 6.0), with 2 % (w/v) glucose and incubated in the absence (*open circles*) or the presence of 1,000 μmol/l Pb (*closed circles*). **a** Viability evaluated by colony-forming units (*cfu*) counting on YPD agar. Each point represents the mean of six independent experiments; standard deviations are presented with 95 % confidence limits (*vertical error bars*). **b** Membrane integrity evaluated using propidium iodide (*PI*), by image-based cytometry. Each point represents the mean of three independent experiments; standard deviations are presented with 95 % confidence limits (*vertical error bars*). In each assay, >10,000 cells were analysed. **c** PI histograms of fluorescence intensity profiles obtained by image-based cytometry at zero time and after 3 h of exposure to 1,000 μmol/l Pb. As a positive control (PI positive cells), cells were heat treated (65 °C, 60 min). The blue line shows the relative fluorescence units (*RFU*) threshold selected. **d** Representative fluorescent images of the conditions shown in **c**

These results argue in favour of the mitochondrial respiratory chain being the main source of Pb-induced ROS in the yeast *S. cerevisiae*. The ρ^0 strain accumulated ROS, although at lower levels than the WT strain (Fig. 2b). This suggests that in addition to the mitochondrial origin, an extra-mitochondrial source of ROS should also be considered.

Because WT cells accumulated more ROS compared to ρ^0 cells, these cells should be more sensitive to Pb stress. Consistent with this possibility, in a clonogenic assay, the WT strain displayed an extremely significant ($P<0.001$) loss of Pb-induced cell viability compared to the ρ^0 strain (Fig. 3). The lower ROS accumulation and sensitivity to Pb stress in

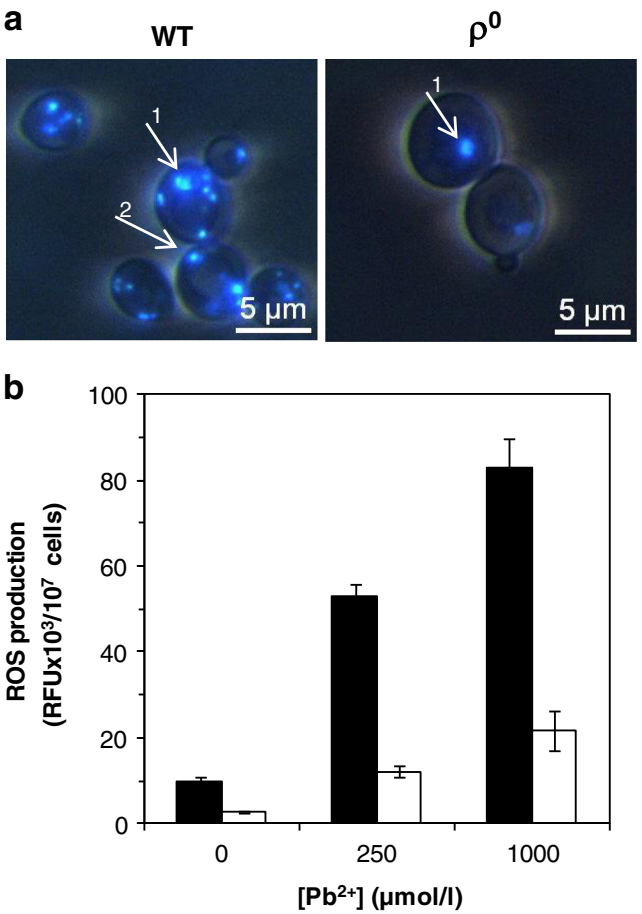

Fig. 2 Comparison of ROS production in *S. cerevisiae* BY4741 (wild type cells [*WT*]) and the respective isogenic ρ^0 mutant cells. **a** Visualization of nuclear (*arrow 1*) and mitochondrial (*arrow 2*) DNA using 4,6-diamidino-2-phenylindole (*DAPI*), by fluorescence microscopy. **b** Cells from WT strain (*dark bar*) or ρ^0 mutant (*white bar*) were pre-loaded with H_2DCFHDA, for 20 min, and subsequently exposed to Pb, in the conditions described in Fig. 1, for 3 h. This is a typical example of an experiment preformed three times. Each *bar* represents the mean of four fluorescent readings. Standard deviations are presented with 95 % confidence limits (*vertical error bars*)

