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Role of Thioredoxin Peroxidase in Aging of Stationary Cultures of *Saccharomyces cerevisiae*

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A soluble protein from *Saccharomyces cerevisiae* acts as a peroxidase but requires a NADPH-dependent thioredoxin system and was named thioredoxin peroxidase (TPx). The role of TPx in aging of stationary cultures of *S. cerevisiae* was investigated in a wild-type strain and a mutant yeast strain in which the *tsa* gene that encodes TPx was disrupted by homologous recombination. The occurrence of oxidative stress during aging of stationary cultures of the yeast has been proposed. Comparison of 5-day-old (young) stationary cultures of *S. cerevisiae* and of cultures aged for 3 months (old) revealed decreased viability, increased generation of reactive oxygen species, modulation of cellular redox status, and increased cellular oxidative damage reflected by increased protein carbonyl content and lipid peroxidation. The magnitude of this stress was augmented in yeast mutant lacking TPx. These results suggest that TPx may play a direct role in cellular defense against aging of stationary cultures presumably, functioning as an antioxidant enzyme.

Keywords: Yeast; Thioredoxin peroxidase; Stationary cultures; Aging; Reactive oxygen species

INTRODUCTION

Reactive oxygen species such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (·OH) are generated *in vivo* from the incomplete reaction of oxygen during aerobic metabolism or from exposure to environmental agents such as radiation, redox cycling agents, or stimulated host phagocytes.[1–3] These oxygen species can cause widespread damage to biological macromolecules leading to lipid peroxidation, protein oxidation and DNA base modifications and strand breaks.[4] There is growing evidence that ROS are directly or indirectly involved in aging.[5]

The yeast *Sacchromyces cerevisiae* is a highly studied unicellular eucaryotes with some remarkable similarities to human cells at the macromolecular and organelle level.[6] When yeast culture enters the stationary phase, cell division stops and cellular metabolism is slowed down. Cells are able to survive in this phase for weeks or months without nutrient supplementation.[7] Oxidative stress is one factor which limits survival in stationary phase since mutants lacking antioxidant enzymes were susceptible to stationary phase death. Therefore, stationary cultures of yeast are particularly useful model for studies of the free radical theory of aging.[8] It has been suggested that the survival of cells in stationary phase yeast cultures may be a model for aging in mammals, particularly for tissues composed of non-dividing cell populations.[6]

Biological systems have evolved an effective and complicated network of defense mechanisms which enable cells to cope with lethal oxidative environments. These defense mechanisms involve antioxidant enzymes, such as superoxide dismutases (SOD), which catalyze the dismutation of O_2^- to H_2O_2 and O_2,[9] catalase and peroxidases which remove hydrogen peroxide and hydroperoxides.[10] Peroxiredoxin forms a large family of newly discovered antioxidant enzymes that act as peroxidases, which reduce hydrogen peroxide and alkyl hydroperoxides to water or the corresponding

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alcohol, respectively.[11] A 25-kDa antioxidant protein from yeast is 2-Cys peroxiredoxin that is able to reduce H_2O_2 using electrons provided by thioredoxin. Thus, it was referred to as thioredoxin peroxidase (TPx).[12]

In the present study, the role of TPx in aging of stationary cultures of *S. cerevisiae* was investigated using the wild-type strain and the TPx-deficient mutant strain, in which the gene encoding TPx (*tsa*) was disrupted. Wild-type and *tsa* mutant cells were expected to exhibit differences with regard to cellular redox status and oxidative damage during aging of stationary cultures. Here we report that TPx may play a direct role in defense against aging of stationary cultures presumably, functioning as an antioxidant enzyme.

MATERIALS AND METHODS

Materials

Hydrogen peroxide, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), β-NADPH, 2,4-dinitophenylhydrazine (DNPH), pyrogallol, phenylmethylsulfonyl fluoride (PMSF) and xylenol orange were obtained from Sigma Chemical Co. (St. Louis, MO). 2′,7′-Dichlorofluoroscin diacetate (DCFH-DA) was purchased from Molecular Probes (Eugene, OR).

