Cu,Zn-superoxide dismutase of *Saccharomyces cerevisiae* is required for resistance to hyperosmosis

Adriana Garay-Arroyo[a], Fernando Lledías[a,b], Wilhelm Hansberg[b], Alejandra A. Covarrubias[a,*]

[a] *Departamento de Biología Molecular de Plantas, Instituto de Biotecnología, Apdo. Postal 510-3, Cuernavaca 62250, Morelos, Mexico*
[b] *Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, México D.F., Mexico*

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Abstract Here we analyzed the role of the antioxidant response in *Saccharomyces cerevisiae* adaptation to hyperosmotic stress. We show that Cu,Zn-superoxide dismutase (SOD1) plays a fundamental role in this adaptation process since under hyperosmosis *SOD1* mutants lead to high protein oxidation levels and show a sensitive phenotype, which is reversed by the addition of *N*-acetylcysteine to the medium. Pretreatment with $MnCl_2$, a superoxide scavenger, improves the survival of the *sod1* strain upon hyperosmosis. Additionally, we show that upon hyperosmotic shock there is a small and transient increase in *SOD1* transcript levels, regulated by the protein kinase A-cAMP and SKN7 pathways.

Key words: Cu,Zn-superoxide dismutase; Protein kinase A pathway; Protein oxidation

1. Introduction

In different organisms, reactive oxygen species (ROS) are generated through normal metabolic activity and under stress situations by a variety of mechanisms [1,2]. One primary product of electron leakage from respiratory chain is the superoxide radical (O_2^-) generated by the one-electron reduction of O_2. This anion disturbs the redox balance of cells by reducing and releasing metal ions, particularly bound Fe(III) or Cu^{2+} in proteins, or by converting to other ROS including hydroperoxyl radical and H_2O_2. Reduced metal ions and H_2O_2 undergo the Fenton reaction generating the most reactive hydroxyl radical ($OH^{\bullet}$). Besides, spontaneous dismutation of O_2^-, iron-catalyzed reaction of O_2^- with H_2O_2, and decomposition of H_2O_2 can give rise to singlet oxygen ($^1O_2$). $OH^{\bullet}$ and $^1O_2$ damage cells by reacting with many cellular molecules (reviewed in [2]). An imbalance between ROS generation and the basal antioxidant cell response leads to an oxidative stress situation in which ROS production is accentuated, triggering an antioxidant defense system [1].

In yeast, common features have been found in cell responses to oxidative and other stress conditions [3]. In particular, a substantial overlap has been detected in gene expression patterns obtained upon osmotic, heat shock, and oxidative stress [3,4]. Upon osmotic shock, a number of genes encoding enzymes implicated in oxidative damage repair are induced, some of them under the control of osmotic signals [3,5]. That is the case of the cytoplasmic catalase gene (*CTT1*), required for detoxification of H_2O_2, whose expression in response to hyperosmosis is dependent on the 'general stress response' and the HOG pathway [6,7].

To get insight into the role of the antioxidative response in the adaptation of yeast cells to osmotic stress, we analyzed the phenotype of different mutants affected in enzymes involved in ROS detoxification, and determined the protein oxidation levels upon osmotic shock. In this work, we show that the antioxidative response is implicated in *Saccharomyces cerevisiae* adaptation to hyperosmotic stress. We present evidence which indicates that Cu,Zn-superoxide dismutase (SOD1) plays a relevant role in this adaptation process since *SOD1* mutants render yeast cells osmosensitive. We also show that the response of the *SOD1* gene to osmotic shock is regulated by the protein kinase A (PKA)-cAMP and SKN7 pathways rather than by the HOG pathway.

