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Superoxide Dismutase 1-mediated Production of Ethanol- and DNA-derived Radicals in Yeasts Challenged with Hydrogen Peroxide

*MOLECULAR INSIGHTS INTO THE GENOME INSTABILITY OF PEROXIREDOXIN-NULL STRAINS**

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Peroxiredoxins are receiving increasing attention as defenders against oxidative damage and sensors of hydrogen peroxide-mediated signaling events. In the yeast *Saccharomyces cerevisiae*, deletion of one or more isoforms of the peroxiredoxins is not lethal but compromises genome stability by mechanisms that remain under scrutiny. Here, we show that cytosolic peroxiredoxin-null cells (*tsa1*Δ*tsa2*Δ) are more resistant to hydrogen peroxide than wild-type (WT) cells and consume it faster under fermentative conditions. Also, *tsa1*Δ*tsa2*Δ cells produced higher yields of the 1-hydroxyethyl radical from oxidation of the glucose metabolite ethanol, as proved by spin-trapping experiments. A major role for Fenton chemistry in radical formation was excluded by comparing WT and *tsa1*Δ*tsa2*Δ cells with respect to their levels of total and chelatable metal ions and of radical produced in the presence of chelators. The main route for 1-hydroxyethyl radical formation was ascribed to the peroxidase activity of Cu,Zn-superoxide dismutase (Sod1), whose expression and activity increased ~5- and 2-fold, respectively, in *tsa1*Δ*tsa2*Δ compared with WT cells. Accordingly, overexpression of human Sod1 in WT yeasts led to increased 1-hydroxyethyl radical production. Relevantly, *tsa1*Δ*tsa2*Δ cells challenged with hydrogen peroxide contained higher levels of DNA-derived radicals and adducts as monitored by immuno-spin trapping and incorporation of ^{14}C from glucose into DNA, respectively. The results indicate that part of hydrogen peroxide consumption by *tsa1*Δ*tsa2*Δ cells is mediated by induced Sod1, which oxidizes ethanol to the 1-hydroxyethyl radical, which, in turn, leads to increased DNA damage. Overall, our studies provide a pathway to account for the hypermutability of peroxiredoxin-null strains.

Living organisms are constantly exposed to oxygen- and nitrogen-derived reactive species that are produced by normal metabolic activity and in response to external stimuli. To protect themselves against the toxicity of these species, aerobic organisms have evolved a range of defense mechanisms (1). Among these, a family of cysteine-based peroxidases, currently named peroxiredoxins, has attracted considerable attention because of its ubiquity and versatility. These enzymes have been shown to detoxify hydrogen peroxide, organic peroxides and peroxynitrite through oxidation of their reactive cysteine residues, which are recycled back by reducing equivalents provided by thioredoxin and other thiol-electron donors (reviewed in Refs. 2–5). In addition to detoxifying peroxides, specific peroxiredoxins have been shown to act as molecular chaperones (6, 7) and to play roles in regulating hydrogen peroxide-mediated cell signaling events (3, 8, 9).

Many organisms have multiple peroxiredoxins. Six peroxiredoxins have been identified in human cells, and five in the yeast *Saccharomyces cerevisiae*. Peroxiredoxin isoforms are distributed to different locations within the cell, and two of the yeast peroxiredoxins, Tsa1 and Tsa2, are cytosolic (10). Tsa1 was the first peroxiredoxin identified in eukaryotes. It is expressed constitutively and corresponds to 0.7% of total soluble protein content in this species. In contrast, the levels of Tsa2 are very low under normal conditions, but are highly induced upon treatment of the yeast with peroxides (11, 12). Tsa1 and Tsa2 share 86% identity in their amino acid sequence, and both react with hydrogen peroxide with second order rate constants similar to those of hemoproteins, such as catalase ($k \sim 10^7\ M^{-1}\ s^{-1}$) (13). Deletion of the *TSA1* gene increases the expression of Tsa2 and other peroxiredoxin isoforms (10, 14, 15), suggesting that these peroxidases have both redundant and non-redundant physiological functions.

The deletion of one or all of the peroxiredoxin genes in *S. cerevisiae* is not lethal, probably because of the resulting increase in the levels of other antioxidant enzymes, such as catalase, cytochrome *c* peroxidase, and Sod1, that has been demonstrated by transcriptional and proteomic analysis (10, 15, 16). A problem arising from this compensatory response, however, is the mutator phenotype of the peroxiredoxin-null strains that display increased spontaneous mutation rates and accumulated gross chromosomal rearrangements (16–19). These consequences are particularly conspicuous in *TSA1*-null cells, suggesting a major contribution of Tsa1 to genome stability. Such protection has been attributed to the ability of Tsa1 to reduce the levels of reactive oxygen species that would other-

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wise oxidize DNA leading to mutations, chromosomal rearrangements and cell death. These genetic and lethality studies, however, did not provide mechanistic insights on how deletion of peroxiredoxin genes leads to DNA damage nor to its molecular nature (16–19).

Here, contrary to the common notion, we show that deletion of both *TSA1* and *TSA2* genes in *S. cerevisiae* increases resistance to toxic concentrations of hydrogen peroxide. In parallel, hydrogen peroxide consumption and ethanol- and DNA-derived radical formation are increased mainly through higher expression of the enzyme Sod1.

EXPERIMENTAL PROCEDURES

Materials—All chemicals were purchased from Sigma-Aldrich, Merck, or Fisher and were analytical grade or better. Yeast nitrogen base (20) was from Difco and media supplements (amino acids, adenine and uracil) were from Sigma-Aldrich or Synth. Desferrioxamine was purchased from Novartis. Bathocuproine disulfonic acid and [2-^{13}C]ethanol were from Sigma-Aldrich. Hydrogen peroxide concentration was determined spectrophotometrically at 240 nm ($\epsilon_{240} = 43.6\ \text{M}^{-1}\ \text{cm}^{-1}$) (21). All solutions were prepared with water purified in a Millipore Milli-Q system. All buffers were treated with Chelex-100 to remove trace amounts of metal ion contaminants prior to use.

S. Cerevisiae Strains and Growth Conditions—The wild-type strain employed was BY4741 (MATα; *his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*), whereas the mutant strains were: *tsa1*Δ (MATα; *his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YML028W::KAN MX4*), *tsa2*Δ (MATα; *his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YDR453C::KAN MX4*), and *tsa1*Δ *tsa2*Δ (MATα; *his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; tsa1::KAN MX4; tsa2::LEU2*). The first three strains were obtained from EUROSCARF, whereas the double mutant (generated according to Ref. 14) was kindly donated by Dr. Jin (University of Hong Kong, China University). Yeasts overexpressing Sod1 were obtained by standard transformation (22) of the BY47411 strain with the YEp600 plasmid containing one copy of the human *sod1* gene with its own promoter. This plasmid was extracted from EGy118 yeasts provided by Dr. Edith B. Gralla. The strains were grown in complete synthetic media (yeast nitrogen base) supplemented with 2% glucose, amino acids, adenine, and uracil under shaking at 300 rpm, 30 °C (11). For all experiments, strains were grown overnight, diluted to $A_{600\ \text{nm}} = 0.2$, and collected as soon as they reached mid-log phase ($A_{600\ \text{nm}} = 0.8$). Growth curves under fermentative conditions were obtained for the four strains by diluting overnight cultures to $A_{600\ \text{nm}} = 0.2$ and by monitoring the optical density at 2-h intervals.

Viability Assay—The viability of the cells was monitored by the number of colony forming units. Cell cultures at mid-log phase of growth were washed with 0.1 M phosphate buffer containing 0.1 mM DTPA,[3] pH 7.4, and resuspended in growth media to a density of 5×10^7 cells/ml. Half of the cell suspension was treated with 1 mM hydrogen peroxide under shaking at 350 rpm for 30 min at 30 °C; the other half was treated with the same volume of buffer and incubated as above. The cell suspensions were then serially diluted, and 100-μl aliquots of $1/10^4$ and $1/10^5$ dilution were plated onto complete synthetic media supplemented with 2% glucose and 2% agar. The plates were incubated at 30 °C for 2 days, and the colony forming unit was number counted. The results shown are expressed as the percentage of colony forming units of hydrogen peroxide-treated cultures in relation to the corresponding controls.