Yeast Strains and Culture Conditions

The wild-type *S. cerevisiae* strain JD7-7C (*mat*α, *ura 3-52*, *leu2*, *trpA*, K^+) and the *tsa* mutant strain, which was created by the integrative disruption method as described,[13] were kindly provided by Dr I.H. Kim (Pai-Chai University, Taejon, Korea). The yeast were grown in liquid YPG medium containing 1% yeast extract, 1% bactopeptone and 2% dextrose at 28°C.

Stationary Cultures

Yeast cultures obtained by growing cells from inoculum for 5 days were assumed as "young" stationary cultures. "Old" stationary cultures were obtained by incubating stationary cultures for 3 months at 28°C.[14] During this time, the cultures were supplied with fresh complete medium two times a week to prevent carbon source depletion. Each time the cells were allowed to sediment and 80 ml of the supernatant of a 110 ml culture was withdrawn and replaced by 80 ml of fresh medium. Cell density did not change significantly during the incubation period.

Cell Viability

Cell viability was estimated using LIVE/DEAD Yeast Viability Kit (Molecular Probes, Eugene, OR). Only metabolically active cells are marked with red fluorescent intravacuolar structures, while dead cells exhibit extremely bright, diffuse and green–yellow fluorescence. The viability of individual yeast cells was assessed by fluorescence microscopy and the relative metabolic activity of yeast cell suspensions was measured in a spectrofluorometer.

Antioxidant Enzyme Assays

The yeast cells were harvested by centrifugation at 3000*g* and resuspended in 600 μl of lysis buffer (20 mM HEPES/1 mM EDTA/2 mM PMSF). To disrupt cells, 1/2 the vol of ice-chilled 0.5 mm-diameter glass beads were added and the microfuge tubes were vortexed for five 2-min intervals interspersed with periods of cooling in an ice bath.[15] Cellular debris was removed by a 10-min centrifugation at 15,000*g*. The supernatant was collected and protein levels were determined by the method of Bradford[16] using reagents purchased from Bio-Rad. Catalase activity was measured in terms of the decomposition of hydrogen peroxide, which was followed directly by the decrease in absorbance at 240 nm.[17] SOD activity in cell extracts was assayed spectrophotometrically using the pyrogallol assay,[18] where one unit of activity is defined as the quantity of enzyme that reduces the superoxide-dependent color change by 50%.

Measurement of *In Vivo* Molecular Oxidation

Intracellular peroxide production was measured using the oxidant-sensitive probe DCFH-DA. Fluorescence was measured using a Shimadzu RF5301 PC spectrofluorophotometer set at an excitation wavelength of 504 nm and an emission wavelength of 524 nm.[19] A 5-mM stock solution of DCFH-DA dissolved in ethanol (20 μl) was added to each culture 30 min before the assay and allowed to incubate at 30°C. After incubation, the cells were placed on ice. They were washed twice in ice-cold distilled water, resuspended in 300 μl of water, and vortexed with glass beads. The supernatant was obtained after centrifugation in a microcentrifuge for 10 min and crude extract (500 μg protein) was suspended in distilled water, and fluorescence was recorded. The 2′,7′-dichlorofluorescein (DCF) fluorescence of individual yeast cells were observed with confocal microscopy. Hydrogen peroxide oxidizes ferrous (Fe^{2+}) to ferric ion (Fe^{3+}) selectively in dilute acid and the resulting ferric ions can be determined using a ferric sensitive dye, xylenol

orange, as an indirect measure of intracellular hydrogen peroxide concentration. Cell-free extracts were added FOX solution (0.1 mM xylenol orange, 0.25 mM ammonium ferrous sulfate, 100 mM sorbitol and 25 mM H_2SO_4) and incubated in a room temperature for 30 min, and absorbance was measured at 560 nm. Hydrogen peroxide was used to draw standard curve as described.[20]

NADPH and GSH Levels

NADPH was measured using the enzymatic cycling method as described by Zerez *et al.*[21] and expressed as the ratio of NADPH to the total NADP pool. The concentration of total glutathione was determined by the rate of formation of 5-thio-2-nitrobenzoic acid at 412 nm ($\varepsilon = 1.36 \times 10^4\,M^{-1}\,cm^{-1}$) as described by Akerboom and Sies,[22] and GSSG was measured by the DTNB-GSSG reductase recycling assay after treating GSH with 2-vinylpyridine.[23] Total GSH level was measured in 0.1 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 0.2 mg NADPH, 30 μg DTNB and 0.12 unit glutathione reductase. GSSG level was measured by the same method as the total GSH level but after treatment of 1 μl of 2-vinylpyridine and 3 μl of triethanolamine for 1 h.

