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Original Contribution

REGULATION OF ANTIOXIDANT ENZYMES GENE EXPRESSION IN THE YEAST *SACCHAROMYCES CEREVISIAE* DURING STATIONARY PHASE

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Abstract—Gene expression of three antioxidant enzymes, Mn superoxide dismutase (MnSOD), Cu,Zn superoxide dismutase (Cu,ZnSOD), and glutathione reductase (GR) was investigated in stationary phase *Saccharomyces cerevisiae* during menadione-induced oxidative stress. Both GR and Cu,ZnSOD mRNA steady state levels increased, reaching a plateau at about 90 min exposure to menadione. GR mRNA induction was higher than that of Cu,ZnSOD (about 14-fold and 9-fold after 90 min, respectively). A different pattern of response was obtained for MnSOD mRNA, with a peak at about 15 min (about 8-fold higher) followed by a decrease to a plateau approximately 4-fold higher than the control value. However, these increased mRNA levels did not result in increased protein levels and activities of these enzymes. Furthermore, exposure to menadione decreased MnSOD activity to half its value, indicating that the enzyme is partially inactivated due to oxidative damage. Cu,ZnSOD protein levels were increased 2-fold, but MnSOD protein levels were unchanged after exposure to menadione in the presence of the proteolysis inhibitor phenylmethylsulfonyl fluoride. These results indicate that the rates of Cu,ZnSOD synthesis and proteolysis are increased, while the rates of MnSOD synthesis and proteolysis are unchanged by exposure to menadione. Also, the translational efficiency for both enzymes is probably decreased, since increases in protein levels when proteolysis is inhibited do not reflect the increases in mRNA levels. Our results indicate that oxidative stress modifies MnSOD, Cu,ZnSOD, and GR gene expression in a complex way, not only at the transcription level but also at the post-transcriptional, translational, and post-translational levels. © 2003 Elsevier Science Inc.

Keywords—Mn superoxide dismutase, Cu,Zn superoxide dismutase, Glutathione reductase, Menadione, Oxidative stress, Proteolysis, Free radicals

INTRODUCTION

Reactive oxygen species (ROS) such as $O_2^{\bullet -}$, H_2O_2, and $HO^{\bullet}$ are produced as normal by-products of aerobic cellular metabolism or through exposure to environmental agents, such as radiation, stimulated host phagocytes, or redox-cycling agents. ROS can cause damage to biological macromolecules [1–3], and cellular injury from ROS has been implicated in the development and progression of several diseases [2,4].

Increases in the levels of ROS, such as those that may occur during periods of oxidative stress, are detected by redox-sensitive regulatory molecules in the cell and trigger a homeostatic response to prevent cellular injury called the oxidative stress response [5]. Included in the cellular oxidative stress response is the regulation of antioxidant enzymes gene expression, leading to increased antioxidant enzymes activities and, therefore, to a faster removal of the oxidants by the cell, protecting the cell against oxidative stress [6–9]. However, the cell response has a complex regulation and one of the main paradoxes is that, in mammalian cells and tissues, increases in antioxidant enzymes mRNAs during oxidative stress do not always correlate with increased activities or protein content of these enzymes [7–11].

In the present work, gene expression of Mn superoxide dismutase (MnSOD), Cu,Zn superoxide dismutase (Cu,ZnSOD), and glutathione reductase (GR) was investigated in stationary phase *Saccharomyces cerevisiae* during oxidative stress induced by menadione. The lower

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eukaryote *S. cerevisiae* is an important model organism for the study of the eukaryotic oxidative stress response [12]. However, most studies of oxidative stress response in *S. cerevisiae* have been performed with cells in the logarithmic phase [13–25] and not in the stationary phase where yeast cells resemble cells of multicellular organisms in important aspects: (i) most energy comes from mitochondrial respiration, (ii) cells are in the G_0 phase, and (iii) damage accumulates over time [26]. Also, with the exception of one study [25], almost no information is available relating the induction of antioxidant enzymes mRNAs with their protein levels and enzymatic activity in *S. cerevisiae*. In this work, transcriptional, post-transcriptional, translational, and post-translational processes are studied in order to elucidate the regulation of antioxidant enzymes gene expression by oxidative stress in *S. cerevisiae*.

