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Evaluation of the Role of Glutathione in the Lead-Induced Toxicity in *Saccharomyces cerevisiae*

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Abstract The effect of intracellular reduced glutathione (GSH) in the lead stress response of *Saccharomyces cerevisiae* was investigated. Yeast cells exposed to Pb, for 3 h, lost the cell proliferation capacity (viability) and decreased intracellular GSH level. The Pb-induced loss of cell viability was compared among yeast cells deficient in *GSH1* (Δ*gsh1*) or *GSH2* (Δg*sh2*) genes and wild-type (WT) cells. When exposed to Pb, Δ*gsh1* and Δ*gsh2* cells did not display an increased loss of viability, compared with WT cells. However, the depletion of cellular thiols, including GSH, by treatment of WT cells with iodoacetamide (an alkylating agent, which binds covalently to thiol group), increased the loss of viability in Pb-treated cells. In contrast, GSH enrichment, due to the incubation of WT cells with amino acids mixture constituting GSH (L-glutamic acid, L-cysteine and glycine), reduced the Pb-induced loss of proliferation capacity. The obtained results suggest that intracellular GSH is involved in the defence against the Pb-induced toxicity; however, at physiological concentration, GSH seems not to be sufficient to prevent the Pb-induced loss of cell viability.

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Introduction

Although lead occurs naturally, its presence in the environment comes, mainly, from human activities, like mining or smelting of ore, manufacture of lead-containing products (such as car batteries), combustion of coal and oil, and waste incineration. Lead is a non-essential metal for biological functions and is classified by the International Agency for Research on Cancer as probable human carcinogen [1].

The yeast *Saccharomyces cerevisiae* is a suitable eukaryotic model organism for studying Pb toxic effects, since its cellular structure and functional organization share many similarities with animal and plant cells. This yeast can be easily manipulated, presents a short generation time and has the genome completely sequenced. In addition, valuable tools are available such as the yeast deletion strain collection for understanding gene functions [22].

In *S. cerevisiae*, Pb inhibits metabolic activity [23] and cell growth, impairs ammonium assimilation and reduces DNA/RNA ratio [4]. In addition, it was shown that Pb induces the intracellular accumulation of reactive oxygen species (ROS), which can be the trigger of programmed cell death by apoptosis [3].

Yeast cells have different protection mechanisms for controlling the levels of ROS, which can be, basically, divided in enzymatic (superoxide dismutases, catalases and peroxidases) and non-enzymatic defences (as glutathione and trehalose) [10, 12].

The tripeptide glutathione (L-γ-glutamyl-L-cysteinyl-glycine) is the main intracellular low molecular mass thiol.

Glutathione is present in cells, principally, as reduced form—GSH [15, 17]. GSH is biosynthesized in two sequential steps: (i) in the first step, the dipeptide L-γ-glutamylcysteine is synthesized, from the L-glutamic acid and L-cysteine, catalyzed by the enzyme L-γ-glutamylcysteine synthetase (*GSH1* gene product); and (ii) in the second step, glycine is added to the C-terminal of γ-glutamylcysteine, by the action of glutathione synthetase (*GSH2* gene product), to synthesize GSH [7, 17].

GSH play different functions in yeast cells such as: (i) radical scavenger, by reacting directly with radical hydroxyl to reduce it to H_2O [7]; (ii) electron donor to glutathione peroxidases (GPX) and glutaredoxins, which catalyzes the reduction of H_2O_2 and organic peroxides to H_2O and the corresponding alcohols, respectively [10]. In these processes, the GSH is oxidized to glutathione disulphide (GSSG). Glutathione can be reduced back by the action of glutathione reductase with the consumption of NADPH, which is essential to the maintenance of a high reduced–oxidized ratio inside the cell [7, 12].

