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Antioxidants protect the yeast *Saccharomyces cerevisiae* against hypertonic stress

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Abstract

Yeast (*Saccharomyces cerevisiae*) mutants lacking CuZnSOD have been reported to be hypersensitive to hypertonic media and to show increased oxidative damage. This study demonstrates that hypertonic medium (containing 0.8 M NaCl) increases the generation of superoxide and other reactive species in yeast cells. Other sequelae of exposure to hypertonic medium include oxidation of cellular low-molecular weight thiols and decrease in total antioxidant capacity of cellular extracts. Δ*sod1* mutant is more sensitive than a wild-type strain to colony growth inhibition on a hypertonic medium. Anaerobic conditions, ascorbate, glutathione, cysteine and dithiothreitol are able to ameliorate this growth inhibition but a range of other antioxidants does not protect. The protective ability of the antioxidants does not correlate with the rate of their reactions with superoxide but seems to be conditioned by low redox potential for one-electron oxidation of free radicals of the antioxidants. It suggests that repair of low-redox potential targets rather than prevention of their damage by superoxide is important in the antioxidant protection against oxidative stress induced by hypertonic conditions.

Keywords: Yeast, *Saccharomyces cerevisiae*, superoxide, superoxide dismutase, osmotic stress, oxidative stress

Introduction

It has been suggested repeatedly that cellular effects of osmotic stress (hypertonic treatment) and heat shock involve oxidative stress. The main reason for such an assumption was the adaptive cross-resistance of cells challenged with low-dose osmotic, heat or oxidative stress [1–4]. In line with these findings, *E. coli sodAsodB* mutants lacking cytosolic superoxide dismutases were demonstrated to be more sensitive to hyperosmotic conditions [5]. It has been recently demonstrated that yeast mutants devoid of CuZn-SOD show increased sensitivity to hyperosmotic stress induced by media high in sorbitol or NaCl. The hyperosmotic shock appears to involve increased generation of reactive oxygen species as judged on the basis of accumulation of protein oxidation products (carbonyl groups) and restoration of growth in hyperosmotic conditions by cell pretreatment with 4 mM $MnCl_2$ or by addition of 30 mM N-acetylcysteine to the medium [6]. This study was aimed at getting further insight into the role of oxidative stress as a possible mediator of hyperosmotic stress, by demonstration of increased production of ROS in yeast subjected to hyperosmotic treatment and by showing the ability of a range of antioxidants to ameliorate the growth inhibition caused by hyperosmotic medium.

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Material and methods

Yeast strains and growth conditions

The following yeast strains were used: wild-type SP-4 (MATα leu1 arg4) and its CuZnSOD disruptant *sod1::natMX* obtained by one-step gene replacement. The SP-4 strain was obtained from crosses between the S2615B strain (Donner Laboratory, University of California at Berkeley) and the 55R5/3c strain (CNRS, Gif sûr Yvette), by screening the progeny of sporulating spontaneously (SP) formed diploids for arginine deficiency (Δ*arg4*) [7]. Yeast were grown either on liquid YPD medium (1% Difco Yeast Extract, 1% Yeast Bacto-Peptone and 2% glucose), with or without addition of 0.8 M NaCl, or, in several dilutions, on plates with solid YPD medium, containing 2% agar and 0.8 M NaCl. Stock solutions of antioxidants were added to sterile media; in the case of solid media, they were cooled to just above the solidification point before addition of antioxidants. All antioxidants stock solutions were freshly prepared before adding to the media. In experiments under anaerobic conditions, the cells were grown on YPD plates under the atmosphere of nitrogen.

Total antioxidant capacity and thiol content

The yeast was grown in liquid YPD to the late logarithmic phase of growth, centrifuged, washed and suspended in 100 mM phosphate buffer, pH 7, with 0.1% glucose and 1 mM EDTA, with or without 0.8 M NaCl, to the density of 1×10^8 cells ml^{-1}. This suspension was incubated at 28°C with shaking. Samples were taken after 15 min, 1, 2 and 3 h of incubation, centrifuged, washed and resuspended in 20 mM phosphate buffer, pH 7, to the density of 1×10^9 cells ml^{-1}, added with an equal volume of ice-cold 5% trichloroacetic acid (TCA), vortexed, placed on ice and centrifuged. The supernatant was used for spectrophotometric assays of thiol content and total antioxidant capacity (TAC). Thiols were estimated with the Ellman reagent [8] and TAC by a modification [9] of the assay of reduction of 2,2′-azinobis(3-ethylbenzthiazoline sulfonate) cation radical ($ABTS^{+\cdot}$) [10,11]. The $ABTS^{+\cdot}$ reduction was read after 10 s (such measurement reflects the content of fast reacting antioxidants) and after 1 min (results of this measurement are contributed partly by slow reacting antioxidants but are more precise) [9,12,13].