MATERIALS AND METHODS

Materials

S. cerevisiae strains used in this work are EG103 (wild-type, genotype MATα leu2-3, 112 his3Δ1 trp1-289 ura3-52 GAL), EG118 (sod1Δ, isogenic to EG103 with sod1ΔA::URA3), EG110 (sod2Δ, isogenic to EG103 with sod2Δ::TRP1) [26], YP250 (wild-type, genotype MATa trp-1Δ1 his3-Δ200 lys2-801 leu2-Δ1 ade2-101 ura3-52), and GRK1 (glr1Δ, isogenic to YP250 with glr1Δ::HIS3) [23]. YPH250 was obtained from the Yeast Genetic Stock Center, University of California at Berkeley. Yeast extract, bactopeptone, and agar were from Difco (Detroit, MI, USA). Horse heart cytochrome *c*, NADPH, menadione, phenylmethylsulfonyl fluoride (PMSF), xanthine oxidase, and xanthine were from Sigma Chemical Co. (St. Louis, MO, USA). Rabbit antihuman Cu,Zn superoxide dismutase and rabbit antirat Mn superoxide dismutase were obtained from Stressgen Biotechnologies (San Diego, CA, USA). Rabbit antibovine glutathione reductase was obtained from Biogenesis Ltd. (Poole, England). Horseradish peroxidase goat antirabbit IgG conjugate antibody was obtained from Zymed Laboratories, Inc. (San Francisco, CA, USA).

Media and growth conditions

S. cerevisiae cells were inoculated at an OD_{600} of 0.1 and cultured in YPD medium (1% yeast extract, 2% bactopeptone, 2% glucose) at 30°C and with shaking at 160 rpm. Stationary phase cells were harvested after 48 h.

Exposure to menadione

To measure cell susceptibility to menadione, cells were harvested (1000 $\times$ *g*, 5 min, 16°C) and resuspended in 100 mM potassium phosphate buffer, pH 7.4, at OD_{600} = 0.5. Menadione was added (final concentration 20, 40, and 60 μM) to the cells and cell survival was monitored by taking samples at several times of exposure, plating aliquots on YPD plates (YPD medium with 2% agar), and counting colonies after 48 h, with the exception of the EG118 strain where colonies were counted after 72 h. For other experiments, stationary phase cells were collected by centrifugation (3000 $\times$ *g*, 5 min, 16°C), washed, and then suspended in 100 mM potassium phosphate buffer, pH 7.4, at OD_{600} = 0.5. A 50 ml aliquot was taken (control) and then menadione (40 μM final concentration) was added to the cells. Aliquots (50 ml) were taken at several times of menadione exposure. Cells were harvested by centrifugation (1000 $\times$ *g*, 5 min, 8°C) and the pellet was washed with 100 mM potassium phosphate buffer (pH 7.4), centrifuged again, and the resulting pellet was used for protein and RNA preparation.

RNA preparation and northern blot analysis

Total RNA from yeast cells not treated and treated with menadione at different times was extracted using the method described previously by Iyer and Struhl [27]. Denatured RNA (10 or 20 μg determined by absorbance at 260 nm) was fractionated by electrophoresis in a 1.5% (w/v) agarose gel with the running buffer (40 mM MOPS, 10 mM sodium acetate, and 1 mM EDTA, pH 8.0) containing 2.2 M formaldehyde and transferred to nylon membrane (Nytran SuPerCharge; Schleicher & Schüll, Dassel, Germany) [28]. RNA blots were hybridized with [α^{32}P]-dCTP radiolabeled probes in 250 mM sodium phosphate buffer, pH 7.2, 7% (w/v) SDS, 1% (w/v) BSA, and 1 mM EDTA, overnight at 60°C. Probed membranes were washed in 20 mM sodium phosphate buffer, pH 7.2, 5% (w/v) SDS, and 1 mM EDTA, autoradiographed with Kodak radiographic film (Biomax MR-1; Eastman Kodak Co., Rochester, NY, USA) for the desired time, and then manually developed.

Autoradiograms were scanned using an LKB 2222-020 Ultra-scan XL laser densitometer (LKB Instruments, Bromma, Sweden) and the signal intensities were processed using the software LKB 24000 GelScan XL (LKB Instruments).

Probes for Cu,ZnSOD, MnSOD, and GR were obtained by PCR with the use of gene-specific oligonucleotides from the sequences obtained in *Saccharomyces* Genome Database (Dept. of Genetics at the School of Medicine, Stanford University—http://genome-www.stanford.edu/Saccharomyces/). The primer sets used for PCR were: 5'-TGCCCCTCTAGCAGTGTTTTGAGC-3' and 5'-CCATCCATACTACGTGGGCACTC-3' for Cu,ZnSOD (YJR104c); 5'-TGCAAGTGGACATTCTT-

GTTGGGG-3' and 5'- AGGCGCGAGTAACGTAG-GAAGCG-3' for MnSOD (YHR008c); and 5'-CCATAG-GTTGAACGGTATATACC-3' and 5'- GTCATGAGA GTTCAGCTTTATACC-3' for GR (YPL091w). The *U3* (SNR17A) probe was used as loading control; the PCR amplification primers for this probe were: 5'-GAATC-CAACTTGGTTGATGAGTCC-3' and 5'-CCATAGAGC-CCTATCCCTTCAAAA-3'.