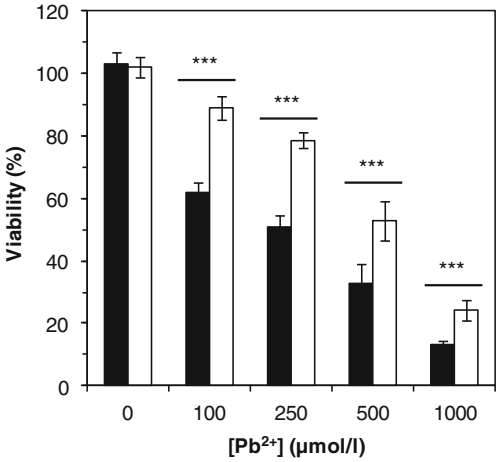

Fig. 3 Comparison of the sensitivity of *S. cerevisiae* BY4741 (wild type cells [*WT*]) and respective isogenic ρ^0 mutant to Pb. Cells from WT strain (*black bar*) or ρ^0 mutant (*white bar*) were exposed to different Pb concentrations for 3 h, as described in Fig. 1. Viability was evaluated by colony-forming units (*cfu*) counting on YPD agar. Each point represents the mean of six independent experiments; standard deviations are presented with 95 % confidence limits (*vertical error bars*). For the Pb concentrations used, the difference between the WT strain and the ρ^0 mutant was tested using an unpaired *t* test. The means with *asterisks* are extremely significantly different (***$P<0.001$)

the ρ^0 mutants strongly suggests that the mitochondrial respiratory chain is the major source of oxidative stress, which causes the Pb-induced loss of yeast proliferative capacity.

Pb-induced production of superoxide anion and hydrogen peroxide

The intracellular accumulation of two of the principal ROS, superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), in yeast cells exposed to Pb were detected with DHE and H_2DCFHDA, respectively. *S. cerevisiae* yeast cells incubated for 3 h with 1,000 μmol/l Pb displayed a red fluorescence (Fig. 4a) due to the intracellular accumulation of $O_2^{\cdot-}$, which causes the oxidation of dihydroethidium to ethidium. Pb-treated cells also accumulated H_2O_2, which causes the oxidation of H_2DCFDA to 2′,7′-dichlorofluorescein, and under these conditions, cells displayed a green fluorescence (Fig. 4a). The double staining protocol with H_2DCFDA and PI allowed verification that the majority of ROS-positive cells were PI negative. Yeast cells incubated in buffer in the absence of Pb (control cells) remained unstained or weakly stained with both fluorescent probes (Fig. 4a), which suggests that intracellular ROS accumulation was highly enhanced by the presence of Pb.

This kinetic study revealed that *S. cerevisiae* exposed to 1,000 μmol/l Pb generated $O_2^{\cdot-}$ after 30–45 min, and the production of H_2O_2 started after 45–60 min (Fig. 4b), which means that the production of $O_2^{\cdot-}$ precedes the production of H_2O_2.

Pb induces the production of respiratory-deficient mutants

Mutations in the mtDNA of normal respiratory-competent *S. cerevisiae* cells result in RD cells, which are unable to grow on non-fermentable carbon sources such as glycerol. This inability to grow on glycerol can be used as a measure of the partial or complete loss of mtDNA (Yazgan and Krebs 2012).

