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Cytosolic thioredoxin peroxidase I is essential for the antioxidant defense of yeast with dysfunctional mitochondria

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Abstract The specific role of cytosolic thioredoxin peroxidase I (cTPx I), encoded by *TSA1* (thiol-specific antioxidant), was investigated in the oxidative stress response of *Saccharomyces cerevisiae*. In most cases, deletion of *TSA1* has showed only a slight effect on hydrogen peroxide sensitivity. However, when the functional state of the mitochondria was compromised, the necessity of *TSA1* in cell protection against this oxidant was much more evident. All the procedures used to disrupt the mitochondrial respiratory chain promoted increases in the generation of H_2O_2 in cells, which could be related to their elevated sensitivity to oxidative stress. In fact, *TSA1* is highly expressed when cells with respiratory deficiency are exposed to H_2O_2. In conclusion, our results indicate that cTPx I is a key component of the antioxidant defense in respiratory-deficient cells. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Thioredoxin peroxidase; Oxidative stress; Gene expression; Mitochondrion; Respiratory deficiency

1. Introduction

Living in an aerobic environment has required the development of sophisticated mechanisms to detect and detoxify reactive oxygen species (ROS), generated as by-products of normal biological reactions, specially oxidative phosphorylation [1]. The maintenance of the redox homeostasis is crucial for many biological processes such as differentiation, regulation of specific genes and signaling pathways [2,3], cell cycle regulation [4], programmed cell death [5] and aging [3]. The protective system against ROS includes enzymatic scavengers such as SOD, catalase and glutathione peroxidase, and non-enzymatic ones, like vitamins C and E, thiol-containing molecules (glutathione, thioredoxin) and transition metals chelators [1].

Peroxiredoxins constitute a group of thiol-dependent peroxidases ubiquitously distributed. In humans, these proteins have been implicated in many different processes such as proliferation [8], differentiation [9], natural killer cytotoxicity [10], apoptosis [11] and mitochondrial permeability transition [12]. Despite the demonstration that this protein decomposes peroxides at the expense of sulfhydryl compounds [7], the relationship between cellular processes and its enzymatic activity remains unknown.

Saccharomyces cerevisiae cytosolic thioredoxin peroxidase I (cTPx I) was the first peroxiredoxin isolated from an eukaryotic cell [6]. During the investigation about the role of cTPx I in yeast oxidative stress response, we found that its gene, *TSA1* (thiol-specific antioxidant), is transcriptionally activated in different situations where cells were exposed to high H_2O_2 concentration. The mitochondrial respiratory chain was disrupted by several means and in all cases the generation of ROS was increased. Moreover, a comparison between a *TSA1* deletion mutant and its corresponding wild-type isogenic strain indicated that this deletion renders cells more sensitive to H_2O_2, remarkably when the functional state of the mitochondria is compromised. Our results indicate that cTPx I has a fundamental importance in the oxidative stress response of cells with dysfunctional mitochondria.

2. Materials and methods

2.1. Yeast strains and growth conditions

The *S. cerevisiae* strains used in this study were JD7-7C (MATα ura3-52 leu2 trpA K+) [13], *tsa1Δ*(MATαura3-52 leu2 trpA K+ tsaΔ::LEU2) [13], W303-1a (MATαade2-1 trp1-1 can1-100 leu2-3,112 his3-11 ura3) and *cox10Δ*(MATα ade2-1 trp1-1 can1-100 leu2-3,112 his3-11 ura3 cox10::HIS3) [14]. Cells were grown at 30°C on YP medium (1% yeast extract, 2% bacto-peptone) with 2% glucose (YPD) or 2% glycerol plus 2% ethanol (YPYE) or even 2% raffinose (YPR). For most analysis, cells were harvested by centrifugation at mid-logarithmic phase, usually at an $OD_{600\,nm}$ between 0.8 and 1.4. The *rho*^{0} (ρ^0) derivatives of the strains JD7-7C and *tsa1Δ* were obtained by growing cells for approximately 14 h in YPD medium containing ethidium bromide at 10 μg/ml. The respiratory deficiency of *rho*^{0} cells were verified by plating isolated colonies on YPYE medium, whereas the absence of mitochondrial DNA was visualized by epifluorescence microscopy, after staining with DAPI (4™,6-diamidino-2-phenylindole, dihydrochloride) in Vectashield antifading solution (Vector Laboratory).