Protein extracts

Crude extracts were prepared by glass bead lysis. Cells were suspended in 100 mM potassium phosphate buffer, pH 7.4, containing 1 mM PMSF with an equal volume of glass beads, and vortexed for 7 cycles of 1 min of vortexing followed by 1 min of cooling on ice. The mixture was then centrifuged at 8000 $\times$ *g* for 20 min at 8°C. The supernatants were used for determinations of total protein according to Peterson [29], enzyme activities, and protein levels by Western blot.

Enzyme activities

Glutathione reductase activity was determined by measuring NADPH oxidation [30]. One unit is defined as the quantity of enzyme oxidizing 1 μmol GSSG/min at 30°C.

Superoxide dismutase activity was measured using the inhibition of cytochrome *c* reduction by superoxide radical [31]. MnSOD activity was determined by adding 2 mM KCN to the SOD assay. The MnSOD was subtracted from total SOD activity to give Cu,ZnSOD activity. One unit of SOD activity is defined as the amount of the enzyme resulting in a 50% inhibition of cytochrome *c* reduction.

SDS-PAGE and Western blot analysis

SDS-PAGE was carried out according to Laemmli [32]; 75 μg of total proteins were denaturated and analyzed in 15% (w/v) polyacrylamide (w/v). For immunodetection, the proteins were transferred to nitrocellulose membrane (0.45 nm; Schleicher & Schüll) in a semidry system using 39 mM glycine, 48 mM Tris, 0.0375% (w/v) SDS, and 20% (v/v) methanol as transfer buffer [33], during 75 min at 0.8 mA/cm^2, not exceeding 25V. Blots were blocked at room temperature for 1 h in PBS (4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 137 mM NaCl, and 2.0 mM KCl, pH 7.4) containing 5% (w/v) fat-free milk powder before incubation with the primary antibody. As primary antibodies, rabbit anti-Cu,Zn superoxide dismutase antibody (anti-SOD1), rabbit anti-Mn superoxide dismutase antibody (anti-SOD2) with a dilution of 1:750 and incubation for 1 h, and rabbit antibovine glutathione reductase antibody with different dilutions (1:500, 1:1000, and 1:2000) and incubation for 1 h were used. After washing five times in PBS containing 0.1% (v/v) Tween-20, the blots were incubated for 1 h with the secondary antibody (horseradish peroxidase goat antirabbit IgG conjugate, 1:3000), washed extensively with PBS, and detected by enhanced chemiluminescence (ECL kit, Amersham, Braunschweig, Germany).

Table 1. Susceptibility of Stationary-Phase Cells to Menadione-Induced Oxidative Stress

Time of exposure (min)	Survival (%) with menadione treatment		
	20 μM	40 μM	60 μM
30	94.5 $\pm$ 11.9	100.8 $\pm$ 9.2	93.7 $\pm$ 5.3
60	94.6 $\pm$ 5.4	95.8 $\pm$ 8.4	87.1 $\pm$ 9.8
90	103.0 $\pm$ 8.5	87.8 $\pm$ 7.0	85.5 $\pm$ 7.9
120	94.7 $\pm$ 7.1	82.7 $\pm$ 5.6	79.9 $\pm$ 9.9

Survival of EG103 cells was calculated in relation to the number of colonies formed without menadione treatment. Results represent the mean $\pm$ SD from five independent experiments.

Protein degradation inhibition with PMSF

Inhibition of protein degradation was carried out by adding to stationary-phase yeast cells PMSF freshly prepared in ethanol, at a final concentration of 1.2 mM, 90 min prior to the addition of menadione (final concentration 40 μM) [34]. Final concentrations of ethanol were 1% (v/v) in control and treated cells. Total protein extracts were prepared and analyzed by Western blot.

Statistical analysis

Data statistical analysis was undertaken using analysis of variance (ANOVA) and the Tukey-Kramer multiple comparisons test.

RESULTS

Susceptibility of stationary-phase cells to menadione stress

Menadione (2-methyl-1,4-naphtoquinone) is a quinone compound that undergoes redox cycling. One-electron transfer, mainly from the mitochondrial respiratory chain [35], forms semiquinone radicals that can rapidly reduce O_2, thereby generating superoxide radical intracellularly and regenerating the quinone. Menadione can also react with thiol-containing compounds such as glutathione, and the glutathionyl-hydroquinone can also redox cycle and generate $O_2^{\bullet -}$ [36]. Thus, either type of redox cycling may result in the intracellular formation of large amounts of $O_2^{\bullet -}$, H_2O_2 due to $O_2^{\bullet -}$ dismutation, and $HO^{\bullet}$ due to Fenton-type reactions.