The results presented above showed that mitochondria are one of the main sources of Pb-induced ROS. Because mtDNA is much more susceptible to oxidative damage than nuclear DNA (Richter et al. 1988), the impact of Pb on mtDNA was investigated by determining the percentage of RD cells after exposure to Pb. Exposure for 3 h to 250 or

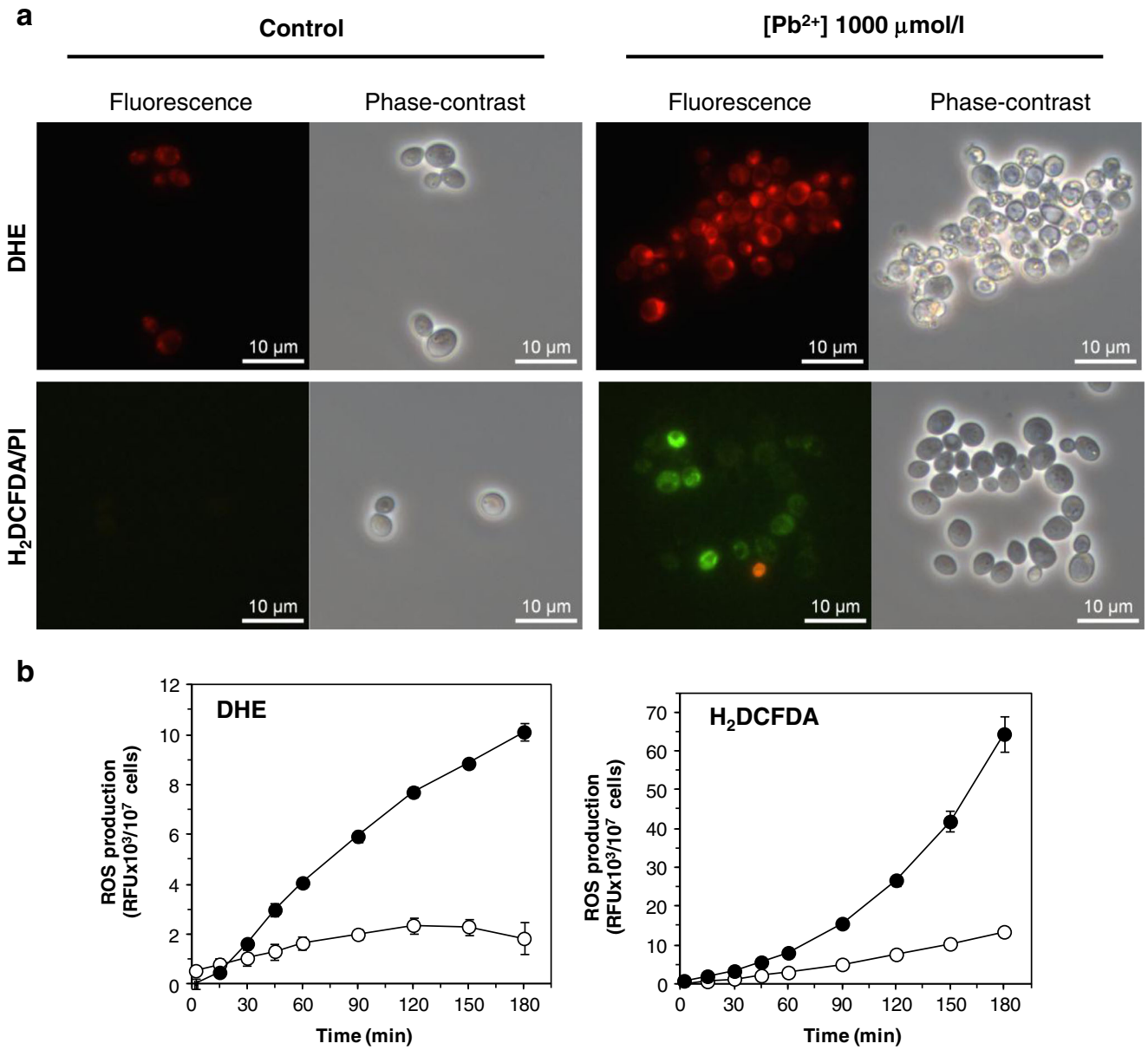

Fig. 4 Reactive oxygen species (*ROS*) production in *S. cerevisiae* BY4741 exposed to Pb. **a** Visualization by fluorescence microscopy of intracellular accumulation of $O_2^{\cdot-}$ (with dihydroethidium [*DHE*]) or H_2O_2 (with 2′,7′-dichlorodihydrofluorescein diacetate [*H_2DCFDA*]). Cells were pre-incubated with DHE or H_2DCFDA and propidium iodide (*PI*) and subsequently exposed to Pb, in the conditions described in Fig. 1 for 3 h. Control: cells incubated in the absence of Pb. **b** Kinetic of $O_2^{\cdot-}$ or H_2O_2 production. Cells were pre-loaded with DHE or H_2DCFDA and subsequently incubated in the presence of 1,000 μmol/l Pb (*closed circles*) or in the absence of Pb (*open circles*). These are typical examples of experiments preformed six times for each fluorescent probe. Each point represents the mean of four fluorescent readings. Standard deviations are presented with 95 % confidence limits (*vertical error bars*); where error bars are not shown they are within the points

1,000 μmol/l Pb induced the formation of ~49 % and 58 % of RD cells, respectively (Fig. 5). For the control cells, the percentage of respiratory-deficient cells (cells incubated for 3 h in buffer in the absence of Pb) was ~2 %, which is within the values described in the literature for the spontaneous formation of these mutants (Clark-Walker et al. 1981). The increased formation of RD mutants strongly suggests that mtDNA is one of the targets of Pb-induced ROS.