Hydrogen Peroxide Consumption—Hydrogen peroxide consumption was determined by the ferrous oxidation xylenol orange assay (FOX) (23). Cells at mid-log phase were treated with 1 mM hydrogen peroxide as above. At the specified times, 50-μl aliquots were diluted with 500 μl of growth media and mixed with 950 μl of FOX solution (100 μM xylenol orange, 250 μM ammonium ferrous sulfate, 100 mM sorbitol, and 25 mM sulfuric acid). After 30-min incubation at room temperature, the absorbance of the samples was read at 560 nm. Calibration was performed with a standard solution of hydrogen peroxide.

Detection of Ethanol-derived Radicals—Production of the 1-hydroxyethyl radical formed from the oxidation of ethanol, a glucose metabolite, was detected by EPR spin-trapping experiments with POBN (24, 25). Cells (5×10^7 cells/ml) in growth media containing glucose were preincubated with 90 mM POBN for 5 min and 1 mM hydrogen peroxide was added under shaking at 30 °C. After 30 min, aliquots were transferred to flat cells, and the EPR spectra were scanned. In some experiments, cells were incubated with POBN as above, and 171 mM [2-^{13}C]ethanol was added together with hydrogen peroxide. In other experiments, cells in growth media containing glucose were preincubated with 2 mM desferrioxamine, 2 mM bathocuproine disulfonic acid, or with 10 mM diethyldithiocarbamate for 30 min or 1 h before addition of the components specified above. Also, cells in media alone were heated at 90 °C for 20 min and brought to 30 °C before addition of glucose, POBN, and hydrogen peroxide. In the case of purified enzymes, 10 μM catalase, horseradish peroxidase, cytochrome *c*, or bovine Sod1 was incubated with 90 mM POBN, 1 mM hydrogen peroxide, 0.1 mM DTPA, and 171 mM ethanol in 100 mM phosphate buffer, pH 7.4, at 30 °C. After 30-min incubation, the samples were transferred to flat cells, and the EPR spectra were scanned. In the case of Sod1, kinetic experiments were also performed. EPR spectra were recorded at room temperature (25 ± 2 °C) on a Bruker EMX spectrometer equipped with an ER4122 SHQ 9807 high sensitivity cavity. Radical adduct quantification was performed by double integration of the EPR spectra and comparison with a standard solution of 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy. Computer simulation analyses of some spectra were performed by using a program written by Duling (26).

Chelatable Iron Ion Measurements—Chelatable iron levels in the strains were determined by low temperature EPR after chelation with desferrioxamine (27, 28). WT and *tsa1*Δ*tsa2*Δ cells were collected at mid-log growth phase. Approximately 10^9 cells of each strain were resuspended in 10 ml of fresh growth medium without glucose, but containing 2 mM desferrioxamine. After 30-min incubation at 30 °C, cells were collected and washed with 10 ml of cold Tris-HCl buffer (20 mM), pH 7.4. The cell pellet was resuspended in 400 μl of the same buffer con-

[3] The abbreviations used are: DTPA, diethylenetriamine-*N*,*N*,*N*,*N*-pentaacetic; FOX, ferrous oxidation xylenol orange assay; DMPO, 5,5-dimethylpyrroline-*N*-oxide; POBN, α-(4-pyridil-1-oxide)-*N*-*tert*-butylnitrone; Tsa1 and -2, cytosolic thioredoxin peroxidases 1 and 2; WT, wild type.

taining 10% glycerol, transferred to a 1-ml disposable syringe, and frozen in liquid nitrogen. The samples were extruded from the syringe into a finger-tip Dewar flask containing liquid nitrogen and examined by EPR at 77 K in the region of $g \sim 4.0$ (28). The concentration of the desferrioxamine-iron complex present in the cell suspensions was obtained by double integration of the EPR signal and comparison with a standard curve constructed with known concentrations of the iron(III)-desferrioxamine complex. This was prepared by mixing different concentrations of ferrous ammonium sulfate with 2 mM desferrioxamine; the complex concentration was determined spectrophotometrically ($\epsilon_{430} = 2865\ M^{-1}\ cm^{-1}$) (29).

Total Iron, Copper, and Zinc Ion Measurements—Levels of iron, copper, and zinc ions in each strain were determined by atomic absorption spectrometry. Approximately 10^9 cells from each strain were washed in metal-free water, resuspended in a solution of nitric acid, hydrogen peroxide, and water (2:1:3, v/v), and digested in a microwave oven. The metal contents of the digested samples were determined in an Analytikjena AG, AAS ZEEnit 60 instrument. Iron, copper, and zinc were detected at 324.8, 248.3, and 213.9 nm, respectively (30).

Total Peroxide Measurements—Levels of total peroxides in WT and *tsa1*Δ *tsa2*Δ strains were determined by the FOX assay (23) as adapted to yeasts (31). Cells (5×10^7 cells/ml) of each strain were washed and resuspended in growth media and treated or not with 1 mM hydrogen peroxide. After 30-min incubation, the cells were washed twice and resuspended in 100 μl of 50 mM phosphate buffer. An equal volume of glass beads (425–600 μm) was added, and the samples were vortexed (1 min) and ice-cooled. After addition of 900 μl of cool methanol containing 4 mM butylated hydroxytoluene, the samples were submitted to two cycles of vortexing (1 min) and ice cooling. Next, the samples were centrifuged (1000 × *g*, 10 min). The supernatants were collected (100 μl) and mixed with 900 μl of the FOX reagent (100 μM xylenol orange, 250 μM ammonium ferrous sulfate, 25 mM sulfuric acid, and 4 mM butylated hydroxytoluene in 90% (v/v) methanol). After 30-min incubation at room temperature, the absorbance of the samples was read at 560 nm. According to previous calibrations performed with standard peroxides, a mean apparent extinction coefficient of $4.5 \times 10^4\ M^{-1}\ cm^{-1}$ was employed (31).

Expression of Sod1—The level of Sod1 expression in WT and *tsa1*Δ *tsa2*Δ strains was determined by Western blot analysis. To this end, cell extracts of the strains were obtained as previously described (11). Briefly, cells collected at mid-log growth phase were washed and resuspended in 50 mM Hepes buffer containing 50 mM NaCl, 2 μg/ml leupeptin, and 1 μg/ml of pepstatin. Next, an equal volume of glass beads (425–600 μm) was added. After 2 cycles of vortexing (6 min) and ice-cooling (6 min), the samples were centrifuged (16,000 × *g*, 5 min). The supernatants were collected and centrifuged again (16,000 × *g*, 30 min) to remove precipitated material. The supernatants are referred to as cell extracts, and their protein contents were determined by the Bradford method with a Bio-Rad Kit. Cell extracts (40 μg of protein) were submitted to electrophoresis (12% acrylamide gel) and transferred onto a nitrocellulose membrane (Hybond-D Extra, Amersham Biosciences). After washing (3 times, 10 min) with TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl), the membrane was blocked with TBST (TBS plus 0.05% Tween 20) containing 5% nonfat milk, at room temperature for 90 min. The primary antibody was anti-human Sod1 (Calbiochem) prepared in TBST containing 0.1% nonfat milk (1:1.500 dilution). Excess antibody was removed by washing three times with TBST. The membrane was then incubated with the secondary antibody (anti-sheep IgG, peroxidase conjugated, Calbiochem) prepared in TBST containing 0.1% nonfat milk (1:7,000 dilution). After 1-h incubation, the membrane was washed three times with TBST and treated for 5 min with the solutions from the kit SuperSignal West Pico Chemiluminescent Substrate (Pierce) and exposed to an x-ray film. Relative quantification of the bands was performed by densitometry (ImageQuaNT V5.2, Molecular Dynamics).

Superoxide Dismutase Activity—Superoxide dismutase activity in WT and *tsa1*Δ *tsa2*Δ cell extracts was determined by native PAGE staining (32) and inhibition of cytochrome *c* reduction (33, 34). After electrophoresis of cell extracts (40 μg of protein) under non-reducing conditions, the gel was incubated in the dark for 20 min with a solution composed of 0.25 mg/ml nitro blue tetrazolium plus 0.1 mg/ml riboflavin. Then, a solution of 10 mg/ml *N,N,N′,N′*-tetramethylethylenediamine was added, and the gel was kept under shaking and light until bands became visible (32). Relative quantification of the bands was performed by densitometry (ImageQuaNT V5.2). The cytochrome *c* reduction assay was performed as previously described (33, 34). The incubations contained 100 μM xanthine and xanthine oxidase in amounts that cause an absorbance change of 0.025/min, 1 unit of catalase ,and cell extracts (0–50 μg protein) in 100 mM phosphate buffer, pH 7.4, 30 °C.