To determine a concentration of menadione that causes oxidative stress without leading to cell death, stationary-phase cells were treated with various concentrations of menadione (20, 40, 60 μM) and cell survival was determined (Table 1). Taking into account the re-

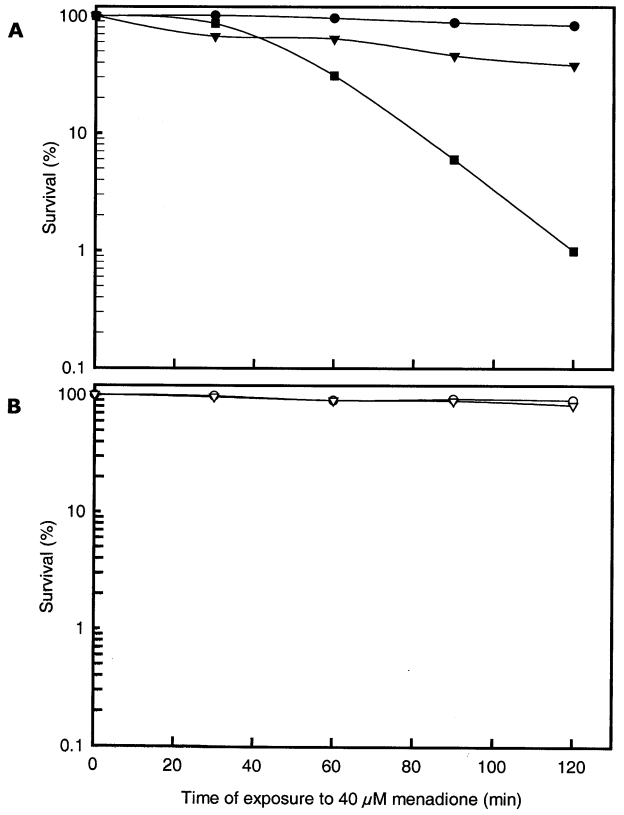

Fig. 1. Susceptibility of wild-type and mutant stationary phase cells to menadione-induced oxidative stress. Survival was calculated in relation to the number of colonies formed without 40 μM menadione treatment. Results represent the median from three independent experiments done in triplicate, with the exception of the EG103 strain where the results are the mean of five independent experiments. (A) Susceptibility of Cu,ZnSOD and MnSOD mutant and wild-type strains: ● EG103 (wt), ■ EG118 (sod1Δ); and ▼ EG110 (sod2Δ). (B) Susceptibility of GR mutant and wild-type strains: ○ YP250 (wt) and ▽ GRK1 (glr1Δ).

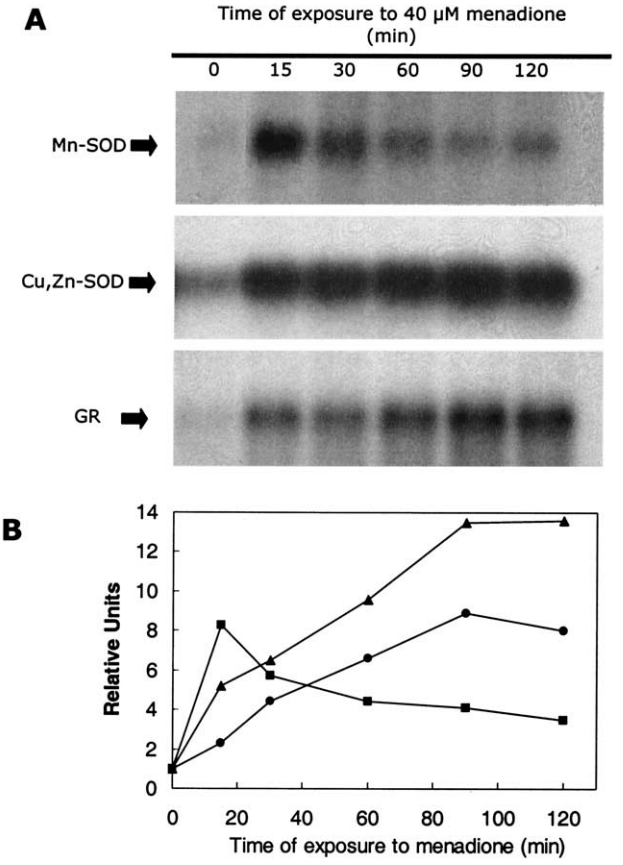

Fig. 2. Time course of the effect of exposure to menadione on steady state mRNA levels of antioxidant enzymes. (A) Northern blot analysis of mRNA levels of Cu,ZnSOD, MnSOD, and GR in EG103 strain. The Northern blots shown are representative of five independent experiments. (B) Quantitative analysis of transcript levels. From experiments shown in (A), transcript levels were quantified by signal intensity analysis, normalized to the levels of U3 transcripts in the sample, and expressed as arbitrary units relative to control: ■ MnSOD; ● Cu,Zn-SOD; and ▲ GR.

sults obtained, 40 μM menadione was used for subsequent studies.