Several works have shown the role of GSH in the protection of the cells against the damage provoked by oxidative stress [11], xenobiotics, carcinogens, radiation [15] and heavy metals [Cd, As(III), Sb(III), Se and Hg] [6, 18, 25]. GSH seems to be a key molecule in the defence against oxidative stress and metal toxicity. The involvement of GSH in metal detoxification can be carried out in several ways: (i) binding to metals and subsequent vacuolar sequestration; (ii) impairing metal-induced oxidative stress, by the mechanisms presented above; and (iii) binding to sulphhydryl groups on proteins (protein glutathionylation), protecting them from irreversible metal binding and/or oxidative damage [25]. However, the role of GSH in Pb stress responses, in the yeast *S. cerevisiae*, is unknown.

The present work aims to examine the role of intracellular GSH in Pb-induced toxicity in *S. cerevisiae*. For this purpose, the loss of viability induced by Pb was evaluated in wild-type (WT) and GSH-deficient mutant strains. In addition, intracellular GSH content of WT cells was modulated by incubation with iodoacetamide (to deplete cellular thiols, including GSH) or with the mixture of amino acids that make up GSH (GSH-enriched cells); in both cases, the influence of the depletion or GSH enrichment on the modification of the susceptibility of yeast cells to Pb was studied.

Material and Methods

Strains, Media and Culture Conditions

Saccharomyces cerevisiae WT yeast strain BY4741 (MATa *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0) and the isogenic mutants Δ*gsh1* and Δ*ghs2* were obtained from EUROSCARF collection and used in this work.

The strains were routinely maintained at 4 °C on YPD agar slants [10 g/l yeast extract (Difco-BD), 20 g/l peptone (Difco-BD), 20 g/l glucose (Merck) and 20 g/l agar (Merck)]. Mutants were maintained under a selective pressure in YPD agar with 0.02 % (w/v) geneticin (Sigma-Aldrich).

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

Treatment of Yeast Cells with Pb

After growth, cells were harvested by centrifugation (2,000×*g*, 5 min), washed twice with deionized water and resuspended in 2-(N-morpholino) ethanesulphonic acid (MES) pH buffer (Sigma-Aldrich, St. Louis, MO, USA) 10 mmol/l, at pH 6.0, with 2 % (w/v) glucose, to $\sim 1 \times 10^7$ cells/ml. MES is a suitable pH buffer for heavy metal toxicity studies because it does not complex lead [20] and yeast cells maintain viability when incubated in this buffer for 48 h [19].

Cell suspensions (40 ml) containing 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. Cells were treated using a Pb concentration range of 0–1,000 µmol/l in order to induce a toxic effect (evaluated by the loss of cell proliferation capacity) in wild-type strain of 0–90 %.

Glutathione Enrichment and Depletion Assays

Enrichment of intracellular glutathione content was carried out as previously described [11]. Briefly, WT yeast cells, 1×10^7 cells/ml, in exponential growth phase, were incubated in the dark, with shaking (150 rpm), at 25 °C, for 1 h, with a mixture containing 0.5 mol/l glucose, 0.01 mol/l $MgCl_2$, 0.02 mol/l L-glutamic acid, 0.02 mol/l L-cysteine, 0.02 mol/l glycine and 0.1 mol/l potassium phosphate buffer (pH 7.4). In the pre-treatment with cysteine, cells were incubated with the mixture described above without L-glutamic acid and glycine.

For depletion of glutathione, 1×10^7 cells/ml, in exponential growth phase, were incubated in the dark, with shaking (150 rpm), at 25 °C, for 1 h, with 1 mmol/l iodoacetamide (Sigma-Aldrich).

After the treatment, cells were washed twice with deionized water, suspended in 10 mmol/l MES buffer, at

pH 6.0, with 2 % (w/v) glucose and exposed to Pb as described above.

Viability Measurement

Viability was determined by plating the cells on YPD agar. Thus, samples (1.0 ml; two–three replicates) were taken at defined intervals of time, serially diluted with sterile deionized water and plated (two replicates of 200 µl of the convenient dilutions). The colonies were counted after 3–4 days of incubation at 25 °C. The % of survivors was calculated using the number of colony-forming units (c.f.u)/ml at zero time as reference (100 %).