Protein Carbonyl Content

The protein carbonyl content was determined spectrophotometrically using the DNPH-labeling procedure as described.[24] The crude extract (~1 mg protein) was incubated with 0.4 ml 0.2% DNPH in 2 M HCl for 1 h at 37°C. The protein hydrazone derivatives were sequentially extracted with 10% (w/v) trichloroacetic acid, treated with ethanol/ethyl acetate, 1:1 (v/v), and reextracted with 10% trichloroacetic acid. The resulting precipitate was dissolved in 6 M guanidine hydrochloride, and the difference spectrum of the sample treated with DNPH in HCl was examined versus a sample treated with HCl alone. Results are expressed as nmol of DNPH incorporated per mg of protein calculated from an absorbability of $21.0\,mM^{-1}\,cm^{-1}$ at 360 nm for aliphatic hydrazones.

Lipid Peroxidation

Thiobarbituric acid-reactive substances (TBARS) were determined as an independent measurement of lipid peroxidation. Samples were evaluated for malondialdehyde (MDA) production using a spectrophotometric assay for TBARS.[25] The extinction coefficient at 532 nm of $153{,}000\,M^{-1}\,cm^{-1}$ for the chromophore was used to calculate the MDA-like TBARS produced.

Replicates

Unless otherwise indicated, each result described in this paper is representative of at least three separate experiments.

RESULTS

As shown in Fig. 1, dead cells exhibit a diffuse yellow–green fluorescence while the metabolically active yeast cells that contain cylindrical, red-fluorescent structure in their vacuoles. The population of dead cells was higher in the old stationary culture of TPx-deficient yeast strain compared to young TPx-deficient or old wild-type strain. The strain exhibiting no TPx activity lost viability more rapidly that the wild-type, indicating that TPx is involved in protecting cells against oxidative stress induced by aging of stationary cultures.

To investigate whether the difference in viability in young and old stationary cultures of wild-type and TPx-deficient cells observed is associated with oxidant formation, the levels of *in vivo* intracellular oxidation in stationary cultures were measured using the oxidant sensitive probe DCFH-DA. Deacylation by esterases to dichlorofluoroscin occurs within the cells, and the non-fluorescent dichlorofluoroscin is subsequently oxidized in the presence of intracellular hydroperoxides and peroxides to the highly fluorescent DCF, which can be evaluated by confocal microscopy or spectrofluorometry.[19] As shown in Fig. 2, DCF fluorescence intensity was significantly increased in old stationary cultures. The increase in DCF fluorescence was augmented in TPx-deficient strain. We also demonstrated the level of intracellular H_2O_2 in stationary

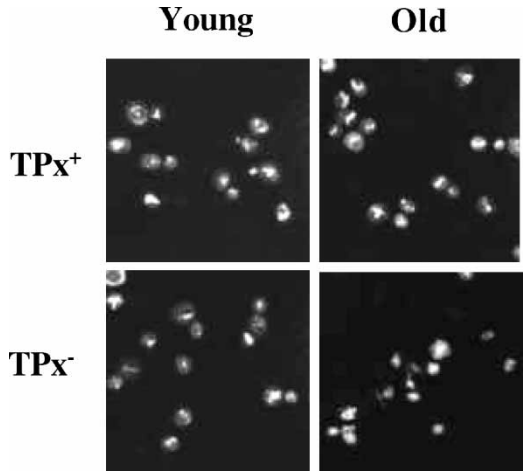

FIGURE 1 Yeast viability assay using LIVE/DEAD Kit. The yeast cells were incubated with cell stain and fluorescence images were obtained under microscopy. Dead cells that exhibit a diffuse yellow-green fluorescence while live cells that contain cylindrical, red fluorescent structure in their vacuole. TPx^+, wild-type; TPx^-, TPx-deficient mutant.