2. Materials and methods

2.1. Yeast strains and growth conditions

The strain used in this study was wild-type RS58 and mutant RS58-*hog1* [8]. Cells were grown as batch cultures in YPDA [8] to an OD_{660} of 0.5 (±0.1). At this point, osmotic stress was applied by addition of sorbitol at a final concentration of 0.5 M. Samples were taken at different times to determine viability and to obtain total RNA and protein. Serial dilutions of cells were grown in plates with different osmotic concentrations or with *N*-acetylcysteine (NAC; 30 mM), a scavenger of reactive oxygen intermediates and precursor of glutathione, as antioxidant [9]. Cells were grown to an optical density of 0.5_{660} in YPD medium at 25°C. Yeast cultures were subjected to $MnCl_2$ pretreatments by the addition of $MnCl_2$ to a final concentration of 4 mM; after 30 min, $MnCl_2$ was removed by washing cells twice with water [1]. Thereafter, yeast cells were grown in solid medium containing NaCl, as indicated. To determine protein oxidation and enzymatic activity, cell samples were washed with water and acetone powders were made. When stress pretreatments were carried out, yeast cells were grown as batch cultures in YPDA to an OD_{660} of 0.5 (±0.1). At this point, pretreatments were achieved for 1 h by addition of either NaCl to a final concentration of 300 mM, or H_2O_2 to a final concentration of 200 μM. Then, cells were centrifuged and resuspended in 3.0 M NaCl solution and incubated for 8 h with vigorous shaking at 25°C. In all cases, viability was measured by plating the appropriate dilution of cells on YPD plates, in duplicate, and expressed as a percentage of the initial colony forming units, before the stress shock. The number of colony forming units was obtained using COVASIAM [10].

*Corresponding author. Fax: (52)-777-313 9988.
E-mail address: crobles@ibt.unam.mx (A.A. Covarrubias).

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2.2. RNA purification and RNA blot hybridization analysis

Total RNA was extracted as indicated by Collart and Oliviero [11]. For RNA blot hybridizations, 10 μg of total RNA was separated by electrophoresis in formaldehyde 1.2% (w/v) agarose gel and blotted to Hybond N^+ membrane (Amersham) following standard protocols. Probes were labeled with a commercial kit (Dupont) using α-^{32}P (3000 Ci/mmol; Amersham). High stringency conditions were used for all hybridizations and washes.

2.3. Hybridization probes

The *SOD1* full-length gene sequence was obtained by polymerase chain reaction (PCR) amplification using specific primers designed according to the known sequences. Primers used were 5′-TTG TGG CCG CAG CCTC CGG C-3′ and 5′-CCC ACC CCA GCA CGC CGG GG-3′. PCR reactions using genomic DNA as template were as follows: 35 cycles of 30 s at 94°C, 30 s at 55°C and 45 s at 72°C. The PCR product was used as probe for RNA blot hybridizations. The *CTT1* probe was obtained from a genomic clone.

2.4. Constructions of yeast strains carrying disrupted genes

Disruptions of the *CTT1*, *CTA1*, *HPA1*, *SOD1* and *SKN7* genes were obtained from Euroscarf Company. The *HOG1* disruption strain was kindly given by S. Hohmann and *BCY1* and *SKO1* disruptions by J. Folch.

2.5. Protein oxidation

Protein oxidation was determined by the number of carbonyl groups in total protein that reacted with phenylhydrazine [12].

2.6. Catalase and SOD activity assays

Catalase activity was determined by the initial rate of dioxygen production using a Clark microelectrode [13]. Units are defined as μmol O_2 produced per min per mg of protein. SOD activity was measured according to Flohé and Otting [14].

3. Results

3.1. Antioxidant response in cell adaptation to hyperosmotic stress

To assess the role of antioxidant response in the adaptation of yeast cells to hyperosmosis, a priming oxidative treatment was imposed to exponentially growing cultures (0.5 ± 0.1 OD_{660}) and compared with a culture primed with NaCl. The oxidative and osmotic pretreatments consisted in 1 h incubation in medium containing 200 μM H_2O_2 or 300 mM NaCl. Thereafter, severe osmotic stress was imposed by incubating cells with 3.0 M NaCl. Non-pretreated cultures showed a dramatic loss of viability under these conditions (0.007 ± 0.001). Primed cells with oxidative pretreatment showed a higher index of survival (25-fold over non-primed cells) and, as expected, cultures subjected to NaCl pretreatment had the highest survival index (470-fold over non-primed cells). These data strengthen the idea that antioxidant enzymes could be part of the adaptive response to hyperosmosis.