Evaluation of Intracellular Reduced Glutathione Content

Intracellular GSH was monitored with monochlorobimane (mBCl, Sigma-Aldrich) as previously described [21] with minor modifications. After exposition to 1,000 µmol/l Pb, for 3 h, cells were washed once with deionized water, suspended at 1×10^7 cells/ml in 0.1 mol/l phosphate buffered saline solution (PBS buffer), at pH 7.0, and incubated with mBCl (in a final concentration of 50 µmol/l), for 2 h, at 25 °C, in the dark. Stock solutions of 50 mmol/l mBCl were prepared in dimethyl sulphoxide (Sigma-Aldrich) and stored at −20 °C; working solutions of 1 mmol/l were prepared in PBS buffer, pH 7.0, just before use. Fluorescence intensity (as relative fluorescent units—RFU) was measured using a microplate reader at fluorescence excitation of 355/40 nm and an emission of 460/25 nm. Fluorescence was normalized (considering cell concentration) and corrected by subtracting cell, buffer and dye autofluorescence.

For epifluorescence microscopy analysis, 1×10^7 cells/ml were suspended in 0.1 mol/l PBS buffer (pH 7.0) and incubated with mBCl (in a final concentration of 100 µmol/l), for 60 min, at 25 °C, in the dark; cells were examined using a Leica DLMB epifluorescence microscope equipped with a HBO—100 mercury lamp and the filter set A [excitation filter (band pass filter, BP) BP 340–380, dichromatic mirror 400 and suppression filter (long pass filter, LP) LP 425], from Leica. The images were acquired with a Leica DC 300F camera using N plan objectives and processed using Leica IM 50-Image manager software.

Reproducibility of the Results

All experiments were repeated, independently, four times. Fluorescence data were expressed as the mean ± standard deviation (SD), presented with 95 % confidence value, of quintuplicate measurements of a typical experiment. The data reported for viability were the mean ± SD, presented with 95 % confidence value of four independent experiments.

Results and Discussion

Pb-induced Loss of Viability in Wild-Type and Glutathione-Deficient Mutants

The exposition of *S. cerevisiae* cells to Pb resulted in a marked intracellular ROS accumulation [3]. GSH is the main redox buffer of the cells being a key factor against the oxidative stress and metals toxicity [25]. In this context, the effect of reduced GSH on the protection against Pb-induced toxicity was evaluated using the WT strain and the knockout mutants devoid in the genes *GSH1* or *GSH2*, responsible for the enzymes L-γ-glutamylcysteine synthetase and γ-glutamylcysteine, respectively.

Saccharomyces cerevisiae WT cells, in exponential phase of growth, when exposed to different Pb concentrations, for 3 h, lose the viability, assessed by c.f.u. counting on YPD plates, in a dose-dependent manner. The survival of WT cells exposed to 500 or 1,000 µmol/l Pb, for 3 h, was 43 or 8 % of cells, respectively (Fig. 1); i.e. these Pb concentrations induced a loss of cell proliferation capacity of 57 and 92 %, respectively.