Discussion

Previous work using a brewing yeast strain of *S. cerevisiae* showed that Pb induced severe oxidative stress, which can trigger the phenotypic hallmarks of programmed cell death by apoptosis (Bussche and Soares 2011). In the present study, the well-known laboratory strain *S. cerevisiae* BY4741 was used as a cell model to evaluate the toxic impact of Pb. This laboratory strain presented a higher sensitivity to Pb than the

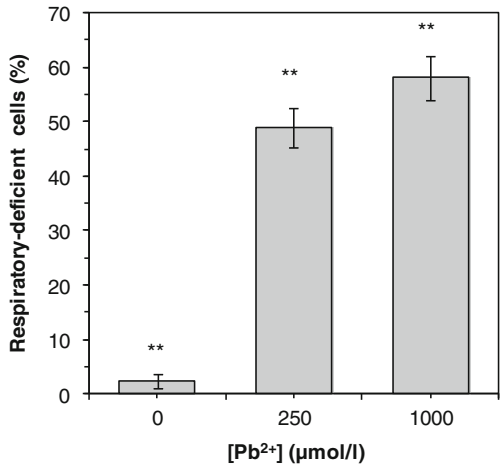

Fig. 5 Formation of Pb-induced respiratory-deficient cells in *S. cerevisiae* BY4741. Cells were exposed to different Pb concentrations for 3 h, as described in Fig. 1. The formation of respiratory-deficient (*DR*) mutants was monitored through their inability to grow on non-fermentable media (YPGly). Each bar represents the mean of seven independent experiments; standard deviations are presented with 95 % confidence limits (*vertical error bars*). The difference in the formation of DR cells in the absence or in the presence of Pb was tested (one-way ANOVA followed by Tukey–Kramer multiple comparison method). The means with *asterisks* are significantly different (**$P<0.01$)

brewing yeast strain. When the two strains were exposed to the same Pb concentration (1,000 μmol/l), the loss of proliferation capacity (~83 %) after 3 h (Fig. 1a) in *S. cerevisiae* BY4741 was similar to that observed with the brewing yeast strain after 6 h (~84 %) (Bussche and Soares 2011). The cell membranes of both the laboratory and industrial strains remained impermeable to propidium iodide during the exposure to 1,000 mmol/l Pb, for 3 h (Fig. 1b). This result shows that the loss of Pb-induced proliferation capacity occurred in the absence of cell membrane damage.