Bicarbonate-dependent Peroxidase Activity—This activity was evaluated in whole cells by EPR monitoring of the oxidation of the spin-trap 5,5-dimethylpyrroline-*N*-oxide (DMPO) to DMPO/•OH radical adduct was monitored (35, 36). Cells (5×10^7 cells/ml) of each strain were resuspended in 100 mM phosphate buffer containing 0.1 mM DTPA, pH 7.4, and incubated with 80 mM DMPO at room temperature for 5 min. After addition of 25 mM sodium bicarbonate and 1 mM hydrogen peroxide, the samples were incubated at 30 °C for 15 min and transferred to flat cells, and their EPR spectra were scanned at room temperature as described above. Quantification of radical adduct was performed by double integration of the EPR spectra and comparison with a standard solution of 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy.

Detection of DNA-derived Radicals—Formation of DNA-derived radicals was monitored by immuno-spin trapping employing DMPO and the antibody against oxidized DMPO adducts (37). Cells (5×10^7 cells/ml) of each strain were washed, resuspended in fresh media, and treated with 100 mM DMPO and 1 mM hydrogen peroxide at 30 °C. After 30-min incubation, the cells were washed with distilled water (2 times) and resuspended in 800 μl of a buffer to digest cell walls (0.9 M sorbitol, 0.1 M EDTA, 50 mM dithiothreitol, and 3.5 μg/ml zymolyase 20T) (38). After 2-h incubation at 37 °C, samples were centrifuged (1,000 × *g*, 10 min), and the spheroplasts obtained were resuspended in lysis buffer (1% SDS, 100 mM NaCl, 25 mM DTPA in 10 mM Tris-HCl, pH 8.0). From this point on, DNA extraction and transfer onto nitrocellulose

membrane followed the previously described protocol (37). DNA extracted from WT cells (100 ng) treated with 50 mM DMPO, 1 mM $CuCl_2$, and 20 μM hydrogen peroxide in phosphate buffer, pH 7.4, for 30 min at 30 °C was employed as a positive control. DNA radicals/DMPO nitrone adducts blotted onto the nitrocellulose membrane were detected with the anti-DMPO nitrone adduct antibody provided by Dr. Ronald P. Mason (37). First, the membrane was blocked for 1 h with phosphate-buffered saline buffer (0.725 M NaCl, 2.7 mM KCl, 80 mM Na_2HPO_4 and 14 mM KH_2PO_4) containing 3% nonfat milk. After washing for 10 min with phosphate-buffered saline supplemented with 0.05% nonfat milk and 0.1% Tween 20, the membrane was incubated with the anti-DMPO/adduct antibody for 1 h. The antibody was prepared in phosphate-buffered saline supplemented with 0.05% nonfat milk and 0.1% Tween 20 (1:10,000 dilution). Excess antibody was removed by three 10-min washes with the same buffer, and the membrane was incubated 1 h with the secondary antibody (anti-rabbit IgG, peroxidase-conjugated). After one phosphate-buffered saline wash, the membrane was incubated with the solutions from the kit SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology) for 5 min and exposed to an x-ray film. Relative quantification of the bands was performed by densitometry (ImageQuaNT V5.2).

Detection of DNA Adducts—To probe for the addition of glucose metabolites to yeast DNA, we monitored incorporation of ^{14}C at the DNA by the strains grown in media supplemented with [^{14}C]glucose (Schwarz/Mann; 1 mCi/ml and 230 mCi/mM). Cells (2×10^8) harvested at the exponential growth phase were resuspended in 500 μl of fresh media containing four times less glucose than usual and supplemented with 50 μCi of [^{14}C]glucose (39). These samples were treated with 1 mM hydrogen peroxide for 30 min at 30 °C, in the absence or presence of POBN (90 mM). DNA from the samples was extracted as above. DNA (100 ng) from each experimental condition was transferred to filter paper that was incubated with scintillation liquid. Radioactivity was measured in a liquid scintillation analyzer (166 TR, Packard).

RESULTS

tsa1Δtsa2Δ Cells Are More Resistant to Hydrogen Peroxide but Metabolize It Faster Producing Ethanol-derived Radicals—As previously reported (11, 14, 16), *tsa1Δ*, *tsa2Δ*, and *tsa1Δtsa2Δ* yeast cells were shown to be viable and to grow similarly to the WT strain in fermentative media (data not shown). However, surprisingly, *tsa1Δtsa2Δ* cells were significantly more resistant than WT cells to toxic concentrations of hydrogen peroxide (Fig. 1*A*). No significant differences were observed between the WT and *tsa1Δ* and *tsa2Δ* cells. The unexpected resistance of the double mutant cells to hydrogen peroxide led us to explore this phenomenon in more detail. First, the consumption of 1 mM hydrogen peroxide by the strains was compared. No significant differences were observed in the rates of hydrogen peroxide consumption by the WT and single mutants (data not shown). The *tsa1Δtsa2Δ* strain, however, consumed hydrogen peroxide considerably faster than the WT strain (Fig. 1*B*), suggesting an increased capacity to metabolize hydrogen peroxide (Fig. 1*A*).

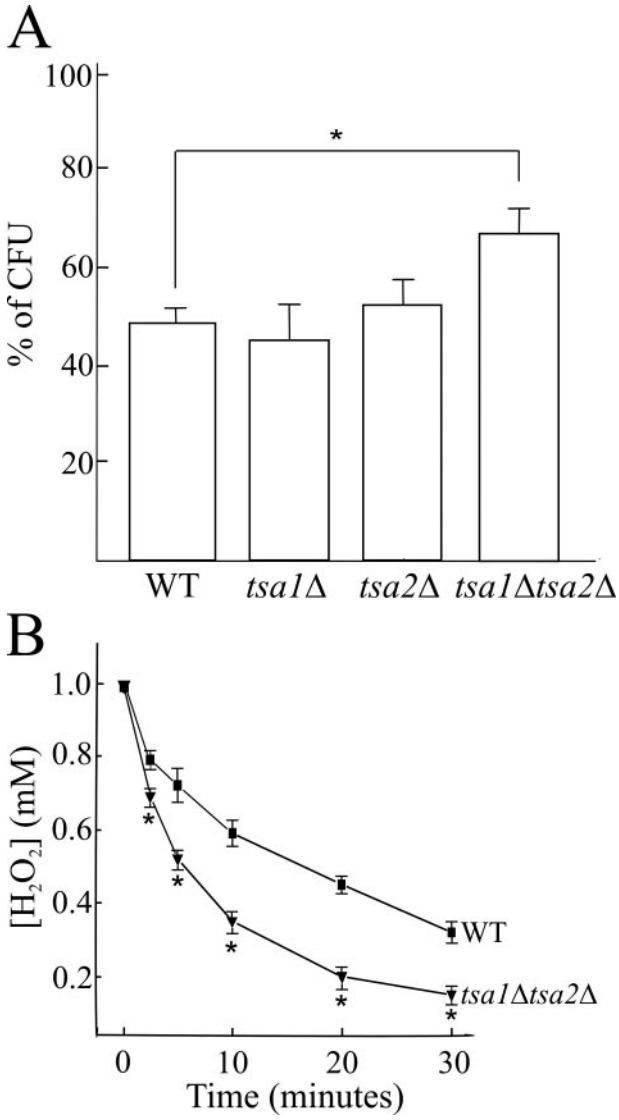

FIGURE 1. **Hydrogen peroxide resistance and consumption by WT, *tsa1Δ*, *tsa2Δ*, and *tsa1Δtsa2Δ* cells.** *A*, survival of the cells (5×10^7 cells/ml) after treatment with 1 mM hydrogen peroxide for 30 min at 30 °C. Treatment and survival monitoring were performed as described under "Experimental Procedures." *B*, rate of 1 mM hydrogen peroxide consumption by the specified yeast strains (5×10^7 cells/ml) monitored by the FOX assay as described under "Experimental Procedures." The values shown correspond to the mean ± standard deviations obtained from three different experiments; *, $p \leq 0.05$, Student's *t* test.