2.2. Plasmids and DNA manipulation

For probe preparation, a 600 bp *Eco*RI/*Xba*I fragment containing the *TSA1* coding sequence was isolated from plasmid pBS-C170S [15]. Plasmidial DNA preparation, gel electrophoresis and purification were all performed using standard methods as described in [16].

2.3. RNA isolation and analysis

Total yeast RNA was extracted by the method of hot acid phenol and Northern blotting was performed as previously described [16]. The *TSA1* ^{32}P-labeled probe was prepared by random primed synthesis [16]. Probed membranes were exposed to Kodak films

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Abbreviations: ROS, reactive oxygen species; cTPx I, cytosolic thioredoxin peroxidase I; TSA, thiol-specific antioxidant; HRP, horseradish peroxidase

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(X-OMAT). Ribosomal RNA, whose abundance is fairly constant under different growth conditions or among the strains derivatives, was used as control of the various total RNA samples loaded into the gels. In some cases, actin was also used as loading control and no significant difference was found (not shown). Quantitation of *TSA1* expression relative to rRNA bands were performed by dosimetry using Image Master VDS software. Normalized results of Northern blotting experiments are graphically represented at the bottom of the corresponding figures.

2.4. Determination of H_2O_2 tolerance

Spot test: cells were first grown in YPD media until a concentration of approximately 10^7 cells per ml, and then diluted to $OD_{600\,nm} = 0.2$. Four subsequent 1/5 dilutions of these cell suspensions were realized and a 12 μl droplet of each was plated on YPD-agar or YPR-agar medium containing 1.2 mM H_2O_2, or 0.1 μg/ml antimycin A or even both agents. Plates were then incubated 2 (YPD) or 4 days (YPR).

The H_2O_2 tolerance was also evaluated by the colony assay where cells were grown on YPD, 30°C until exponential phase ($OD_{600\,nm} = 1.0$), treated with 0,1 μg/ml antimycin A alone or associated to 0.5 mM H_2O_2, during 1 h. Cells were then diluted and plated onto YPD-agar. Colonies were counted after 48 h of growth at 30°C.

2.5. Determination of H_2O_2 generation

H_2O_2 produced by *S. cerevisiae* cells was detected by the horseradish peroxidase (HRP)–scopoletin method [17]. Yeast cells from different strains were grown in YPD medium as described previously, harvested, suspended in appropriate buffer (0.1 M Tris–HCl pH 7.5, 1 mM glucose, 137 mM NaCl, 2.7 mM KCl) containing 0.1% digitonin and incubated at room temperature for 30 min for permeabilization. Cells at the concentration of approximately 7.2×10^6 cells/ml were added to the same buffer containing 1 μM HRP, 1 μM scopoletin, 5 mM ATZ and, only in the positive control reaction mixture, 0.1 μg/ml antimycin A. Fluorimetric measurement of scopoletin oxidation was assessed on a Hitachi 4500 spectrofluorimeter with excitation and emission wavelengths of 380 and 465 nm, respectively.

3. Results

3.1. ROS induces TSA1 transcription

Studies performed in cell-free systems have demonstrated that cTPx I possesses thiol peroxidase activity [7,18]. To learn more about the physiological role of cTPx I in whole cells, the transcription of *TSA1* gene was analyzed by Northern blot. Initially, it was investigated the effect of H_2O_2 on *TSA1* transcription. When cells were grown in YPD (glucose), the maximum *TSA1* mRNA levels were observed 30 min after H_2O_2 addition, at the concentration of 1 mM, reaching around two- to five-fold the basal levels. Therefore, the time course of *TSA1* induction after H_2O_2 exposition is closely related to the periods required for the adaptation of yeast to oxidative stress [19].