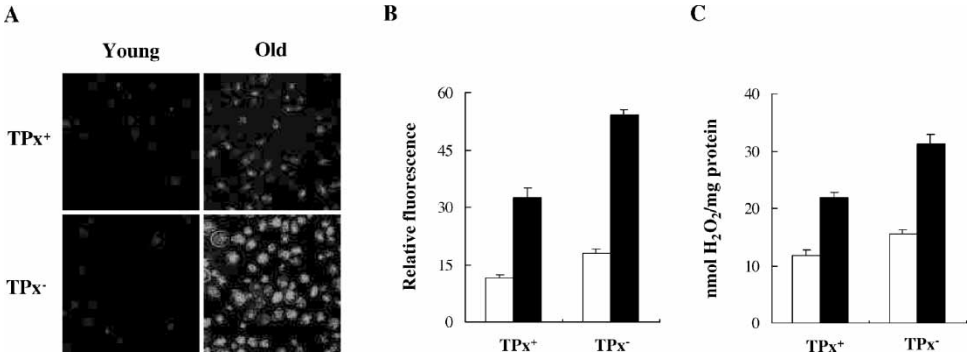

FIGURE 2 (A) Measurement of *in vivo* molecular oxidation by DCF fluorescence. The pseudocolor images of DCF fluorescence by ROS were analyzed with the confocal microscopy. (B) DCF fluorescence was measured in cell-free extract from young and old stationary cultures of yeast strains. Fluorescence was recorded at an excitation wavelength of 504 nm and an emission wavelength of 524 nm. Open and shaded bars represent young and old stationary cultures, respectively. Each value represents the mean ± SD from four independent experiments. (C) Intracellular hydrogen peroxide level of young and old stationary cultures of yeast strains. The formation of hydrogen peroxide was determined with xylenol orange. Open and shaded bars represent young and old stationary cultures, respectively. Each value represents the mean ± SD from five independent experiments.

cultures of yeast strains. The intracellular H_2O_2 level was significantly increased during aging of stationary cultures and such increase was more pronounced in TPx-deficient strain.

We compared redox status in wild-type yeast and yeast strain deficient TPx during prolonged incubation of stationary cultures. One important parameter of GSH metabolism is the ratio GSSG/GSSG + GSH (GSHt), which may reflect the efficiency of GSH turnover. Although the change in the level of GSHt was not pronounced, the ratio of [GSSG]/[GSHt] was significantly higher in old stationary cultures. The magnitude of this increase was augmented in TPx-deficient strain (Fig. 3A). This data indicate that GSSG in old stationary cultures of wild-type and TPx-deficient strains was not reduced as efficiently as in young stationary cultures of yeast strains. This result suggests that decrease in the efficiency of GSH recycling may be responsible for the higher concentration of intracellular peroxides. NADPH, required for GSH generation by glutathione reductase, is an essential factor for the cellular defense against oxidative damage. Although the total NADP pool did not change significantly, the ratio for [NADPH]/[$NADP^+$ + NADPH] was decreased during aging of stationary cultures, however, the decrease in this ratio was much more pronounced in TPx-deficient strain (Fig. 3B).

Because antioxidant systems work as a team, the question of whether the aging and the lack of cellular TPx activity induced concomitant alterations in the detoxifying systems against ROS was investigated. As shown in Fig. 4, SOD activity decreased considerably during aging of stationary cultures of both wild-type and TPx-deficient strains. In contrast, catalase activity was increased in aged stationary cultures of both wild-type and TPx-deficient strains.

We performed carbonyl content measurements for protein oxidation in stationary cultures of yeast whether to estimate aging and TPx expression affected sensitivity to protein damage (Fig. 5). Oxidative stress is known to introduce carbonyl groups into the amino acid side chains of proteins.[24]