Hydrogen peroxide consumption by yeast cells may produce radical intermediates (25). Thus, radical production by the strains while metabolizing 1 mM hydrogen peroxide was compared by EPR spin-trapping experiments with POBN. After 30 min of incubation, radical production was marginal except for *tsa1Δtsa2Δ* cells that yielded a clear EPR signal whose parameters ($a_N = 15.8$ G; $a_H = 2.6$ G) are characteristic of the POBN/•1-hydroxyethyl radical adduct (POBN/•$CHOHCH_3$) (Fig. 2) (24, 25). To confirm radical identity, we performed parallel experiments where 171 mM [2-^{13}C]ethanol was added together with hydrogen peroxide to the incubations containing *tsa1Δtsa2Δ* cells, growth media, glucose, and POBN. In these cases, the 6-line spectrum detected in the presence of glucose alone was substituted by a 12-line spectrum (Fig. 2). Computer

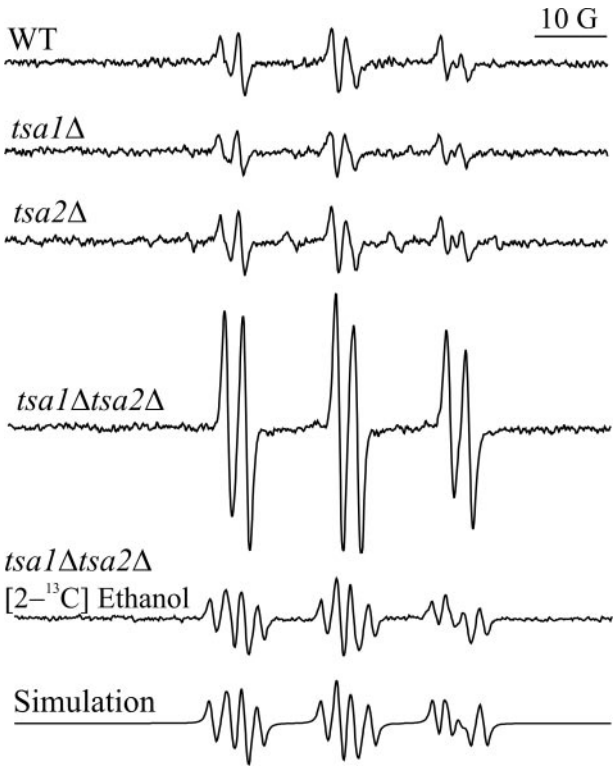

FIGURE 2. **Representative spectra of POBN radical adducts produced by WT, *tsa1*Δ, *tsa2*Δ and *tsa1*Δ*tsa2*Δ cells treated with 1 mM hydrogen peroxide.** Cells (5×10^7 cells/ml) in growth medium containing 2% glucose were treated with 90 mM POBN and 1 mM H_2O_2 for 30 min at 30 °C. Aliquots were transferred to flat cells, and the EPR spectra were scanned at room temperature. The fifth spectrum was obtained from incubations of *tsa1*Δ*tsa2*Δ cells under the same conditions except for the addition of 171 mM [2-^{13}C]ethanol together with hydrogen. The sixth spectrum is the computer simulation of the fifth considering the POBN/$^{\bullet}$CHOHCH$_3$ (a_N = 15.8 G; a_H = 2.6 G) and POBN/$^{\bullet}$^{13}CHOH^{13}CH$_3$ radical adduct (a_N = 15.5 G; a_H = 2.6 G; a_{13C} = 4.2 G) in yields of 30 and 70%, respectively. Instrumental conditions: microwave power, 20 milliwatts; modulation amplitude, 1 G; time constant, 82 ms; scan rate, 2.4 G s^{-1}; gain, 7.1×10^5; number of averaged scans, 4.

simulation showed that the latter is an EPR composite spectrum of the POBN/$^{\bullet}$CHOHCH$_3$ and POBN/$^{\bullet}$^{13}CHOH^{13}CH$_3$ radical adduct (a_N = 15.5 G; a_H = 2.6 G; a_{13C} = 4.2 G) (24) in relative yields of 30 and 70%, respectively (Fig. 2). Detection of the hyperfine splitting of ^{13}C from ethanol unambiguously proves that the main radical produced by *tsa1*Δ*tsa2*Δ cells challenged with hydrogen peroxide is the 1-hydroxyethyl radical.

The spin-trapping experiments were repeated several times, and the results were consistent. Considerable yields of the POBN/$^{\bullet}$1-hydroxyethyl radical adduct were obtained only in cultures of the *tsa1*Δ *tsa2*Δ strain and were dependent on all incubation components, that is, yeast cells, glucose, and hydrogen peroxide (Fig. 2). Thus, despite being more resistant to hydrogen peroxide, the *tsa1*Δ*tsa2*Δ strain removes it faster and produces the 1-hydroxyethyl radical.

Routes for 1-Hydroxyethyl Radical Formation—The source of the 1-hydroxyethyl radical in yeast cultures metabolizing glucose should be its metabolite ethanol. Indeed, fermentative yeasts accumulate ethanol, because glucose represses respiration and mitochondrial biogenesis through several signaling pathways (40). To produce the 1-hydroxyethyl radical, ethanol has to be oxidized by the hydroxyl radical or by a hydroxyl radical-like oxidant (Reaction 1), whose production is likely to depend on Fenton chemistry, that is, on hydrogen peroxide decomposition by redox-active transition metal ions (Reaction 2) (24, 41, 42).

$$CH_3CH_2OH + HO^{\bullet} \rightarrow CH_3C^{\bullet}HOH + H_2O$$

REACTION 1

$$H_2O_2 + Me^{n+} \rightarrow HO^{\bullet} + H_2O + Me^{(n+1)+}$$

REACTION 2

Although intracellular levels of transition metal ions are tightly controlled, regulatory mechanisms can be altered by stressed conditions such as those likely to result from deletion of genes encoding antioxidant enzymes, including Tsa1 and Tsa2. Redox-active iron ions in cells become more accessible to chelators, and their levels can be estimated by complexation with desferrioxamine and EPR analysis (see "Experimental Procedures") (27, 28). The levels of the iron(III)-desferrioxamine complex, however, were higher in WT than in *tsa1*Δ*tsa2*Δ cells (1.00 ± 0.05 and 0.28 ± 0.03 μg/g of cell pellet, respectively) (Fig. 3, *inset*, and Table 1). Relevantly, parallel experiments showed that preincubation of *tsa1*Δ *tsa2*Δ cells with 2 mM desferrioxamine, or 2 mM bathocuproine before the addition of hydrogen peroxide did not prevent POBN/1-hydroxyethyl radical adduct production, although its yield decreased (~30%) (Fig. 3). Total iron content in WT and *tsa1*Δ*tsa2*Δ cells was determined by atomic absorption spectrometry, and the same trend of chelatable iron ion levels was observed. Total iron content was higher in WT than in *tsa1*Δ*tsa2*Δ cells (Table 1). In contrast, total copper content was higher in *tsa1*Δ*tsa2*Δ cells, whereas total zinc content was similar. Although copper content is higher in *tsa1*Δ*tsa2*Δ cells, chelators of copper I and II (1) inhibited radical formation to ~70% of control levels (Fig. 3). Taken together, these results (Fig. 3 and Table 1) argue against iron and copper ion-mediated Fenton chemistry being the main route for 1-hydroxyethyl radical formation (Reactions 1 and 2).

Because peroxiredoxins are important to detoxify organic peroxides, deletion of their genes may lead to accumulation of organic peroxides and their derived peroxyl radicals (ROO$^{\bullet}$), which can oxidize ethanol to the 1-hydroxyethyl radical ($k \sim 1 \times 10^4$ M^{-1} s^{-1}) (43). However, levels of total peroxides present in WT and tsa1Δtsa2Δ cells were similar under basal conditions or after treatment with hydrogen peroxide (Table 1). Thus, organic peroxides and their derived radicals are unlikely to be major sources of the 1-hydroxyethyl detected in *tsa1*Δ*tsa2*Δ cells.