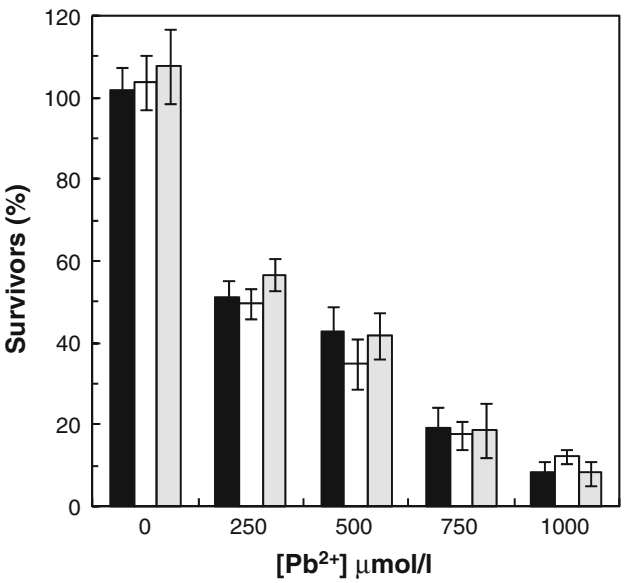

Fig. 1 Comparison of the susceptibility to Pb of wild-type and GSH-deficient mutant strains of *S. cerevisiae*. Cells in exponential phase of growth of the wild-type strain BY4741 (*dark bar*) or the isogenic GSH-deficient mutant strains Δ*gsh1* (*white bar*) and Δ*gsh2* (*grey bar*) were treated with different Pb concentrations for 3 h. Viability was estimated by c.f.u. counts. Each bar represents the mean of four independent experiments. Standard deviations are presented with 95 % confidence limits (*vertical error bars*)

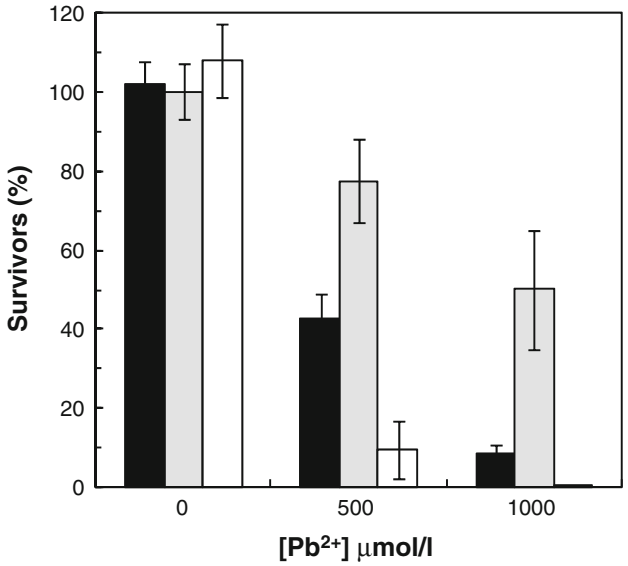

Fig. 2 Impact of iodoacetamide or amino acids mixture constituting GSH on the viability of Pb-exposed cells of *S. cerevisiae*. Cells of the wild-type strain BY4741, in exponential phase of growth, were incubated with 1 mmol/l iodoacetamide (*white bar*) or with a mixture containing 0.5 mol/l glucose and 0.02 mol/l L-glutamate, L-cysteine and glycine (*grey bar*), for 1 h, at 25 °C, before being exposed to Pb, for 3 h. As control, cells of the wild-type strain BY4741 in exponential phase of growth (non-treated cells—*black bar*) were exposed to Pb for 3 h. Viability was estimated by c.f.u. counts. Each bar represents the mean of four independent experiments. Standard deviations are presented with 95 % confidence limits (*vertical error bars*)

Yeast cells lacking *GSH1* gene (Δ*gsh1*) are completely avoided of GSH; cells lacking *GSH2* gene (Δ*gsh2*) are able to synthesize L–glutamylcysteine, from L-glutamic acid and L-cysteine, but not GSH. The exposition of Δ*gsh1* or Δ*gsh2* mutant strains, to increasing Pb concentrations, originated a marked loss of cell proliferation. Nevertheless, Δ*gsh1* and Δ*gsh2* strains did not display an increased loss of viability compared to WT strain (Fig. 1).

Change in the Thiol Compounds Content Modify the Sensitivity to Pb

In order to test if the GSH was not involved as defence mechanism or was not present in enough amount to protect yeast cells against Pb-induced toxicity, cells from WT strain, in exponential phase of growth, were treated, for 1 h, with 1 mmol/l iodoacetamide (thiol-depleted cells) or with an amino acids mixture constituting GSH (GSH-enriched cells), washed and subsequently exposed to Pb.