Wild-type and mutant cells in stationary phase were treated with 40 μM menadione and cell survival was determined to check their susceptibility to menadione stress (Fig. 1). There was no difference in the susceptibility to menadione-induced oxidative stress between wild-type and GR-deficient cells (Fig. 1B). GR mutant cells in stationary phase had been previously shown to be more sensitive to millimolar concentrations of hydrogen peroxide than wild-type cells [37]. The fact that we found no differences in cell survival indicates that either the levels of GSSG being produced due to menadione redox-cycling are not high enough to alter cell viability in the GR mutant strain, or that there are cellular compensatory mechanisms when this enzyme is not present. However, MnSOD- and particularly Cu,ZnSOD-deficient cells displayed higher susceptibility to menadione than wild-type cells (Fig. 1A). The higher susceptibility of Cu,ZnSOD-deficient cells to menadione indicates that this enzyme has a key role in the antioxidant defense against menadione-induced oxidative stress when compared to MnSOD.

Menadione increases differentially antioxidant enzymes mRNA steady state levels

S. cerevisiae cells in stationary phase were treated with menadione and steady state mRNA levels of MnSOD, Cu,ZnSOD, and GR were determined to find whether cells were reacting to oxidative stress by changes in antioxidant enzyme expression. As can be seen in Fig. 2, for all three enzymes studied exposure to menadione increased mRNA steady state levels. For both GR and Cu,ZnSOD, mRNA steady state levels increased reaching a plateau at about 90 min of exposure to menadione. However, the induction in GR mRNA levels was higher (about 14-fold after 90 min) than that of Cu,Zn-

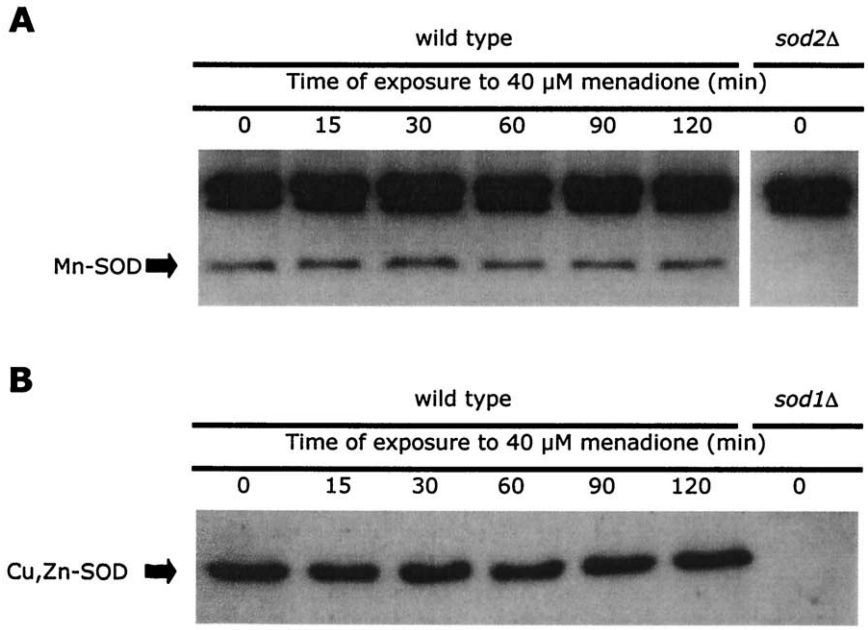

Fig. 3. Time course of the effect of exposure to menadione on steady state MnSOD and Cu,ZnSOD levels determined by Western blot. MnSOD (A) and Cu,ZnSOD (B) were identified with rabbit anti-MnSOD and anti-Cu,ZnSOD antibodies and the specificity of the identification was confirmed using the sod2Δ and sod1Δ mutant strains, respectively. The upper bands observed in (A) are due to nonspecific reaction of the rabbit anti-MnSOD antibody with yeast proteins. The Western blots shown are representative of three independent experiments.