3.2. Osmosensitive phenotype of mutant strains affected in ROS detoxification

To characterize the participation of the antioxidant response in yeast adaptation to hyperosmosis, we analyzed the phenotype under osmotic stress of five mutant strains affected in genes whose products are involved in enzymatic ROS detoxification (CTA1, CTT1, HPA1, SOD1 and SOD2), and of two strains affected in genes encoding transcription factors implied in yeast antioxidant response (Skn7p and Yap1p). Cells were plated onto solid media containing different sorbitol (1.6, 1.8, 2.0, 2.2, 2.5 M) or NaCl (0.4, 0.6, 0.8, 1.0, 1.2 M) concentrations and growth was scored. *sod1* cells exhibited a slow growth rate in 1.8 M sorbitol, in 1 M NaCl (Fig. 1A), and in 0.6 M NaCl (Fig. 1B), denoting a salt- and osmosensitive phenotype. No effect on growth was detected under lower concentrations of sorbitol or NaCl. The other six mutant strains had wild-type sensitivity to these osmotic stress conditions. In another assay, which involved mild hyperosmotic pretreatment (300 mM NaCl) followed by a severe osmotic stress (3 M NaCl) [6], *sod1* cells also showed increased sensitivity, four-fold higher than *ctt1* cells (data not shown, [6]. This was not the case for the other five mutants.

To confirm that superoxide radical was involved, we analyzed the effect of pretreatments with $MnCl_2$, an O_2^- scavenger, on the osmosensitive *sod1* strain. Pretreatment with 4 mM $MnCl_2$ for 30 min led to a three-fold increase in the survival of *sod1* cells upon osmotic stress (Fig. 2A). The addition of NAC, a widely used antioxidant [15,16], showed a stronger reversion of the salt- and osmosensitive phenotypes of *sod1* mutant than $MnCl_2$ (Fig. 2B).

3.3. Regulation of SOD1 transcript during hyperosmosis

Total RNA was obtained from yeast cultures subjected to osmotic shock (0.5 M sorbitol) for the time periods indicated and *SOD1* transcript was analyzed. *SOD1* transcript level increased (40%) in response to osmotic treatment. Maximal transcript accumulation was observed after 20 min, and then, after 60 min the initial level was attained (Fig. 3). A similar accumulation pattern was found for *CTT1* transcript

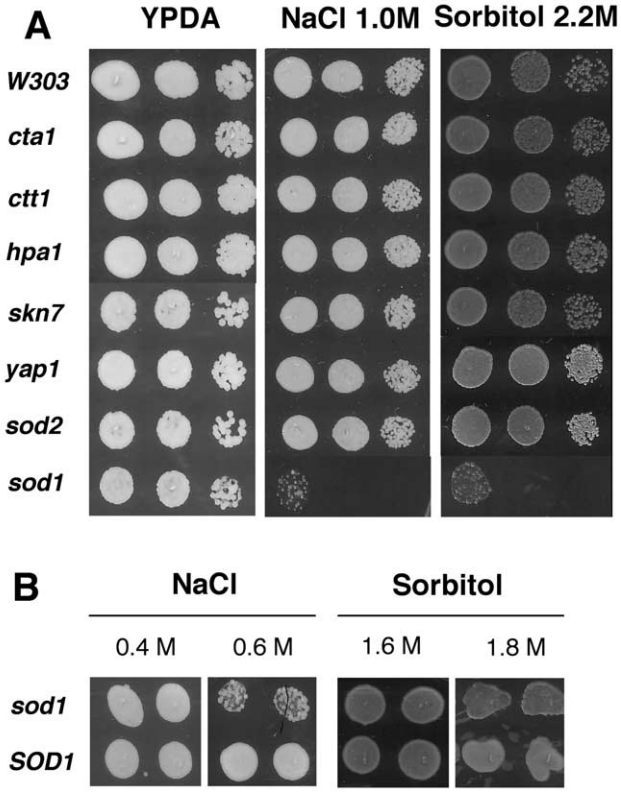

Fig. 1. Cellular role of *SKN7*, *YAP1*, *CTA1*, *CTT1*, *HPA1*, *SOD2* and *SOD1* genes during hyperosmosis. A: Deletion of *CTA1*, *CTT1*, *HPA1*, *SOD2*, *SKN7* or *YAP1* does not affect growth in high osmolarity medium. Deletion of the *SOD1* gene renders cells osmosensitive. B: This is evident only when the yeast cells were grown under high osmolarity. Results in this figure were reproduced in at least three independent experiments.

as well as for several other osmoresponsive genes (data not shown) [8,17].