On the other hand, the higher content of copper in *tsa1*Δ*tsa2*Δ cells compared with WT cells led us to consider the possibility of increased expression of Sod1 providing a route for ethanol oxidation to the 1-hydroxyethyl radical. In fact, deletion of the *TSA1* gene has previously been shown to increase the mRNA levels of Sod1, catalase, and cytochrome peroxidase among other enzymes (10, 15, 16). In addition, Sod1 displays a peroxidase activity, which may oxidize ethanol to 1-hydroxyethyl radical, although this process has been considered inefficient (35, 44). These previous studies, however, were performed in the presence of bicarbonate buffer, which modulates Sod1 peroxidase activity to produce the carbonate radical (35, 36, 45),

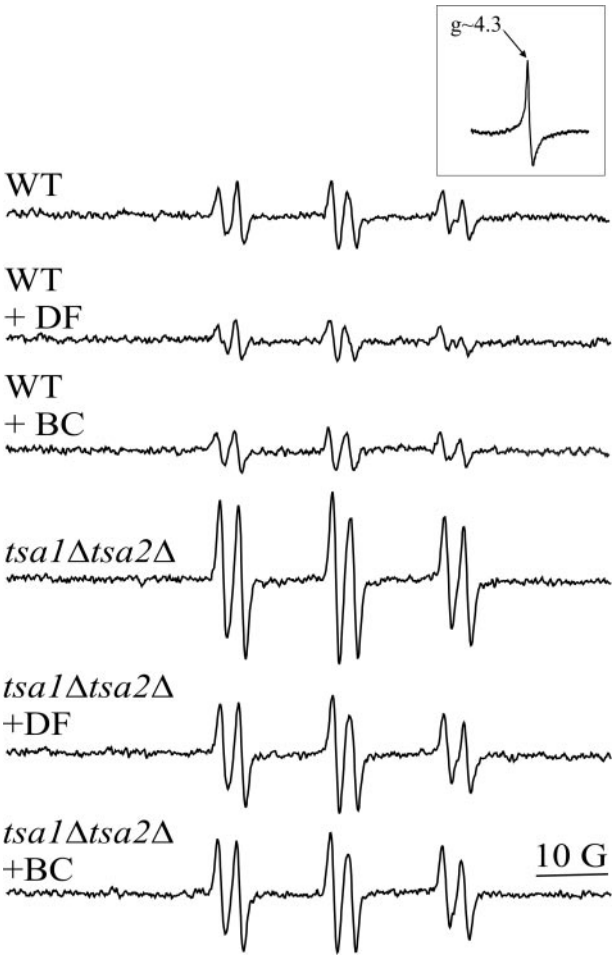

FIGURE 3. **Effects of desferrioxamine and bathocuproine on the yields of the POBN/1-hydroxyethyl radical adduct produced by WT and *tsa1*Δ*tsa2*Δ cells treated with 1 mM hydrogen peroxide.** Cells (5×10^7 cells/ml) in growth medium were treated with 2 mM desferrioxamine or 2 mM of bathocuproine for 30 min before the addition of 2% glucose, 90 mM POBN, and 1 mM H_2O_2. After 30-min incubation at 30 °C, aliquots were transferred to flat cells, and the EPR spectra were scanned at room temperature. Instrumental conditions: microwave power, 20 milliwatts; modulation amplitude, 1 G; time constant, 82 ms; scan rate, 2.4 G s^{-1}; gain, 7.1×10^5; number of averaged scans, 4. The *inset* shows a typical low temperature EPR spectrum of the Fe(III)-desferrioxamine complex obtained from the WT strain to determine the levels of chelatable iron content as described under "Experimental Procedures." Instrumental conditions: microwave power, 20 milliwatts; modulation amplitude 5 G; time constant 163.84 ms; scan rate, 2.4 G s^{-1}; gain 1.12×10^5.

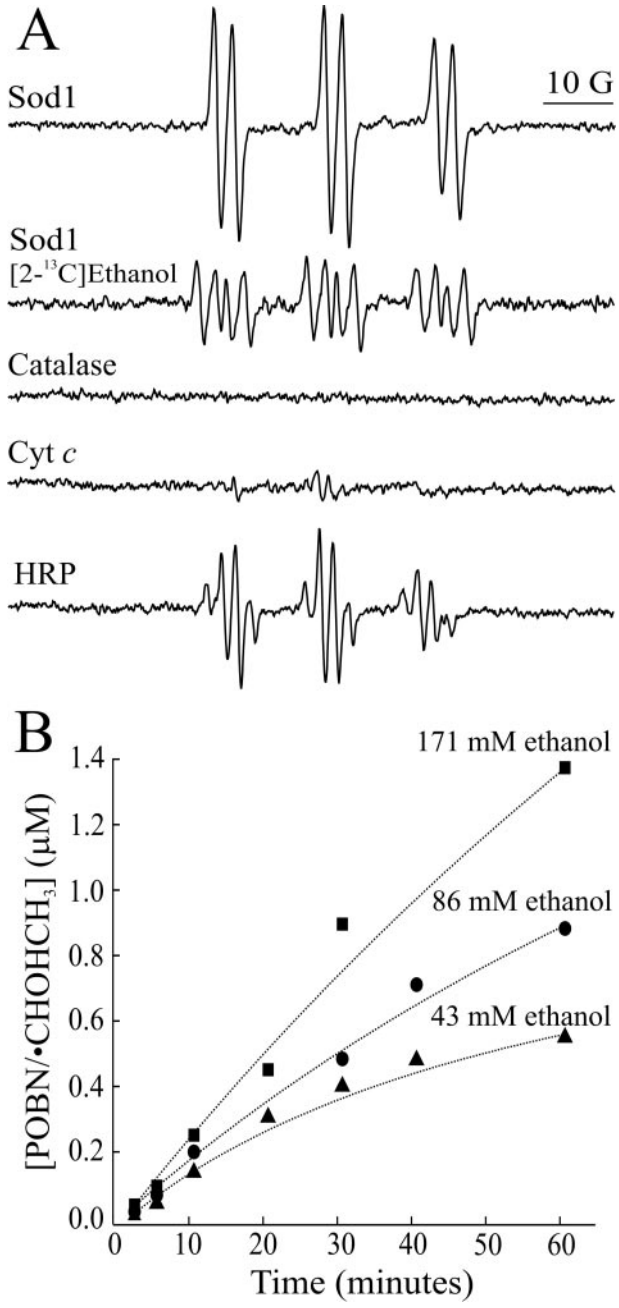

FIGURE 4. **Yields of the POBN/1-hydroxyethyl radical adduct produced from diverse enzymes treated with hydrogen peroxide and ethanol after 30 min (*A*) and from Sod1 as a function of time (*B*).** *A*, the specified enzymes (10 μM) were incubated with 1 mM H_2O_2, 90 mM POBN, 0.171 M ethanol, and 0.1 mM DTPA in 100 mM phosphate buffer, pH 7.4, for 30 min at 30 °C. As shown in the figure, experiments with Sod1 were performed with unlabeled and labeled ethanol ([2-^{13}C]ethanol). Aliquots were transferred to flat cells, and the EPR spectra were scanned at room temperature. *B*, Sod1 was incubated as in *A* in the presence of the specified concentrations of ethanol. At the specified times, aliquots were removed and treated with 10 units of catalase to stop the reaction. The samples were transferred to flat cells, and the EPR spectra were scanned at room temperature. Instrumental conditions: microwave power, 20 milliwatts; modulation amplitude, 1 G; time constant, 82 ms; scan rate, 2.4 G s^{-1}; gain, 7.1×10^5; number of averaged scans, 4.

TABLE 1

Basal levels of desferroxamine-chelatable iron ion (Fe(III-DF), total iron, copper, and zinc ions, and of total peroxides in WT and *tsa1*Δ*tsa2*Δ cells

Chelatable iron ions were determined by low temperature EPR; total iron, copper and zinc ion contents were determined by atomic absorption spectrometry; and total peroxides were determined by the FOX assay (see "Experimental Procedures"). The shown values are expressed as micrograms of ion/g of cell pellet and nanomoles of peroxide/g of cell pellet, and correspond to the mean ± S.D. values obtained from three different experiments.