SOD mRNA levels (about 9-fold after 90 min). Exposure to menadione induced a different pattern of response for MnSOD mRNA levels, with a peak at about 15 min (about 8-fold higher) followed by a decrease in the mRNA levels to a plateau at about 4-fold higher than the value in nontreated cells.

The increased mRNA steady state level of antioxidant enzymes could be caused by an increased transcription rate, an increased mRNA stability, or both. To address the mechanism for these increased mRNA steady state levels, mRNA's stability was studied by incubating cells with 100 μg/ml of the transcriptional inhibitor 1,10-phenanthroline [38] for several times up until 120 min, in the absence and presence of menadione. Exposure to menadione stabilized Cu,ZnSOD mRNA but destabilized GR and MnSOD mRNAs (results not shown). This suggests that an increased transcription rate is responsible for the increased mRNA steady state levels of MnSOD and GR and that for Cu,ZnSOD the increased mRNA levels are probably due both to an increased transcription rate and an increased mRNA half-life.

Menadione does not increase antioxidant enzymes protein levels and enzymatic activities

To determine whether changes in protein levels and enzyme activity paralleled changes in message levels, the effects of exposure to menadione on the activities and protein levels of antioxidant enzymes were examined.

As can be seen in Fig. 3, exposure to menadione did not change the steady state levels of MnSOD and Cu,Zn-SOD as revealed by Western blot analysis. The antibody used for GR did not recognize yeast GR (results not shown).

As shown in Fig. 4, Cu,ZnSOD- and GR-specific activities remained essentially unaltered during exposure to menadione. However, exposure to menadione for 15 min decreased MnSOD-specific activity to about half the value observed in the absence of menadione. This decreased MnSOD activity was maintained during all times of exposure to menadione studied. In *S. cerevisiae*, Cu,ZnSOD and GR are cytosolic enzymes, whereas Mn-SOD is located in the mitochondrial matrix. Two causes may explain MnSOD sensitivity to menadione: (i) Mn-SOD, being in the mitochondria, is more exposed to menadione-induced oxidative stress, since ROS generated by menadione redox cycling heavily target mitochondrial enzymes causing their inactivation [39]; and, (ii) MnSOD has an intrinsically higher sensitivity to oxidative damage than Cu,ZnSOD and GR, since Mn-SOD was found to be inactivated in vivo in other models of oxidative stress [10,40].

Antioxidant enzymes protein synthesis and degradation

Mild oxidative stress induced by menadione increases intracellular proteolysis by modifying cellular proteins, thus increasing their proteolytic susceptibility, whereas

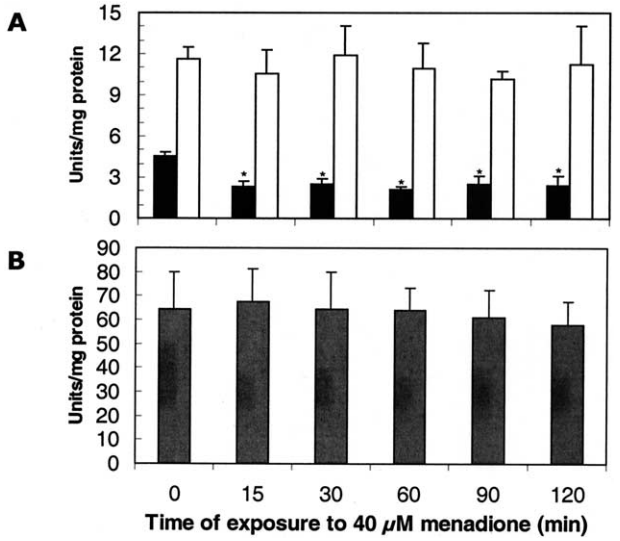

Fig. 4. Effect of menadione exposure on MnSOD, Cu,ZnSOD, and GR activities. (A) MnSOD- (filled bars) and Cu,ZnSOD- (empty bars) specific activities in EG103 strain. (B) GR-specific activity in EG103 strain. Data are the mean ± SD from at least five experiments; $*p < .001$ vs. control.

severe oxidative stress diminishes intracellular proteolysis, probably due to the generation of severely damaged cell proteins that can not be easily degraded or by damaging proteolytic enzymes [41]. Considering that increased mRNA levels of MnSOD, Cu,ZnSOD, and GR (Fig. 2) were not followed by increases in their protein levels (Fig. 3) and activities (Fig. 4) and that the MnSOD decreased activity (Fig. 4) may be caused by oxidative damage, a hypothesis was put forward: exposure to menadione was resulting in an increased rate of synthesis of the three antioxidant enzymes, but their rate of degradation was also increased due to oxidative damage. This increased degradation rate would explain why increased mRNA levels did not result in increased steady state protein levels of the antioxidant enzymes. So, if this hypothesis was correct, then the steady state protein levels of the antioxidant enzymes should increase after exposure to menadione in the presence of proteolysis inhibitors.