The leakage of electrons from the mitochondrial respiratory chain is pointed out as the main source of endogenous ROS in *S. cerevisiae*. However, *S. cerevisiae* is known to tolerate the loss of mtDNA (Yazgan and Krebs 2012), which makes this organism a useful model to evaluate how the mitochondrial respiratory chain is involved in Pb-induced oxidative stress. Thus, to evaluate whether mitochondrial function was the source of Pb-induced oxidative stress and, consequently, was associated with Pb toxicity, cells from an RD ρ^0 strain (lacking mtDNA) were used. These ρ^0 cells displayed a lower intracellular Pb-induced ROS accumulation (Fig. 2b) and a higher resistance to Pb (Fig. 3) compared with the respective WT strain. This result is evidence that mitochondrial function plays an important role in Pb-induced ROS generation. The higher resistance of the ρ^0 strain to Pb-induced stress is in agreement with work that reported that a mutant lacking mtDNA was more resistant to oxidative stress by the ROS-generating agent linoleic acid hydroperoxide (Evans et al. 1998). Ludovico et al. (2002) also reported that the ρ^0 mutant was more resistant to acetic acid-induced death compared to the wild-type strain.

Pb-induced ROS production was studied using two fluorescent probes, DHE and H_2DCFDA, which allowed the detection of the generation of $O_2^{\cdot -}$ and H_2O_2, respectively. In this approach, it was also possible to verify that the intracellular accumulation of $O_2^{\cdot -}$ precedes the triggering of H_2O_2 accumulation in *S. cerevisiae* cells exposed to Pb (Fig. 4b). These results are in agreement with literature on the sequence of endogenous ROS formation. Thus, $O_2^{\cdot -}$ is the first ROS produced as result of the reaction of the high-energy electrons leaked from the mitochondrial chain with molecular oxygen; the $O_2^{\cdot -}$ is then converted to H_2O_2 by the enzyme mitochondrial superoxide dismutase (Sod2) (Herrero et al. 2008).

Intracellular ROS accumulation induced by the presence of redox-inactive metals, such as Cd, Hg or Pb, can be explained by several indirect mechanisms such as the displacement of redox-active metals from cellular binding sites or depletion of non-enzymatic (such as thiol-containing compounds) antioxidant defences (Avery 2001; Jomova and Valko 2011; Flora et al. 2012). Consistent with this possibility, it was found that Pb decreased the intracellular level of reduced glutathione in yeast cells (Perez et al. 2013). Another possible explanation for the induction of intracellular ROS accumulation by Pb is through the inhibition of the enzymes associated with antioxidant defences (Avery 2001; Jomova and Valko 2011; Flora et al. 2012). It is known that H_2O_2 accumulation can lead the attack on the thiol groups of enzymes (Bartosz 2009; Avery 2011).

Mitochondria have defence mechanisms, such as mitochondrial superoxide dismutase, to scavenge the ROS produced (Herrero et al. 2008). However, when ROS are overproduced, the defence mechanisms become overwhelmed and a chain of events can originate the loss of part or all of the mtDNA. Consistent with this possibility, it was shown that the exposure to Pb induced the formation of RD mutants (Fig. 5). These mutants are unable to grow on non-fermentable carbon sources such as glycerol due to the mutations in mtDNA and consequent loss of mitochondrial function. Together, these results suggest that the mitochondria are one of the sources and, simultaneously, one of the targets of Pb-induced ROS.

In conclusion, the results obtained confirmed that the exposure of *S. cerevisiae* yeast cells to 1,000 μmol/l Pb for 3 h induced the loss of proliferation capacity without damaging membrane integrity. Mitochondrial respiration is one of the main sources of Pb-induced ROS because the isogenic derivative ρ^0 strain, lacking mtDNA and consequently respiratory competence, displayed a lower intracellular ROS accumulation and a higher resistance to Pb compared to the WT strain. The kinetic analysis of ROS production verified that the production of $O_2^{\cdot -}$ precedes the production of H_2O_2 in *S. cerevisiae* cells exposed to Pb, which is consistent with the possibility of $O_2^{\cdot -}$ generation results from the leakage of

electrons from the mitochondrial respiratory chain. The mitochondria contribute to and are one of the targets of Pb toxicity because the intracellular Pb-induced ROS accumulation provoked mutations at the mtDNA level, with the consequent formation of RD cells.

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