Glucose represses the expression of genes in *S. cerevisiae* involved in mitochondrial biogenesis, respiratory metabolism and antioxidant defense [20]. Therefore, it was also analyzed the effect of H_2O_2 induction in cells growing in non-fermentative carbon source, glycerol/ethanol. The maximum increase in *TSA1* expression occurred faster (15 min) than in cells growing in glucose and after 60 min of treatment returned to the basal levels. These results indicate that *TSA1* is responsive to H_2O_2 treatment in all the situations analyzed.

3.2. Participation of cTPx I in the oxidative stress response of cells with dysfunctional mitochondria

It is well established that the majority of intracellular ROS production is derived from the leaking of electrons from the mitochondrial transport chain, mainly at complex I (NADH dehydrogenase) and at complex III (ubiquinone-cytochrome *c* reductase) [3], and several antioxidant enzymes may act in their detoxification. On the other hand, mitochondrial function has been demonstrated to improve resistance to oxidative stress in yeast [21]. Here it was evaluated the participation of cTPx I in the antioxidant response of cells with normal or impaired mitochondrial function growing under repressing (glucose) or derepressing (raffinose) conditions (Fig. 2).

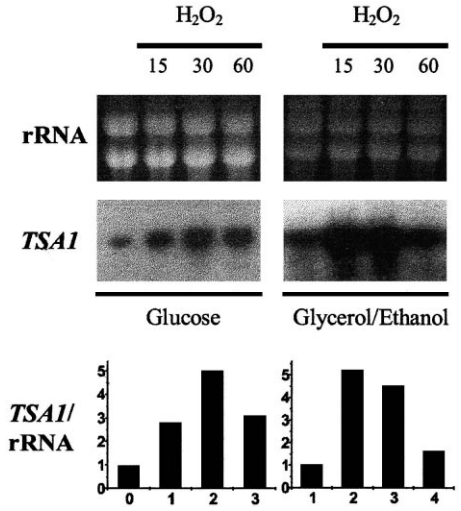

Fig. 1. Effect of H_2O_2 on *TSA1* expression. Northern blot analysis of RNA isolated from mid-logarithmic yeast strain JD7-7C exposed to 1 mM H_2O_2 during times (min) and carbon sources indicated in the figure.

In glucose-containing medium, the rho^+ strain with *TSA1* deletion was only slightly more sensitive to H_2O_2 than the corresponding wild-type (Fig. 2A). Antimycin A, a drug that inhibits respiratory complex III, alone did not interfere with growth of any strain. However, association of antimycin A with H_2O_2 led to severe growth retardation of *tsa1Δ* strain relative to wild-type, indicating a prominent role of cTPx I in ROS detoxification under respiratory deficiency. To confirm this trend, the same experiment was performed using rho^0 cells, an alternative method to disrupt mitochondrial function. It was repeatedly observed that deletion of *TSA1* led to a faster growth of the rho^0 cells in YEPD (glucose). Addition of H_2O_2 reverted the relative growth between the two strains; wild-type cells grew faster than the *tsa1Δ* ones. As expected and in contrast to respiratory-competent strains, presence of antimycin A did not have any additional effect on the growth rates of the rho^0 cells under any treatment.

The same experiment was performed in medium containing raffinose as the sole carbon source, a sugar that does not repress respiration. In contrast to the results obtained with cells grown in glucose, deletion of *TSA1* did not reduce resistance to H_2O_2 in respiratory-competent cells, indicating that other mitochondrial-related defenses overcome cTPx I role in this case. Treatment of antimycin A alone led to a growth retardation independent of cTPx I, however addition of H_2O_2 under this condition clearly showed the requirement of cTPx I for growth.