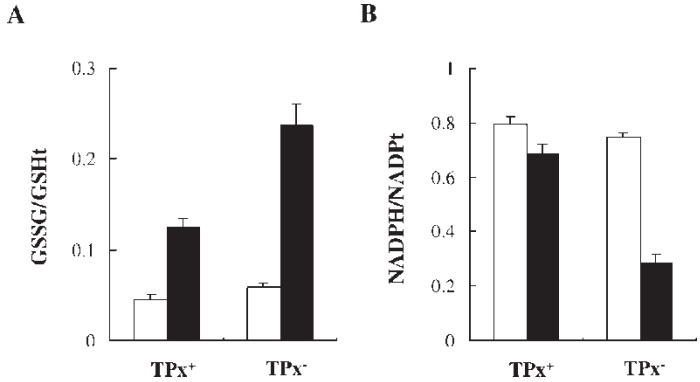

FIGURE 3 The cellular redox status of young and old stationary cultures of yeast strains. (A) Ratios of GSSG versus total GSH pool. Open and shaded bars represent young and old stationary cultures, respectively. Each value represents the mean ± SD from five independent experiments. (B) Ratios of NADPH versus NADP pool. Open and shaded bars represent young and old stationary cultures, respectively. Each value represents the mean ± SD from five independent experiments.

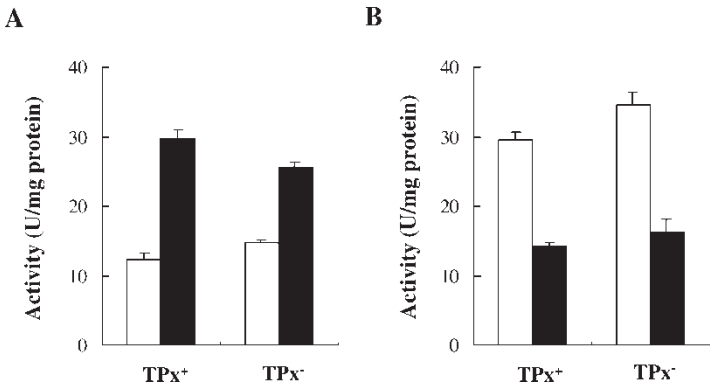

FIGURE 4 Activities of catalase (A) and SOD (B) in young and old stationary cultures of yeast strains. Open and shaded bars represent young and old stationary cultures, respectively. Each value represents the mean ± SD from five independent experiments.

The level of protein carbonyl group was increased during aging of stationary cultures. The increase was higher in the TPx-deficient strain that in the wild-type strain. As an indicative marker of oxidative damage to cells, the occurrence of lipid peroxidation was estimated by measuring the formation of TBARS. A higher increase of TBARS was seen in aged stationary cultures and the magnitude of this increase was augmented in stationary cultures of TPx-deficient strain.

DISCUSSION

As in higher eucaryotes, the unicellular yeast *S. cerevisiae* undergoes an age-dependent increase in cell dysfunction and mortality rates. Yeast cells have been used as a model system for studying two different aspects of life-span: (1) replicative life-span, measured as the number of buds generated by each mother cells; and (2) chronological life-span, measured as the ability of stationary phase cultures (non-dividing) to maintain viability over time.[8] It has been proposed that stationary phase yeast cells are a good model system for aging of somatic cells of higher eukaryotic organisms, as both are postmitotic cells.[26] In recent years a variety of experimental data has been suggesting a key role of antioxidant defenses in the extension of the life-span in post-mitotic yeast cells.[8] For instance, the decreased life-span was observed in mutant yeast deficient in CuZnSOD or MnSOD.[26]

Since TPx exhibits its antioxidant activity only in the thiol-containing metal-catalyzed oxidation system, the function of TPx was initially suggested as a sulfur radical scavenger.[27] However, in the presence of thiol, TPx acts as an antioxidant protein in an ascorbate containing oxidation system.[28] This result suggested that TPx may require reduced thiol as a reducing equivalent to remove reactive oxygen species. It has been reported that a thiol group of cysteine in TPx is involved in catalysis[13] and chemical modification with thiol-specific reagents such as N-ethylmaleimide inhibits the antioxidant activity of TPx,[29] presumably through the modification of Cys47-SH, which is the primary site of TPx