The HOG pathway is the best-characterized regulatory system for osmoresponsive genes in *S. cerevisiae*. Mutations in this mitogen-activated protein kinase render cells osmosensitive [3]. A strain containing a *HOG1* disruption was used to determine *SOD1* transcript levels during osmotic shock. SOD1 transcript accumulation in *hog 1* was similar to wild-type, indicating that *SOD1* osmotic response is HOG-independent. Consistent with this finding, a mutant strain in the *SKO1* gene, which encodes one of the six transcriptional factors that control a subset of the Hog1p target genes [18], does not show significant changes in *SOD1* transcript accumulation levels in response to hyperosmosis (Fig. 3).

The cAMP-PKA pathway negatively controls the expression of osmoresponsive genes. Mutations in the PKA regulatory subunit gene (*bcy1*) result in high cAMP-independent PKA activity, and in repression of several osmoresponsive genes [3]. Fig. 3 shows that the absence of BCY1 abolishes the *SOD1* transcript osmotic response.

The MSN2–MSN4 transcription factors mediate the response to osmotic stress of many genes [19]. In a double *msn2–msn4* mutant, no accumulation of *SOD1* transcript was detected in response to hyperosmosis (Fig. 3).

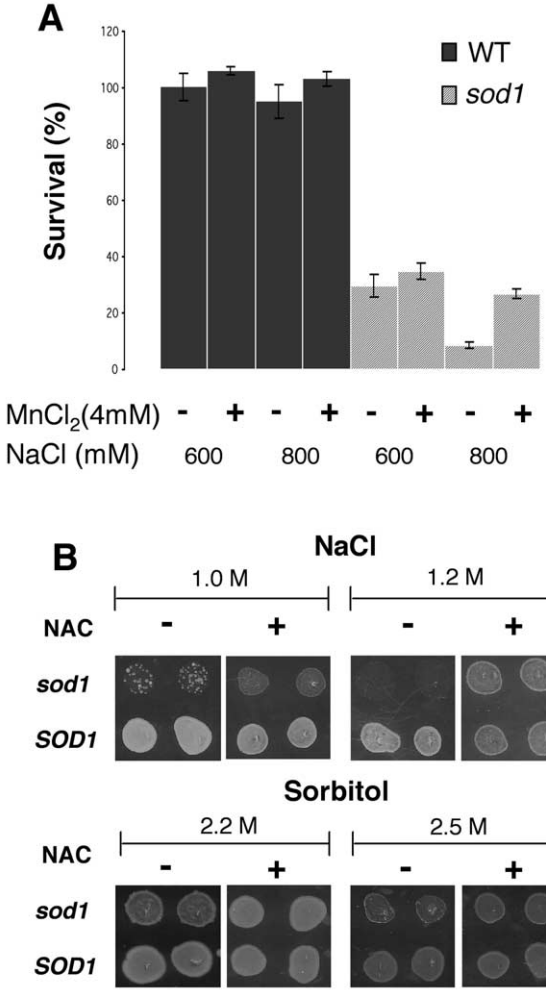

Fig. 2. Effect of antioxidants on the osmosensitivity of *sod1* yeast cells. S.D. was obtained from three independent experiments ($P < 0.05$). A: $MnCl_2$ treatment increases survival of *sod1* cells under hyperosmosis. B: Osmosensitivity of *SOD1* mutant is reversed by the addition of NAC.