Chemical induction of GSH depletion can be carried out, basically, through two mechanisms: (i) reaction with GSH, i.e. GSH is depleted due to the reaction with chemicals like diethylmaleate or iodoacetamide; and (ii) by inhibition of GSH biosynthesis with an enzyme inhibitor,

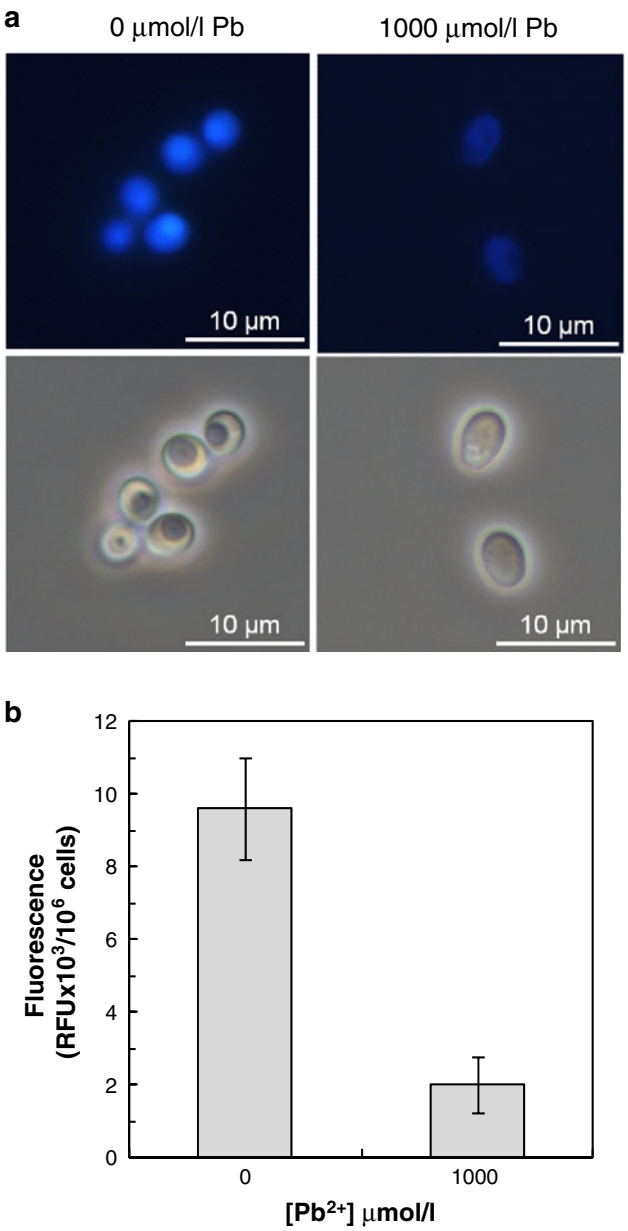

Fig. 3 Pb exposition induced the decrease of intracellular glutathione content in *S. cerevisiae*. Cells in exponential phase of growth were incubated for 3 h in the absence or presence of 1,000 µmol/l Pb; subsequently, cells were washed and incubated with monochlorobimane, for 1 h. Untreated cells exhibited fluorescence (due to the formation of bimane-glutathione conjugates), which was absent in cells exposed to Pb (**a**). Quantification of fluorescence (**b**). This is a typical example of an experiment performed four times; each bar represents the mean of five fluorescent readings. Standard deviations are presented with 95 % confidence limits (*vertical error bars*)

such as L-buthionine sulphoximine [13, 15]. With the aim to test the effect of the absence of GSH, on Pb toxicity, by a different way (GSH biosynthesis inhibition was tested with Δ*gsh* mutant strains), yeast cells were GSH depleted by treatment with iodoacetamide; this compound was selected due to its efficiency in GSH depletion, in a short