The importance of cTPx I on yeast defense against oxidative stress when mitochondria were not functional was confirmed by the cell colony assay (Fig. 2B). Deletion of *TSA1* provoked a remarkable sensitivity to oxidation when cells were simultaneously treated with antimycin A and H_2O_2. In

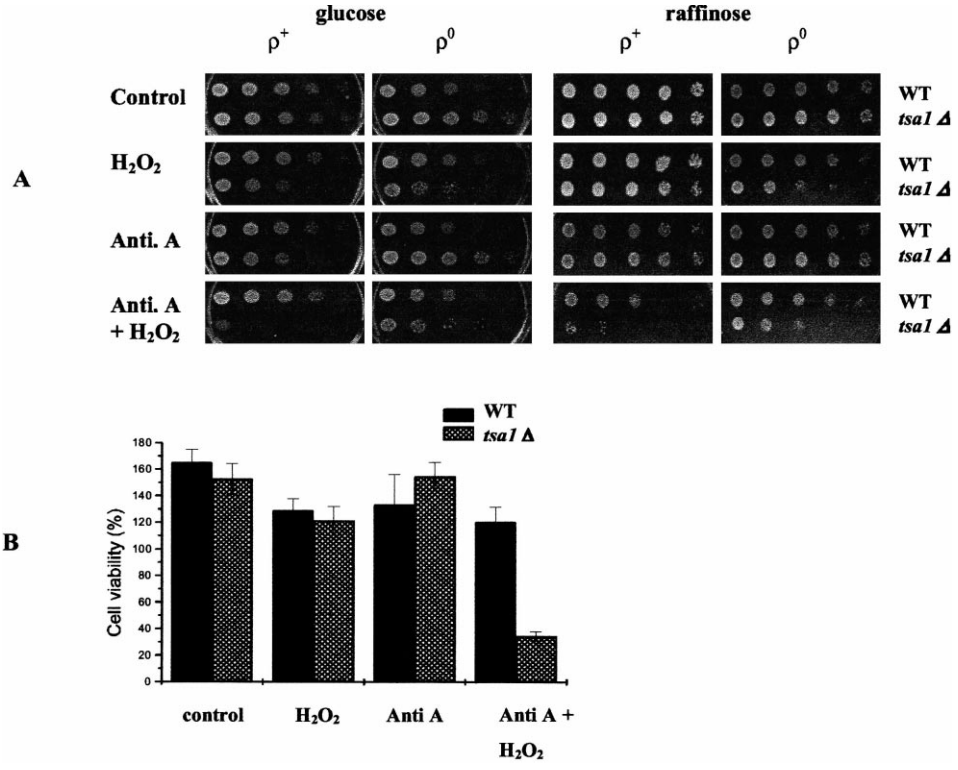

Fig. 2. H_2O_2 tolerance of respiratory-competent and respiratory-incompetent yeast cells. A: Spot test. H_2O_2 tolerance was evaluated by different growth rates of cells of the strains JD7-7C (WT) and *tsa1Δ* on YP agar plates containing the agents described in the figure. B: Colony assay. H_2O_2 tolerance was reflected by the number of colonies of the strains JD7-7C (WT) and *tsa1Δ*, counted after 48 h of growth on YPD-agar plates. Cells were treated before plating. Percentage survival is expressed related to the number of colonies at the beginning of the experiment (100%).

conclusion, our results showed that cTPx I has a key role in the defense of cells with dysfunctional mitochondria against H_2O_2.

3.3. *TSA1* transcription in cells with dysfunctional mitochondria exposed to H_2O_2

Since respiratory-deficient cells presented high dependence on cTPx I for detoxification of H_2O_2, it was evaluated whether this condition raised signals for the regulation of the *TSA1* gene. Therefore, *TSA1* transcription was analyzed under the same situations described in Fig. 2.