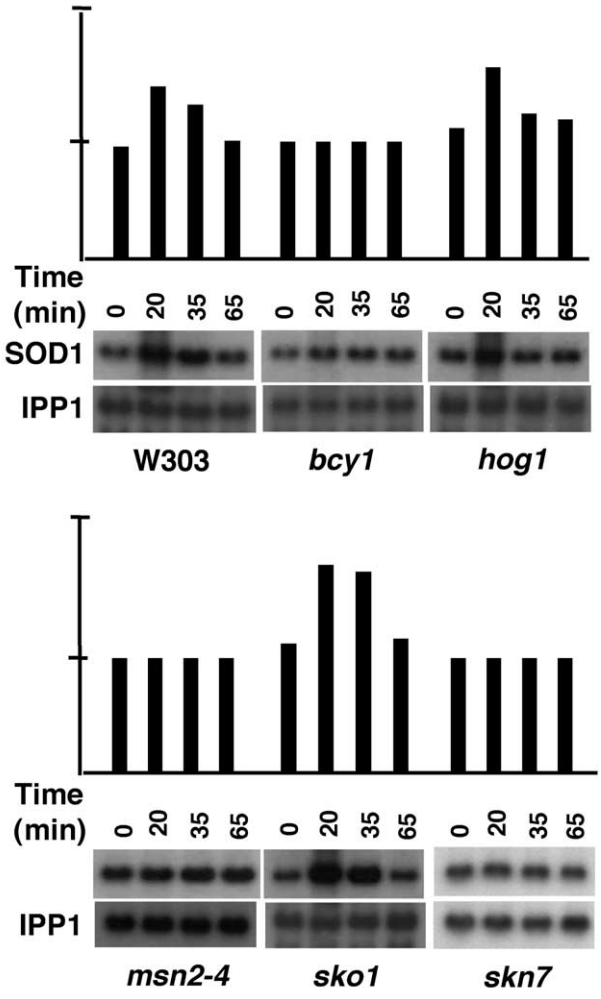

Fig. 3. Effect of mutations in *HOG1*, *BCY1*, *MSN2–MSN4*, *SKO1* and *SKN7* genes on the accumulation of *SOD1* transcript upon osmotic shock. Wild-type, *hog1*, *bcy1*, *msn2–msn4*, *sko1* and *skn7* strains were subjected to 0.5 M sorbitol treatment during the indicated periods (20, 35, 65 min). *IPP1* transcript, encoding inorganic pyrophosphatase, was used as a loading reference. *SOD1* transcript levels were determined using the NIH image 1.62 software and were normalized relative to the intensity of *IPP1* and *SOD1* (control condition) transcripts. The results in this figure are representative of at least three independent experiments.

Transcription factors Yap1p and Skn7p are implicated in the signal transduction pathway that controls oxidative stress response in *S. cerevisiae* [20]. However, several lines of evidence indicate that the Skn7p response regulator is also involved in the HOG pathway, in cell wall integrity pathways, cell cycle-dependent transcription and cellular responses to hypo-osmotic stress [3,21]. Fig. 3 shows that Skn7p is required for *SOD1* gene expression in response to hyperosmosis.

3.4. CTT1 and SOD1 activities in response to hyperosmosis

CTT1 transcript was accumulated after 20 min of a hyperosmotic treatment (0.5 M sorbitol) (Fig. 4). In agreement, CTT1 activity started to increase within the first 20 min of hyperosmosis, attaining a maximum at 35 min, and a high activity level was maintained during the next 30 min (Fig.