Generation of reactive oxygen species

Cells were washed and suspended in 100 mM phosphate buffer pH 7 with 0.1% glucose, 1 mM EDTA, without or with 0.8 M NaCl, to the density of 1×10^8 cells ml^{-1}. Generation of superoxide was assessed with dihydroethidine (HE; Molecular Probes, OR, USA; 18.9 μM) [14] and generation of peroxides with 2′,7′-dichlorodihydrofluorescein acetate (H_2DCF-DA; Sigma, Poznan, Poland; 27 μM). Kinetics of fluorescence increase, due to oxidation of 2′,7′-dichlorodihydrofluorescein (H_2DCF) to 2′,7′-dichlorofluorescein (DCF), was measured using a Hitachi F2500 fluorescence spectrophotometer. Measurement conditions were: $\lambda_{ex} = 518$ nm, $\lambda_{em} = 605$ nm, for HE, and $\lambda_{ex} = 495$ nm, $\lambda_{em} = 525$ nm, for H_2DCF-DA; temperature, 28°C.

All results represent mean ± SD from at least three experiments or consistent results of a typical experiment reproduced at least three times.

Results and discussion

The rate of superoxide generation in 100 mM phosphate buffer, estimated by oxidation of HE, was higher in the CuZnSOD disruptant than in the isogenic wild-type (WT) strain. This rate was several-fold increased by placing the cells in the hypertonic medium, containing 0.8 M NaCl, both in the WT strain (about 5-fold) and in the Δ*sod1* strain (more than 11-fold). Considering the differences in the basal rates of superoxide generation, the Δ*sod1* cells were found to produce about 4-fold more superoxide than WT cells in the hypertonic medium (Figure 1).

The rate of oxidation of H_2DCF in cellular systems appears to reflect the generation of mainly hydrogen peroxide [15,16]. The rate of H_2DCF oxidation was elevated in the Δ*sod1* strain and significantly augmented in the hypertonic medium in both the WT and Δ*sod1* strain (Figure 2). The effect of

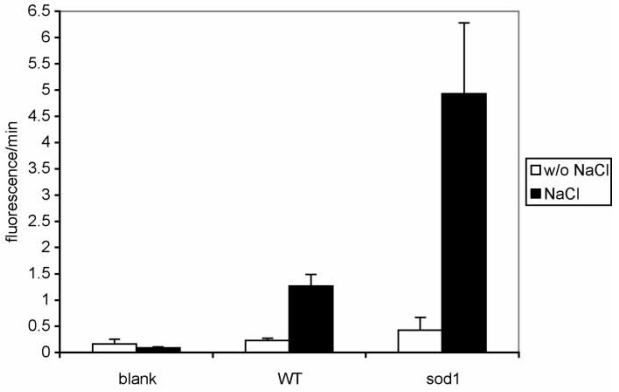

Figure 1. Superoxide production by *S. cerevisiae* cells in 100 mM phosphate buffer (with 0.1% glucose and 1 mM EDTA) and in the hypertonic medium (the buffer containing 0.8 M NaCl) estimated by the rate of increase of HE fluorescence. Cells grown in YPD medium were suspended in the phosphate buffer containing or not containing 0.8 M NaCl, immediately added with HE and placed in a spectrofluorimeter. Initial rate of fluorescence increase was measured (within 5 min). Mean values ± SD from three independent experiments. Blank: probe oxidation in the media in the absence of cells.