Strain	Fe(III)-DF	Iron	Copper	Zinc	Peroxides[a]
		μg/g			nmol/g
WT	1.00 ± 0.05	104 ± 2	1.11 ± 0.02	22 ± 5	75 ± 5
*tsa1*Δ*tsa2*Δ	0.28 ± 0.03	64 ± 7	1.58 ± 0.04	23 ± 3	95 ± 10

[a] Total peroxides were also determined in both cells after treatment with 1 mM hydrogen peroxide and the values obtained were 100 ± 11 and 94 ± 8 for nanomoles/g for WT and *tsa1*Δ*tsa2*Δ cells, respectively.

which is not an efficient oxidant of alcohols (46). Thus, it became relevant to compare the ability of purified enzymes, such as bovine Sod1, catalase, cytochrome *c*, and horseradish peroxidase, to oxidize ethanol to the 1-hydroxyethyl radical in the presence of hydrogen peroxide in phosphate buffer con-

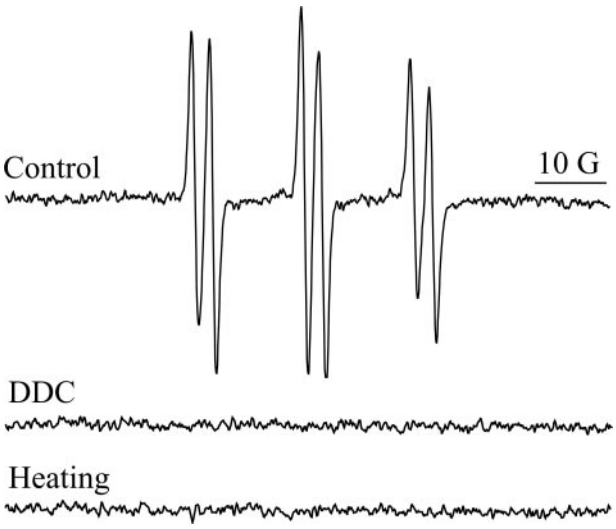

FIGURE 5. **Effects of diethyldithiocarbamate (*DDC*) and heating on the yields of the POBN/1-hydroxyethyl radical adduct produced by WT and *tsa1*Δ *tsa2*Δ cells treated with 1 mM hydrogen peroxide.** Cells (5×10^7 cells/ml) in growth medium were pre-treated with 10 mM diethyldithiocarbamate for 1 h or pre-heated at 90 °C and brought to room temperature before the addition of 2% glucose, 90 mM POBN, and 1 mM H_2O_2. After 30-min incubation at 30 °C, aliquots were transferred to flat cells, and the EPR spectra were scanned at room temperature. Instrumental conditions: microwave power, 20 milliwatts; modulation amplitude, 1 G; time constant, 82 ms; scan rate, 2.4 G s^{-1}; gain, 7.1×10^5; number of averaged scans, 4.

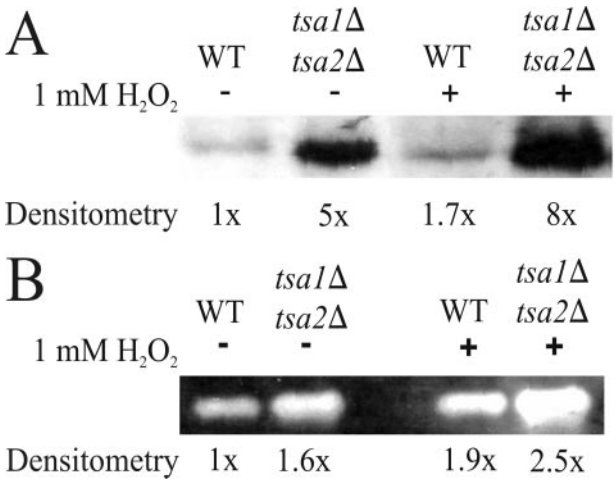

FIGURE 6. **Levels of Sod1 expression and superoxide dismutase activity in cells extracts of WT and *tsa1*Δ*tsa2*Δ strains.** *A*, Western blot analysis of Sod1 expression in the extracts of strains untreated or treated with 1 mM hydrogen peroxide before protein extraction. Treatment and analysis were performed as described under "Experimental Procedures." *B*, superoxide dismutase activity in native PAGE of WT and *tsa1*Δ *tsa2*Δ extracts (40 μg) obtained before and after treatment with 1 mM hydrogen peroxide (30 min, 30 °C). Analysis was performed as described under "Experimental Procedures."

taining DTPA. Considerable yields of the POBN/•1-hydroxyethyl radical adduct were produced only in the presence of Sod1 (Fig. 4). This was confirmed by the 12-line spectrum produced in incubations containing [2-^{13}C]ethanol, which is characteristic of the POBN/•^{13}CHOH^{13}CH$_3$ radical adduct (a_N = 15.5 G; a_H = 2.6 G; a_{13C} = 4.2 G) (24). The radical adduct detected in the presence of horseradish peroxidase is an oxidation product of the spin trap POBN as demonstrated by control experiments in the absence of ethanol (data not shown). Production of the POBN/•1-hydroxyethyl radical adduct by Sod1/hydrogen peroxide was shown to be ethanol- and time-dependent (Fig. 4*B*). This indicates that ethanol oxidation to the 1-hydroxyethyl radical depends mainly on the peroxidase activity of Sod1 and not from Fenton chemistry resulting from enzyme inactivation and copper liberation from its active site (47). These results confirm that biological oxidation of ethanol to the 1-hydroxyethyl radical is likely to be mediated mainly by the hydroxyl radical and hydroxyl radical-like oxidants (24, 41, 42). Indeed, the peroxidase activity of Sod1 produces a hydroxyl-like oxidant, which is able to oxidize ethanol, whereas catalase, horseradish peroxidase, and cytochrome *c* use hydrogen peroxide to oxidize substrates through ferryl states that are not efficient one-electron oxidants of ethanol (Fig. 4).

To further establish a role for Sod1 in the production of 1-hydroxyethyl radicals by the *tsa1*Δ*tsa2*Δ strain, cells were pre-treated with 10 mM diethyldithiocarbamate, a Sod1 inhibitor and copper chelator (48), or heated to promote enzyme inactivation. Cells submitted to both treatments failed to produce POBN/•1-hydroxyethyl radical adduct upon incubation with glucose, hydrogen peroxide, and POBN, in contrast with untreated cells (Fig. 5). These results indicate that 1-hydroxyethyl radical formation depends on an enzymatic activity, most likely the Sod1 peroxidase activity (Fig. 4). This conclusion was reinforced by the 5-fold higher level of Sod1 expression in the *tsa1*Δ*tsa2*Δ cells compared with the WT cells (Fig. 6*A*). This higher expression was confirmed by comparing the superoxide dismutase activity of the extracts monitored by both native PAGE stain (Fig. 6*B*) and inhibition of cytochrome *c* reduction (Fig. 7*A*) (32–34). Likewise, the bicarbonate-dependent peroxidase activity, characteristic of the Sod1 enzyme (35, 36, 45), was higher in *tsa1*Δ*tsa2*Δ cells (Fig. 7*A*, *inset*). Next, we transfected WT yeast to overexpress human Sod1. These cells displayed superoxide dismutase and bicarbonate-dependent peroxidase activity similar to those of the *tsa1*Δ*tsa2*Δ cells (Fig. 7*A*). Likewise, they produced similar yields of the 1-hydroxyethyl radical adduct (Fig. 7*B*), further demonstrating the role of Sod1 in radical production.

Independent of the employed methodology, the increase in superoxide dismutase and bicarbonate-dependent peroxidase activity in *tsa1*Δ*tsa2*Δ cells compared with WT cells was roughly the same (~2-fold) (Figs. 6*B* and 7*A*). However, the increase in Sod1 contents was higher (~5-fold) (Fig. 6*A*) indicating that not all the expressed enzyme is active, probably because total copper content increased less (<2-fold) (Table 1) than overall Sod1 expression (49).