time [16]. The treatment of yeast cells with iodoacetamide and subsequent staining with mBCl allowed to verify a severe reduction of intracellular thiol content, including GSH, as no fluorescence was observed in iodoacetamide-treated cells. This observation is in agreement with the results presented in the literature which described that the treatment with iodoacetamide originates a reduction of the GSH to <5 % of normal levels [16]. On the contrary to GSH-deficient mutants, WT cells pre-treated with iodoacetamide displayed an enhanced sensitivity to Pb compared to non-treated cells. The survival of thiol-depleted cells to the exposition to 500 or 1,000 μmol/l Pb, for 3 h, was 10 % or <1 %, respectively (Fig. 2). Iodoacetamide is an alkylating agent, which binds covalently to thiol groups [8]. Thus, in addition to GSH depletion, iodoacetamide can also irreversibly inhibit other thiol compounds and enzymes associated with the defence against the oxidative stress; these facts can explain the hypersensitivity of iodoacetamide-treated cells to Pb.

The incubation of WT cells with glucose and L-glutamic acid, L-cysteine and glycine lead to an increase of intracellular levels of GSH [11]. In the presence of 500 or 1,000 μmol/l Pb, for 3 h, the GSH-enriched cells presented a higher viability, comparatively to non-treated cells (Fig. 2); under these conditions, a survival of 78 and 50 %, respectively, was observed. The pre-treatment of WT cells with glucose and L-cysteine did not protect the cells against the Pb-induced loss of viability, since a similar % of survival to non-treated WT cells was observed. Together, these data suggest the involvement of GSH in the defence against the Pb-induced toxicity, for instance, as co-factor of enzymatic defences against oxidative stress (such as GPX enzymes).

Pb Reduces Intracellular Glutathione Level

Redox-inactive metals, as is the case of Pb, can produce ROS by indirect mechanisms such as the displacement of redox-active metals from cellular binding sites, the inhibition of specific enzymes associated with antioxidant defences or by depleting pools of antioxidants, such as GSH [2, 25].

The intracellular GSH content in *S. cerevisiae* cells was evaluated using mBCl, a cell-permeant probe. Once inside the cell, the probe is essentially nonfluorescent until conjugated; mBCl reacts with reduced glutathione and forms fluorescent bimane-glutathione (B-SG) adducts [9]. Cells, in the absence of Pb, exhibited a typical fluorescence due to the formation of B-SG adducts. On the contrary, in Pb-treated cells (1,000 μmol/l Pb, for 3 h) only a faint fluorescence was observed (Fig. 3a). As described above, similar images of Pb-treated cells were obtained with iodoacetamide-treated cells. The quantification of the fluorescent signal produced due to the formation of B-SG adducts in cells non-treated or treated with 1,000 μmol/l Pb, for 3 h, showed a marked reduction (∼94 %) of fluorescence in Pb-treated cells (Fig. 3b). These results suggested a reduction of intracellular GSH in Pb-exposed yeast cells, which is in agreement with the data presented in the literature, that describe a GSH decrease in kidney, liver and brain cells of rats exposed to lead [5, 24]. It is known that several metals including Hg, Cd and Pb present high affinity to thiol group [14]. The reduction of GSH observed can be due to the affinity of the thiol group of GSH to Pb. Once in the cytosol, Pb can be complexed by GSH, decreasing the level of GSH.

Conclusions

The lack of *GSH1* or *GSH2* genes did not enhance the susceptibility of the yeast cells to the toxic action of Pb. However, the manipulation of intracellular GSH content modified, markedly, the viability in Pb-exposed cells. In addition, the exposition to Pb decreased the intracellular GSH level. These results suggest that intracellular GSH is involved in the Pb detoxification; however, at physiological concentration, GSH seems not enough, per se, to halt the Pb-induced loss of cell viability.

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