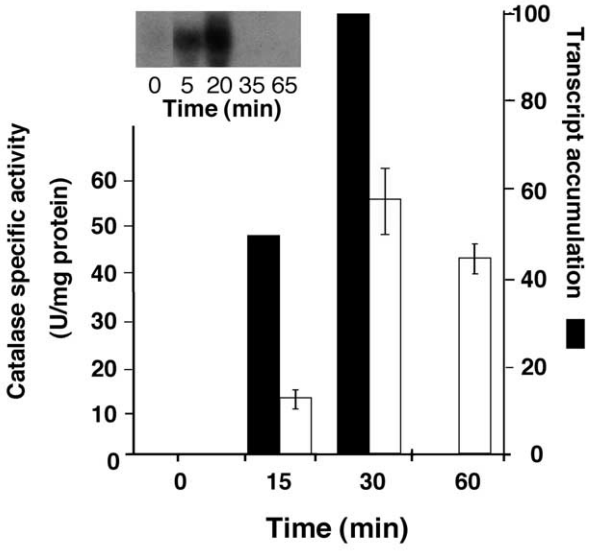

Fig. 4. Transcript and activity levels of CTT1 in response to hyperosmosis. Total RNA was obtained from RS58 wild-type strain grown to an OD_{660} of 0.5 on YPDA and stressed with 0.5 M sorbitol for the times indicated. CTT1 transcript levels were obtained as in Fig. 3. Total catalase activity was determined as described in Section 2. Units are defined as μmol O_2 produced per min per mg of protein. S.D. was obtained from at least four independent experiments ($P < 0.05$).

4). This CTT1 response to hyperosmotic stress was not detected in a *hog1* strain (data not shown).

In the case of *SOD1*, a high specific activity was detected under non-stress conditions, but no changes were detected in response to hyperosmosis in wild-type and in mutant strains (*bcy1*, *hog1*, *msn2–msn4*, *skn7*, *sko1*) (data not shown).

3.5. Protein oxidation in response to hyperosmotic stress

Oxidative damage to proteins can be evaluated by titration of carbonyl groups generated in some amino acid side chains during stress conditions [22]. Carbonyl accumulation in total protein was determined during osmotic stress in wild-type, *hog1* and *sod1* strains in an RS58 background. Wild-type strain showed a decrease in carbonyl content after 35 min of hyperosmotic treatment, reaching lower levels than under non-stressed conditions (Fig. 5). Total protein oxidation in *hog1* was maintained at similar levels during hyperosmotic stress whereas the *sod1* strain showed the highest carbonyl content, 2.5-fold increase after 65 min treatment (Fig. 5).

4. Discussion

The results in this study show that the antioxidative response plays a relevant role in *S. cerevisiae* adaptation to hyperosmosis. Our data indicate that SOD1 constitutes a fundamental factor in this adaptation process since the absence of this enzyme leads to osmosensitivity in yeast cells and confirm that oxidative stress is a cause of lethal damage during hyperosmotic stress conditions. From the different genes of the antioxidant response tested, *CTT1* and *SOD1* were found to be more involved in the defense against the damage caused by hyperosmosis. The requirement of CTT1 is evident when yeast cells are exposed to a severe osmotic stress after pretreatment with a mild stress (this work and [6]). The absence of SOD1 has a major effect considering that, in contrast to the *cct1* mutant, osmosensitivity in the *sod1* mutant was detected in high osmolarity media without any pretreatment suggesting that, under aerobic conditions, superoxide-derived oxidative damage plays a major role during hyperosmosis in yeast. This hypothesis is supported since, under these stress conditions, the carbonyl content in the *sod1* mutant is higher than in *hog1* or wild-type cells (see Fig. 5). The presence of SOD1 activity, even under non-stress conditions, avoids the superoxide toxicity to lethal levels. Once the superoxide is dismutated to peroxide, this can be detoxified by different activities such as catalases and peroxidases. This may explain why a *sod1* mutant shows a more pronounced osmosensitivity than a mutant lacking CTT1.

SOD1 and CTT1 activities are also present under mild hyperosmotic stress; however, the physiological relevance of SOD1 and CTT1 is mainly upon severe hyperosmosis, indicating that this leads to higher ROS accumulation levels than