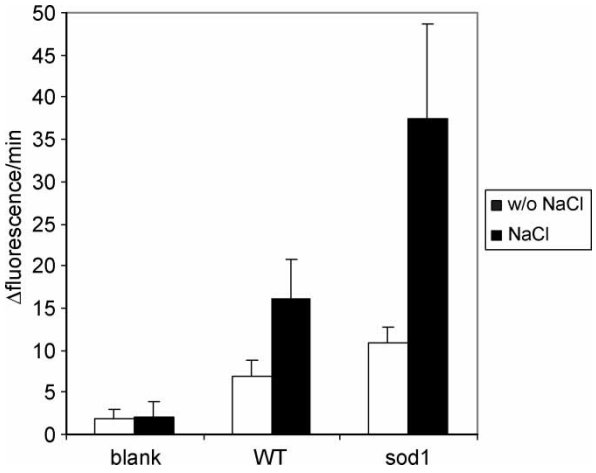

Figure 2. Peroxide production by *S. cerevisiae* cells in 100 mM phosphate buffer (with 0.1% glucose, 1 mM EDTA) and in the hypertonic medium (the buffer containing 0.8 M NaCl) estimated by the rate of increase of DCF fluorescence. Cells grown in YPD medium were suspended in the phosphate buffer containing or not containing 0.8 M NaCl, immediately added with H_2DCF-DA and placed in a spectrofluorimeter. The rate of fluorescence increase was measured within 30 min; maximal rate recorded within this time was assumed to be a measure of the rate of peroxide production. Mean values ± SD from three independent experiments. Blank: probe oxidation in the media in the absence of cells.

the hypertonic medium on HE and H_2DCF oxidation was clearly dependent on the phase of growth of the yeast, early exponential cultures being more sensitive to the hypertonic treatment than late exponential cultures, and stationary cultures showing much lower sensitivity (not shown).

Oxidative stress imposed on the yeast cells in the hypertonic medium can be expected to lead to depletion of cellular antioxidants. Indeed, the level of acid-soluble thiols (contributed mainly by glutathione) was progressively decreasing when the cells were incubated in the hypertonic medium (Figure 3).

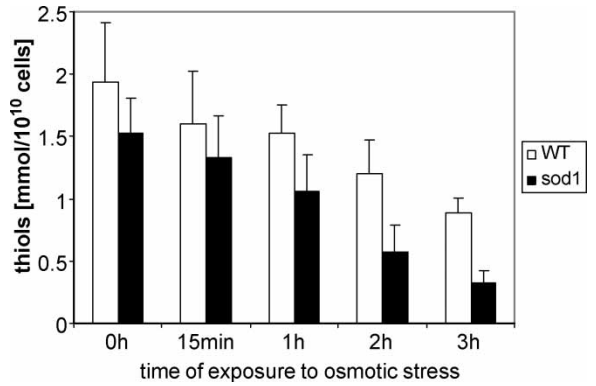

Figure 3. Decrease of thiol content in the acid-soluble fraction of yeast cells (WT and Δ*sod1*) during incubation in the hypertonic medium. No significant changes in the thiol level were observed during parallel incubation of cells in the control medium (not shown). Mean values ± SD from three independent experiments.

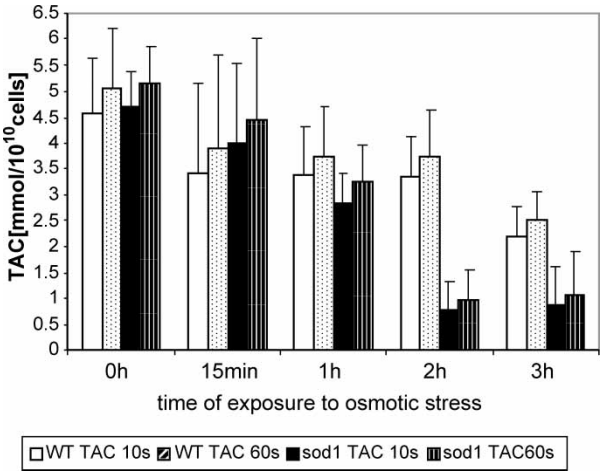

Figure 4. Decrease of TAC of the acid-soluble fraction of yeast cells (WT and Δ*sod1*) during incubation in the hypertonic medium. TAC was estimated by reduction of $ABTS^{+\cdot}$ measured after 10 s (results reflecting the activity of fast-reacting antioxidants) and after 60 s (results more reproducible but contributed by slow-reacting antioxidants). No significant changes TAC were observed during parallel incubation of cells in the control medium (not shown). Mean values ± SD from three independent experiments.

Total antioxidant capacity (TAC) of the acid-soluble fraction of the cells decreased in parallel to the thiol content (Figure 4), in agreement with our previous results showing that TAC of yeast [13] and mammalian [9] cell extracts is determined mainly by its thiol content. No decrease in the thiol level or TAC was observed under these conditions in the absence of 0.8 M NaCl (not shown).