To test whether there was an increase in MnSOD and Cu,ZnSOD protein levels when proteolysis was inhibited, we added PMSF to cells prior to menadione exposure. PMSF, a serine protease inhibitor, was chosen to inhibit proteolysis since none of the known proteasome inhibitors can enter wild-type yeast cells [34] and the vacuoles have a major role in yeast proteolysis. When PMSF was added to cells, an increase in the protein levels of both SODs was observed (Fig. 5), indicating that PMSF was inhibiting proteolysis of both enzymes. However, the increase in Cu,ZnSOD protein levels was more pronounced (Fig. 5). Furthermore, exposure to menadione in the presence of PMSF induces an approximately 2-fold increase in Cu,ZnSOD protein levels compared to those in the presence of just PMSF. This indicates that the rate of Cu,ZnSOD synthesis is increased and that an increased proteolysis rate, probably due to oxidative damage to the enzyme, is responsible for the maintenance of steady state Cu,ZnSOD protein levels in the presence of menadione (Fig. 3). In the case of MnSOD, no changes were found in its protein levels after

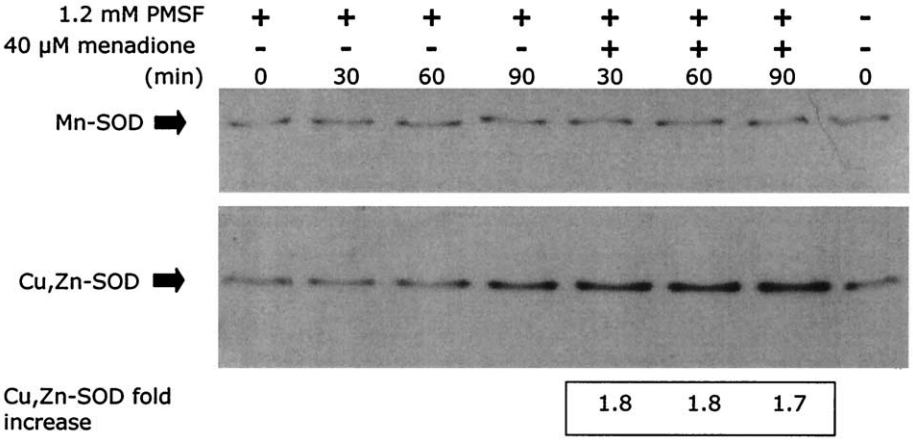

Fig. 5. Effect of menadione exposure and proteolysis inhibition by PMSF on steady state MnSOD and Cu,ZnSOD levels determined by Western blot. PMSF (1.2 mM in ethanol) was added to EG103 cells and incubated for 90 min prior to the addition of 40 μM menadione (time 0). Cells were further incubated with or without menadione and aliquots were analyzed at the indicated times (min). Control cells were incubated with the same concentration of ethanol used in PMSF-treated cells for 90 min. MnSOD was detected with rabbit anti-Mn superoxide dismutase antibody and Cu,ZnSOD detection with rabbit anti-Cu/Zn superoxide dismutase antibody. Fold increase in the ratio of Cu,ZnSOD protein levels in the presence of menadione relative to the Cu,ZnSOD protein levels in the absence of menadione at the same incubation times is shown. The Western blots shown are representative of three independent experiments.

exposure to menadione in the presence of PMSF compared to exposure to just PMSF (Fig. 5). The results in Figs. 3 and 5 can be explained if both MnSOD protein synthesis and degradation rates are unaltered by exposure to menadione. The results in Fig. 5 also indicate that probably for both enzymes there is a decreased translational efficiency, since increases in protein synthesis as measured by protein levels when proteolysis is inhibited do not reflect the increases in mRNA levels found in Fig. 2.

DISCUSSION

Our results in stationary phase *S. cerevisiae* cells exposed to menadione show that the increases found in MnSOD, Cu,ZnSOD, and GR mRNA steady state levels were not paralleled by increased protein levels and enzymatic activities. As can be seen in Table 2, oxidative stress modifies MnSOD, Cu,ZnSOD, and GR gene expression in a complex way, not only at the transcription level but also at the post-transcriptional, translational, and post-translational levels. Gene expression of all three antioxidant enzymes seems to be differentially regulated during menadione-induced oxidative stress. This differential regulation may be associated with the relative importance of the three enzymes for cell survival during menadione-induced oxidative stress. In fact, for Cu,ZnSOD, a fundamental enzyme for cell survival, gene expression is under a complex regulation since there are increases in the transcription rate, mRNA stability, translation rate, and proteolysis rate, resulting in the maintenance of steady state protein levels and activity. The increase in mRNA stability besides transcription rate indicates that oxidative stress can exert regulatory actions through different mechanisms.