Production of DNA-derived Radicals and Adducts—Like other alkyl radicals, the 1-hydroxyethyl radical is known to attack DNA to produce DNA-derived radicals and DNA-8-alkylguanine adducts (24, 50, 51). To monitor the production of DNA adducts under our experimental conditions, [^{14}C]glucose was added to the cultures, and the amount of ^{14}C incorporated to DNA ([^{14}C]DNA) was determined after 30-min incubation. As shown in Fig. 8*A*, *tsa1*Δ*tsa2*Δ cells treated with hydrogen peroxide contained about three times more [^{14}C]DNA than WT cells. Relevantly, [^{14}C]DNA production in *tsa1*Δ*tsa2*Δ cells was inhibited by the spin-trap POBN (~30%) indicating that a considerable fraction of ^{14}C incorporated into DNA comes

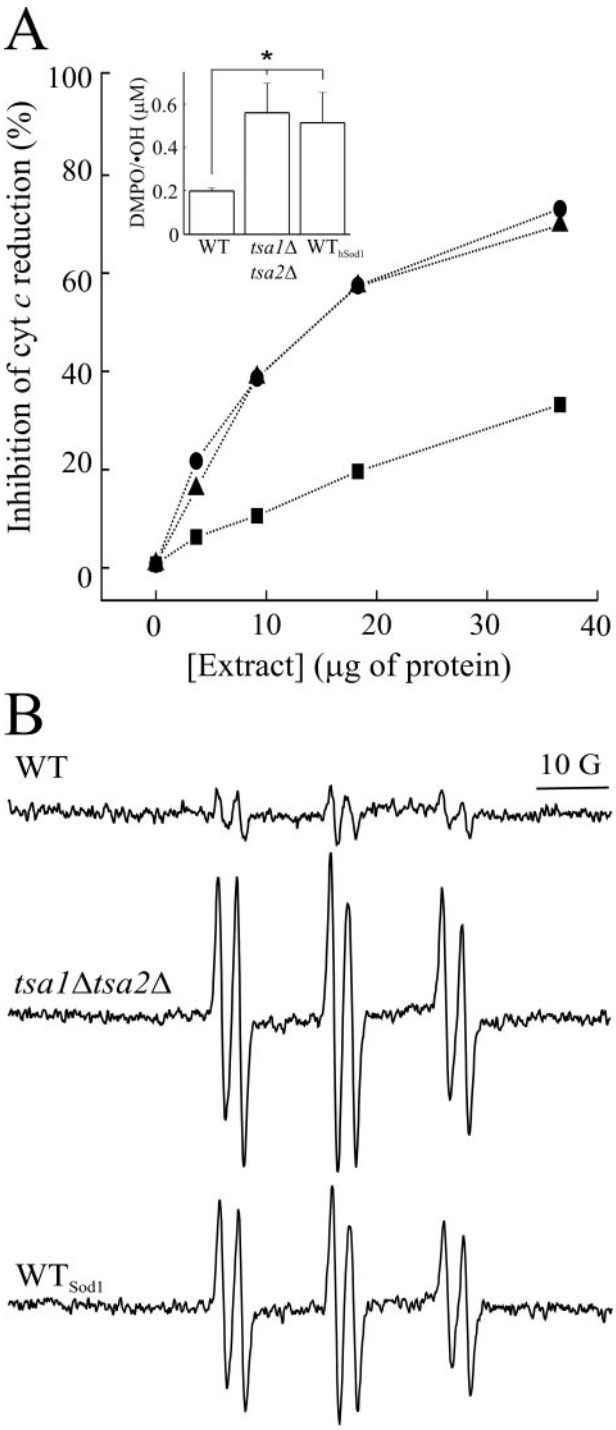

FIGURE 7. **Superoxide dismutase and bicarbonate-dependent peroxidase activity (*A*) and POBN/1-hydroxyethyl radical adduct production (*B*) in WT, *tsa1Δtsa2Δ*, and WT_{hSod1} strains.** *A*, superoxide dismutase activity was monitored as the percentage of inhibition of cytochrome *c* reduction by WT (■), *tsa1Δtsa2Δ* (●), and WT_{hSod1} (▲) cell extracts as a function of total protein concentration. The *inset* shows the bicarbonate-dependent peroxidase activity of the specified cells monitored by DMPO oxidation. Cells were resuspended in 100 mM phosphate buffer containing 0.1 mM DTPA, pH 7.4, and incubated with 80 mM DMPO for 5 min before addition of 25 mM bicarbonate and 1 mM hydrogen peroxide. After 15-min incubation at 30 °C, aliquots were transferred to flat cells, and the EPR spectra were scanned. The values shown correspond to the mean ± S.D. obtained from three different experiments; *, $p \leq 0.05$, Student's *t* test. *B*, representative spectra of POBN radical adducts produced by WT, *tsa1Δtsa2Δ*, and WT_{hSod1} cells treated with 1 mM hydrogen peroxide. Cells (5×10^7 cells/ml) in growth medium containing 2% glucose were treated with 90 mM POBN and 1 mM H_2O_2 for 30 min at 30 °C.

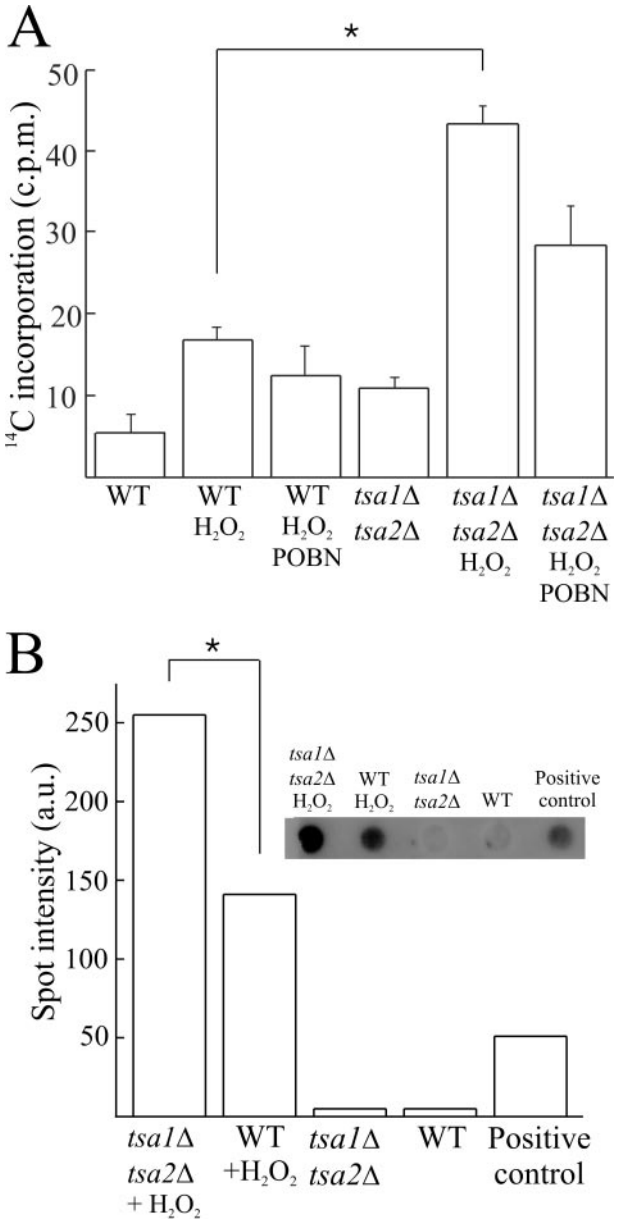

FIGURE 8. **Levels of DNA-derived adducts and radicals in WT and *tsa1Δtsa2Δ* cells treated with hydrogen peroxide.** *A*, incorporation of ^{14}C into the DNA of WT and *tsa1Δtsa2Δ* cells metabolizing ^{14}C-labeled glucose in the presence or absence of 1 mM hydrogen peroxide and 90 mM POBN as specified. The values shown correspond to the mean ± S.D.s obtained from three different experiments; *, $p \leq 0.05$, Student's *t* test. *B*, levels of DNA-derived radicals in WT and *tsa1Δtsa2Δ* cells treated or not with hydrogen peroxide monitored by immuno-dot blot with the antibody anti-DMPO nitrone adduct. The positive control corresponds to purified DNA (100 ng) extracted from the WT strain treated with 50 mM DMPO, 1 mM $CuCl_2$, and 20 μM hydrogen peroxide in phosphate buffer, pH 7.4, for 30 min at 30 °C. *Bars* represent the spot intensity as determined using the program ImageQuaNT 5.1 (Molecular Dynamics). The results are representative of two independent experiments.

from a radical metabolite of glucose, most likely, the 1-hydroxyethyl radical (Fig. 2). Attack of radicals on DNA was also indicated by parallel experiments with the antibody anti-DMPO, which reveals the amount of DNA-derived radicals that are

Instrumental conditions: microwave power, 20 milliwatts; modulation amplitude, 1 G; time constant, 82 ms; scan rate, 2.4 G s^{-1}; gain, 7.1×10^5; number of averaged scans, 4.

trapped by DMPO (37). Indeed, treatment of *tsa1*Δ*tsa2*Δ cells with hydrogen peroxide produced about two times as many DNA-derived radicals as WT cell treatment (Fig. 8*B*). Taken together, these results associate the higher production of the 1-hydroxyethyl radical by *tsa1*Δ*tsa2*Δ cells (Fig. 2) with increased damage to their DNA (Fig. 8).