In glucose-containing media, addition of H_2O_2 increased *TSA1* transcription independently of the functional state of mitochondria. However, when cells were grown on raffinose, *TSA1* induction after H_2O_2 treatment was considerably higher in respiratory-incompetent cells than in those where respiratory capacity was preserved (Fig. 3). This fact indicates that absence of functional mitochondria may generate an additional signal that triggers *TSA1* induction beyond the level achieved by the presence of H_2O_2 alone. This signal could be the H_2O_2 concentration itself.

3.4. Cellular generation of H_2O_2 in cells with dysfunctional mitochondria

It is well established that the generation of ROS by normal mitochondria is significantly enhanced when terminal steps of the respiratory chain are blocked [22]. Hence it was investigated whether respiratory-deficient cells could present increased H_2O_2 internal levels, which could contribute to the lower oxidative stress resistance (Fig. 2) and additional increase in *TSA1* transcription (Fig. 3) of these cells. Antimycin A treatment or *COX10* deletion led to higher H_2O_2 formation levels than their corresponding control or wild-type cells (Fig. 4) according to previous studies [22–26]. However, little is known about ROS generation in *rho*^{0} cells. These cells have normal levels of flavoproteins and ubiquinone ([24] and refer-

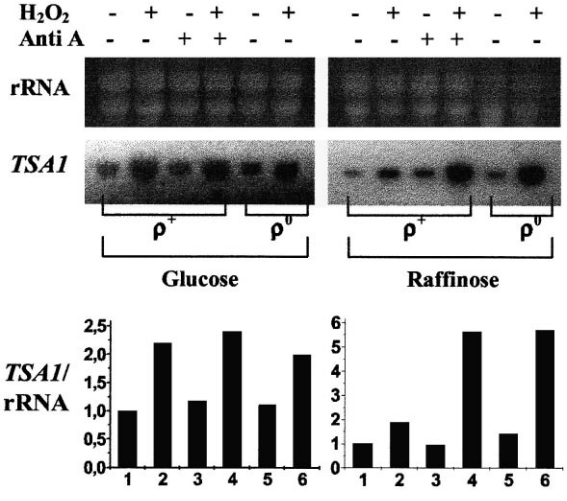

Fig. 3. Effect of H_2O_2 on *TSA1* expression in respiratory-competent and incompetent yeast cells. Northern blot analysis of RNA isolated from mid-logarithmic yeast strains JD7-7C (ρ^+) and its ρ^0 derivative, following treatment with 1 mM H_2O_2 alone or associated with 0.1 μg/ml antimycin A. All the treatments were performed during 1 h.

ences cited herein), which can donate electrons to oxygen leading to superoxide generation, but cannot donate electrons to cytochrome oxidase, which is dysfunctional in these cells due to the lack of subunits encoded by mitochondria [27]. In fact, *rho*^{0} cells generated more H_2O_2 than their corresponding *rho*$^+$ cells, although in an apparent slower rate, compared to antimycin A treatment (Fig. 4). These experiments were also performed in the *tsa1Δ* strain. Deficiency of cTPx I did not alter significantly the release of H_2O_2 (data not shown). This is in agreement with the results described in Fig. 2 showing that deletion of *TSA1* gene do not alter considerably the tolerance of cells for disruption of respiratory chain. Jiang et al. [28], have also detected more ROS generation in mammalian *rho*^{0} cells. The increased production of ROS was attributed to increased activity of enzymes such as nitric oxide synthase, xantine oxidase and lipoxygenases [28]. Regardless where or how ROS are produced in *rho*^{0} cells, they are really elevated in comparison to *rho*$^+$ cells. Therefore, cTPx I had an important protective role in cells with dysfunctional mitochondria where the levels of ROS are high.

4. Discussion

The antioxidant defense system of yeast comprises many different antioxidant enzymes, among other components. Besides cTPx I, yeast has other enzymes that decompose peroxides such as catalase A, catalase T, cytochrome *c* peroxidase and glutathione peroxidase (reviewed in [20]). It is not known what are the specific roles for these enzymes; each one may be responsible for the decomposition of peroxide at a particular compartment or at a particular situation. In order to obtain some clues about the physiological role of cTPx I in yeast, we have measured the transcription of *TSA1* gene and evaluated the effect of the absence of cTPx I in cell survival in different conditions.