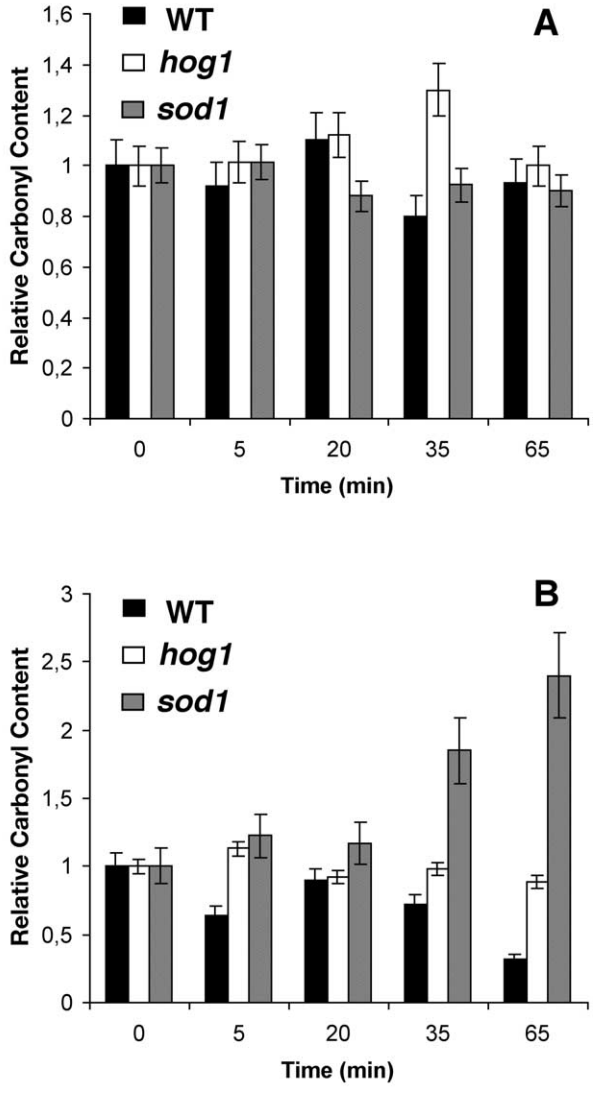

Fig. 5. Carbonyl content in total protein from wild-type, *hog1* and *sod1* cells upon hyperosmotic conditions. A: Non-stress conditions (YPDA). B: Hyperosmotic conditions (YPDA+0.5 M sorbitol). S.D. was obtained from at least four independent experiments ($P < 0.05$).

those present under mild conditions. Protein oxidation levels determined in yeast cultures upon severe hyperosmosis (1 M sorbitol or higher) support this assumption (unpublished results).

The involvement of SOD1 in the adaptive response to ionic stress in yeast is suggested since the sensitivity shown by the *sod1* mutant in media containing NaCl in a lower osmolality (from 0.6 M = −27 bar) than sorbitol (1.8 M = −66 bar) indicates a contribution of the ionic component. This may be inducing higher ROS levels or may have an inhibitory effect on the oxidative stress defense systems. Accordingly, *sod1* cells also show sensitivity when exposed to 100 mM LiCl (data not shown).

The accumulation of ROS and their involvement in lethal damage to yeast cells during hyperosmosis was confirmed since treatments with NAC suppress the osmosensitivity of *sod1* cells. In particular, the fact that $MnCl_2$ treatments led to a three-fold increase in *sod1* survival upon osmotic stress validates the involvement of superoxide toxicity and supports a physiological role of SOD1 in ROS detoxification under this stress condition. A direct demonstration of increased ROS generation during hyperosmosis is shown by the high carbonyl content detected in *sod1* and *hog1* cells, in agreement with the *HOG1* dependence of *CTT1* and other oxido-reductases [5,21]. Accordingly, *hog1* and *sod1* mutants show the highest carbonyl content after 65 min of stress treatment, when wild-type cells present the most efficient antioxidant response, as indicated by their low carbonyl content. This response occurred behind the accumulation of osmoresponsive transcripts and when the highest CTT1 activity levels were detected (see Fig. 3).

Even though many microarray studies have been done to characterize yeast gene expression in response to stress conditions [23,24], little is known about SOD1 gene regulation under hyperosmosis. In this work we also demonstrate that, even though *SOD1* transcript accumulates to constant and detectable levels under non-stress conditions, higher accumulation levels (40% induction) are reliably detected in response to osmotic shock. Accumulation of the *SOD1* mRNA appears to be negatively regulated by the 'general stress response' pathway, via PKA, since the *SOD1* transcript does not accumulate in the absence of the PKA regulatory subunit (Bcy1p). Accordingly, *SOD1* osmotic response is mediated by the Msn2–4 transcription factors. Involvement of Skn7p in *SOD1* transcription regulation in response to hyperosmosis suggests that this transcriptional factor may modulate the osmoresponse of this gene by sensing ROS accumulation or cell wall modifications induced by this condition.

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