DISCUSSION

Our results demonstrated that deletion of both the *TSA1* and *TSA2* genes in *S. cerevisiae* significantly increases cellular resistance to toxic concentrations of hydrogen peroxide while increasing its consumption rate (Fig. 1). These results contrast with previous studies reporting that *tsa1*Δ, *tsa2*Δ, and *tsa1*Δ*tsa2*Δ strains are more susceptible than the WT strain to hydrogen peroxide (14, 16). In another study employing various experimental conditions, *tsa2*Δ cells were shown to be more resistant than WT (11), although to a lesser extent than reported here for the double mutant. These tolerance assays, however, were performed by spot tests, in which the solid media may affect hydrogen peroxide metabolism by the strains. In synthetic liquid media, the resistance of *tsa1*Δ*tsa2*Δ cells to 1 mM hydrogen peroxide (Fig. 1) suggests that catalase, which is induced in these cells (15, 16), is the main enzyme responsible for decomposing these toxic oxidant concentrations. In agreement, the consumption of hydrogen peroxide by cell extracts of the *tsa1*Δ and *tsa2*Δ strain was shown to be almost completely inhibited by azide (11). However, our results indicate that part of the hydrogen peroxide consumption by *tsa1*Δ*tsa2*Δ cells (Fig. 1*B*) is also mediated by induced Sod1 (Figs. 5–7). Although catalases are efficient enzymes with specific activity values around 17,750 units/nmol (52), their levels in extracts of WT and *tsa1*Δ and *tsa2*Δ cells in exponential growth under fermentative conditions (high glucose concentrations) are low (11). Indeed, catalase activity values ranging from 0.38 to 1.3 units/mg have been reported for yeast extracts (11, 53), which corresponds to $\sim 5 \times 10^{-5}$ nmol catalase/mg. In contrast, Sod1 is an abundant enzyme, and its level in extracts of *tsa1*Δ*tsa2*Δ cells can be estimated as $\sim 7 \times 10^{-1}$ nmol Sod1/mg from the data in Fig. 7*A* (67 units/mg of protein) and the specific activity reported for the bovine liver enzyme (~90 units/nmol) (54).

Thus, the higher level of Sod1 in *tsa1*Δ*tsa2*Δ cells (Figs. 6 and 7) should favor the occurrence of its peroxidase activity (Fig. 4), which oxidizes the glucose metabolite ethanol to the 1-hydroxyethyl radical (Fig. 2). This radical, direct or indirectly, leads to the production of DNA-derived radicals and adducts (Fig. 8). Thus, our studies provide a pathway to account for the hypermutability of peroxiredoxin-null yeast cells while performed with cells challenged with toxic concentrations of hydrogen peroxide (1 mM). This high concentration was probably required to make it possible to detect significant differences among the strains regarding hydrogen peroxide resistance and consumption (Fig. 1) and radical production (Figs. 2 and 8*B*). Indeed, the difficulties involved in the precise quantification of oxidants and radicals in cells and cell cultures are well known (36, 41, 42, 46). In unstressed cells, it has been reported that the mutation rates of the studied strains follow the order WT ≈ *tsa2*Δ < *tsa1*Δ < *tsa1*Δ*tsa2*Δ (16). This trend can be considered similar to the one observed here, if the sensitivities of the methodologies available to measure mutation rates and radical production are taken into account.

Generation of the 1-hydroxyethyl radical in *tsa1*Δ*tsa2*Δ cells challenged with hydrogen peroxide was attributed mainly to the peroxidase activity of the enzyme Sod1, although the participation of Fenton chemistry to a small extent (~30%) is apparent (Fig. 3). 1-Hydroxyethyl radical might be formed through hydrogen abstraction by peroxyl radicals, but no quantitative evidence is this direction was obtained (Table 1). In contrast, the major role of Sod1 was established by several lines of evidence (Figs. 2–7 and Table 1). Among them, the similar properties of WT overexpressing human Sod1 and *tsa1*Δ*tsa2*Δ cells in regard to the superoxide dismutase activity, bicarbonate-dependent peroxidase activity, and 1-hydroxyethyl radical production were particularly convincing (Fig. 7).

The results demonstrated that increased 1-hydroxyethyl radical production by *tsa1*Δ*tsa2*Δ cells increased damage to their DNA with formation of DNA-derived radicals and adducts. Both of these lesions can be promoted directly by the 1-hydroxyethyl radical (24, 50, 51). In fact, the inhibition of [^{14}C]DNA production by the spin-trap POBN argues for a direct participation of the 1-hydroxyethyl radical in forming DNA adducts. At this point, however, we cannot exclude the formation of DNA adducts by 1-hydroxyethyl radical metabolites, such as acetaldehyde (55–57). Likewise, the formation of DNA-derived radicals may result from secondary radicals arising from processes triggered by the 1-hydroxyethyl radical, such as lipid peroxidation (42, 58). It is noteworthy that production of the 1-hydroxyethyl radical close to DNA is feasible, because Sod1 is mainly a cytosolic enzyme but is also found in the nucleus, peroxisomes, and mitochondrial intermembrane space of eukaryotic cells (59). Here, we measured overall expression and activity of Sod1, which was augmented in *tsa1*Δ*tsa2*Δ cells even in the absence of a hydrogen peroxide challenge (Fig. 6). Thus, the molecular events proposed here may also occur in unstressed cells. Relevantly, a higher yield of DNA adducts was obtained in unstressed *tsa1*Δ*tsa2*Δ compared with unstressed WT cells, although the difference was not statistically significant (Fig. 8*A*). The peroxidase activity of Sod1 is increased in the presence of carbon dioxide to produce the carbonate radical, which is also able to produce DNA radicals (60, 61). Consequently, the peroxidase activity of Sod1 may also be involved in promoting DNA mutations in peroxiredoxin-null yeast cells grown aerobically (18).

The signaling pathway responsible for the elevated levels of Sod1 in *tsa1*Δ*tsa2*Δ cells is not known but probably involves Yap1 and Skn7 regulons, because the *SOD1*, *TSA1*, and *TSA2* genes are under their control (62). On the other hand, deletion of both *TSA1* and *TSA2* genes is likely to result in higher steady-state levels of hydrogen peroxide, which, in turn, activate both Yap1 and Skn7 leading to increased Sod1 expression. Another possibility is that induction of *SOD1* could be a response of yeast to increased levels of copper ions (Table 1), probably mediated by Ace1 transcriptional factor (63). Other transcriptional regulators such as Hap1 might also be involved in the induction of Sod1 in *tsa1*Δ*tsa2*Δ cells.

Sod1 is considered one of the most important antioxidant defenses because it efficiently catalyzes the dismutation of

superoxide anion into hydrogen peroxide plus molecular oxygen (59). For instance, the *sod1*Δ yeast mutant presents aerobic spontaneous mutation rates that are 3- to 4-fold higher than for the WT strain (63, 64). On the other hand, higher expression levels of Sod1 in mammalian cells have been frequently associated with deleterious effects (65), including increased DNA damage in cells defective for DNA repair (66–69). Although many investigators attribute these deleterious effects to Sod1 increasing cellular steady-state concentrations of hydrogen peroxide, they are most likely due to the enzyme competing with other metalloproteins for metal cofactors and to the extraneous activities of Sod1 (65). Indeed, Sod1 displays catalytic activities other than superoxide dismutation, such as superoxide oxidase, peroxynitrite synthase, thiol oxidase, and peroxidase activity (35, 36, 44, 45). Among these, our studies of WT and *tsa1*Δ*tsa2*Δ yeast cells challenged with hydrogen peroxide emphasize the importance of the peroxidase activity, because the mutant cells presented increased levels of DNA damage (Fig. 8) in parallel with increased levels of 1-hydroxyethyl radical formation (Fig. 2), Sod1 expression (Fig. 6), and peroxidase activity (Fig. 7*A*). Other extraneous Sod1 roles, such as activation and control of specific DNA repair mechanisms, were not examined here but deserve future consideration (69).

In summary, our studies examined the relative resistance of *tsa1*Δ*tsa2*Δ cells to hydrogen peroxide and provided a sequence of molecular events whereby the compensatory response of *S. cerevisiae* to deal with *TSA1* and *TSA2* gene disruption leads to hypermutability of the strain. In parallel, they present a framework to examine some of the deleterious effects that have been frequently associated with the overexpression of Sod1 in cells and animal models.

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