Our results indicated that cTPx I has an important antioxidant role in *S. cerevisiae*. In fact, when cells were exposed to high H_2O_2 concentrations *TSA1* was transcriptionally activated (Fig. 1) and cells were slightly more sensitive to this oxidant (Fig. 2). All the procedures used in this study to disrupt electron transport chain led to small augments in ROS production (Fig. 4), which were not accompanied by an increase in cell mortality (Fig. 2) and were independent of the presence of cTPx I (not shown). However, when the loss of mitochondrial function was associated with H_2O_2 treatment both the sensitivity of *tsa1Δ* cells (Fig. 2) and *TSA1* expression in wild-type strain (Fig. 3) were increased considerably. It appears that the augment in ROS production by disruption of mitochondrial electron transport can be coped by any of the several antioxidant enzymes present in yeast. However, when H_2O_2 concentration is further increased by external addition of this oxidant, the survival of respiratory-incompetent cells seems to be very dependent on cTPx I.

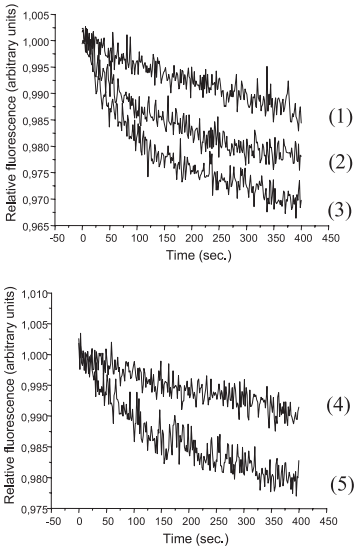

Fig. 4. H_2O_2 generated by *S. cerevisiae* cells by the HRP–scopoletin method. Fluorimetric measurement of scopoletin oxidation due to the H_2O_2 generated by cells of the strains JD7-7C (1 and 3), ρ^0JD7-7C (2), W303-1a (4) and *cox10*Δ (5). Antimycin A at 0,1 μg/ml was added only in (3) (Section 2.5).

Respiratory-deficient cells present increased sensitivity to oxidative stress [21,29], but are still able to mount an inducible adaptive response to H_2O_2 [21]. The reason for this increased sensitivity is not clear, but Grant et al. [21] proposed that it could be due to a defect in energy-requiring processes, like detoxification of ROS or repair of oxidatively damaged molecules. Alternatively, it could be related to the higher levels of H_2O_2 generated by *rho*^{0} cells or by *rho*$^+$ cells treated with antimycin A (Fig. 4). Another suggestion to explain this phenomenon could involve mitochondrial functions related to the metabolism of various compounds with signaling properties, such as heme [30], Ca^{2+} [31] or even ROS [2,3,32]. The loss of function of this organelle could alter the concentrations of these species which could lead to alterations in the expression profile of antioxidant genes, electing cTPx I as a major enzyme to counteract the oxidizing environment.

Recently, two genome-wide studies of cell responses to mitochondrial dysfunction were published, one performed on glucose medium [33] and other on raffinose [34]. Alterations in the expression of genes involved in metabolic remodeling were found but no significant differences in the expression of antioxidant genes were observed. However, these studies were performed without any exposition of cells to an oxidant, hence the oxidative stress response of respiratory-deficient cells could not be evaluated appropriately. We are currently investigating this response in a genome-wide scale.

Mitochondrial defects occur in a wide variety of human degenerative diseases, aging and cancer [21–23]. Understanding how cells with these defects respond to environmental changes or stress situations should provide significant insight to improve treatment of these disorders, aiming replacement of defective functions in the case of preserving cell life (diseases), or even taking advantages of these defects to selectively destroy the cells (cancer). Our results indicate that peroxiredoxins, specially those with high similarity to yeast cTPx I, could be important targets for these studies.

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