For MnSOD there is an increased transcription rate and decreased mRNA stability, resulting in increased mRNA levels; but, since the translation and proteolysis rates are maintained, protein levels are unchanged and the enzyme is partially inactivated as shown by the decreased activity. For GR there is an increased transcription rate and decreased mRNA stability, and unchanged enzymatic activity. Our results indicate that the study of gene expression regulation by oxidative stress is intricate because, besides regulatory actions, nonspecific damaging effects are at work and these damaging effects may preclude the effect of regulatory responses required by the cell to overcome oxidative stress efficiently.

Table 2. Gene Expression of MnSOD, Cu,ZnSOD, and GR After Exposure to Menadione

	MnSOD	Cu,ZnSOD	GR
mRNA steady state level	↑	↑	↑
mRNA stability	⓪	⓪	⓪
Transcription rate[a]	⓪	⓪	⓪
Protein steady state level	=	=	?
Activity	↓	=	=
Translational efficiency[b]	⓪	⓪	?
Protein synthesis rate[c]	=	↑	?
Proteolysis rate[c]	=	↑	?

Regulatory actions are indicated within squares and putative damaging effects are indicated within circles.

[a] Inferred from mRNA steady state levels and mRNA stability.

[b] Inferred from mRNA and protein steady state levels.

[c] Inferred from the protein steady state levels in the presence and absence of PMSF.

Antioxidant enzymes-increased gene expression has been postulated to play a major role in the oxidative stress response by allowing an increased content and activity of antioxidant enzymes, and some studies of the effect of oxidative stress in mammalian cells have found increases in antioxidant enzymes activity and protein level that follow increases in mRNA levels [6,42], which are in agreement with this point of view. However, other studies with mammalian cells and tissues have found a noncorrelation between the increases found in antioxidant enzymes mRNA and activities or protein content of these enzymes under oxidative stress induced by hyperoxia [7,10], peroxynitrite [11], hydrogen peroxide [8], and paraquat [9]. Suggestions have been made that there is a translational block for the synthesis of antioxidant enzymes during oxidative stress [7–10]. In this perspective, the increased mRNA levels of antioxidant enzymes found during oxidative stress may occur to compensate for the decrease of translational efficiency and to maintain the protein levels and enzymatic activities.

Our findings indicate that a decrease in translational efficiency is probably occurring for both MnSOD and Cu,ZnSOD during menadione-induced oxidative stress (Table 2). However, our results for Cu,ZnSOD also show that menadione-induced oxidative stress, besides decreasing translational efficiency, increases the proteolysis rate, and that the increased input from transcription and translation may just be compensating for this increased proteolysis. This increased turnover that prevents a decrease in steady state protein levels and activity, which could exacerbate oxidative stress-mediated cytotoxicity, allows the maintenance of cell viability. So, this work shows for the first time that an apparent lack of cell response to low oxidative stress (maintenance of protein levels and activity) may, in fact, be the result of a highly dynamic response in terms of gene expression to keep protein homeostasis. Still, when the rate of inactivation exceeds that of transcription and translation, as found in the case of MnSOD where the enzymatic activity is partially lost, this compensatory response may be inadequate. Thus, the loss of cell viability found after 90 min of menadione treatment is probably related to mitochon-

drial damage accumulated over time due to the loss of adequate protection by MnSOD.

In conclusion, results from this study provide further understanding of the mechanisms of antioxidant enzyme regulation and show that oxidative stress may inactivate antioxidant enzymes in vivo without loss of cell viability, therefore explaining paradoxical results. Also, our findings indicate that assessing cell capacity to overcome oxidative stress by increases in transcription rates or mRNA steady state levels is not a good approach, since mRNA levels may be increased without increases in protein levels. On the other hand, maintenance of protein levels and activities can also be misleading since it can cover up a highly dynamic response of the cell that keeps homeostasis. Any meaningful study of oxidative stress-induced gene expression must be integrative, following gene expression at all levels from gene to protein.

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ABBREVIATIONS

BSA—bovine serum albumin
Cu,ZnSOD—copper,zinc superoxide dismutase
GR—glutathione reductase
HEPES—N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
MnSOD—manganese superoxide dismutase
MOPS—morpholinepropanesulfonic acid
PBS—phosphate-buffered saline
PMSF—phenylmethylsulfonyl fluoride
ROS—reactive oxygen species
SDS—sodium dodecyl sulfate