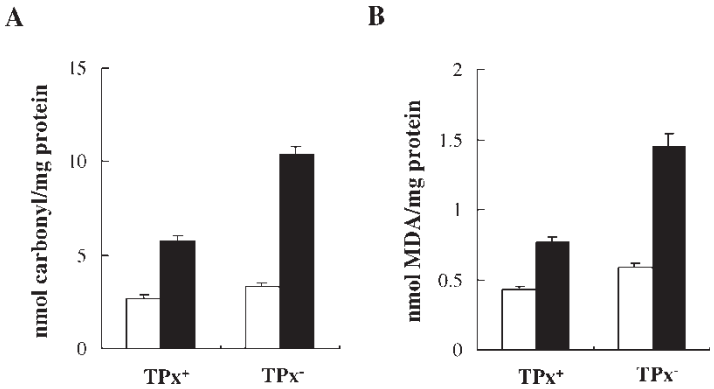

FIGURE 5 (A) Protein carbonyl content of young and old stationary cultures of yeast strains. Protein carbonyls were measured in cell-free extracts by the method of Levine *et al.*[24] with the use of DNPH. Open and shaded bars represent young and old stationary cultures, respectively. Each value represents the mean ± SD from five independent experiments. (B) Lipid peroxidation of young and old stationary cultures of yeast strains. The level of MDA accumulated in the stationary cultures was determined using a TBA assay. Open and shaded bars represent young and old stationary cultures, respectively. Each value represents the mean ± SD from five independent experiments.

catalysis. The antioxidant activity of TPx is restored by a cellular reducing catalyst such as a NADPH-dependent thioredoxin system through a redox control mechanism. The key function of this protein is the removal of hydrogen peroxide, acting as a peroxidase.[11,12] It has been shown that the *tsa* mutant strain was more sensitive than the wild-type strain to oxidative stress induced by *tert*-butyl-hydroperoxide, hydrogen peroxide, cumene hydroperoxide, and a redox-cycling agent, menadione, strongly suggesting an antioxidant role for TPx.[13,15] A database search revealed a number of proteins from a variety of organisms that are similar to TPx. These homologous proteins were named the peroxiredoxin family.[11,30] This protein family is widespread in nature and their high degree of conservation suggests a biological importance of this type of enzyme. In addition to TPx, this family includes, the AhpC subunit of *Salmonella typhimurium* alkyl hydroperoxide reductase,[31] a mitochondrial protein induced during the differentiation of Friend erythroleukemia cells[32] which is also known as SP22,[33] two proteins showing a natural killer cell-enhancing action *in vitro* and named NKEF-A and B,[34,35] DirA, a major iron-repressible polypeptide of *Corynebacterium diphtheriae*,[36] and tryparedoxin peroxidase of the *Crithidia fasciculate* peroxidase system.[37] Although there is ample room for functional diversification within the peroxiredoxin family, the known major functions of peroxiredoxins are mediated by their antioxidant role. In order to elucidate the functions of these proteins, the continued characterization of the individual peroxiredoxin family members is required.

Our results indicate that the increased production of ROS, concomitant with a modulation of redox status and the accumulation of oxidized proteins and lipids seems to be key factors in aging of stationary cultures of yeast. The occurrence of oxidative stress and accumulation of oxidative damage during long-term incubation of stationary cultures of yeast were augmented in mutant deficient in TPx. Despite their role in the cellular defense mechanism, the antioxidant enzymes are susceptible to inactivation by ROS. Previous studies have demonstrated that oxidative processes result in the loss of key antioxidant enzymes,[38,39] which may exacerbate oxidative stress-mediated cytotoxicity. However, it is also possible that prokaryotes and eucaryotes compensate for inactivation of antioxidant enzymes by an enhanced expression. Induction of antioxidant proteins in response to oxidative stress in *Escherichia coli*, *S. typhimurium*,[40,41] yeast[42] and mammalian cells[43] is well known. Our results show that SOD is inactivated during aging of stationary cultures of yeast. SOD is known to be susceptible to inactivation by exposure to hydrogen peroxide. The increase in catalase activity may represent a stress response. It is well documented that this enzyme is easily inducible in yeast by various types of stress,[42,44] possibly in a compensatory mechanism against elevated ROS.

In conclusion, results of this study indicate that a deficiency of TPx increased sensitivity of yeast cells to oxidative stress induced during aging of stationary cultures. Results provide support for the role of TPx as an important antioxidant enzyme to protect cells against aging of stationary cultures of yeast.

Acknowledgements

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