It has been reported that hypertonic medium hampers the growth of colonies of yeast Δ*sod1* mutant [6]. We observed also growth restriction of the yeast by 0.8 M NaCl which in the liquid YPD medium was seen both for WT yeast and for the Δ*sod1* mutant (Figure 5) while only for the mutant on agar plates (Figure 6). Since the plates were read after 48 h while the growth curves were for only up to 24-h measurements, the delay in yeast growth could be apparently seen in the liquid medium but colonies hampered in growth could catch up on the solid medium.

The growth inhibition induced by the hypertonic medium was ameliorated by anaerobic conditions (Figure 6). Antioxidants such as ascorbate, glutathione, cysteine and dithiothreitol were also protective (Figure 7). These findings indicate that the growth inhibition by osmotic stress not only involves but is also mediated by oxidative stress. However, a range of other antioxidants including Trolox, Tempo, Tempol and melatonin was without effect (Table I). The selectivity of action of antioxidants is puzzling. One possible reason for the lack of effect of some antioxidants could be that they did not enter yeast cells. However, it does not seem probable as these compounds are mostly uncharged and moderately

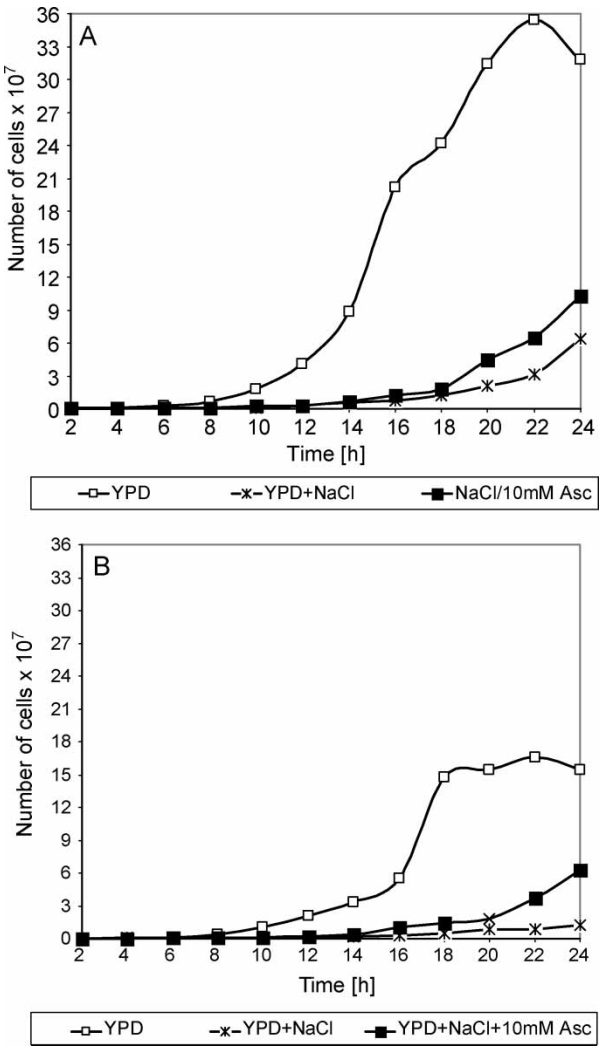

Figure 5. Growth curves of WT yeast (A) and Δ*sod1* mutant (B) in control YPD medium and in the hypertonic medium (YPD medium with 0.8 M NaCl) and in the hypertonic medium with 10 mM ascorbic acid. Cell concentration was determined by counting the cells in a Malassez chamber at appropriate times.

hydrophobic. There is circumstantial evidence for the penetration of numerous among the compounds studied into the yeast cells (e.g. intracellular reduction of nitroxides; not shown).

If the growth inhibition is mediated by superoxide, one should expect that antioxidants could substitute for superoxide dismutase by scavenging superoxide. In such a case, a correlation of the protective effect with the rate constants for the reaction with superoxide of the antioxidants studied should be expected. However, comparison of the rate constants of antioxidants for their reactions with superoxide with their ability to protect the Δ*sod1* yeast against hypertonic stress (Table II) does not show any relationship.

Another features of antioxidants could be taken into account to explain their selectivity in protection against hyperosmotic stress. A simple explanation would be that the action of an antioxidant on the cellular level is a result of a protective effect and possible toxic effects of the compound or of its metabolites. Therefore, the effects of all antioxidants tested on the growth of the Δ*sod1* strain on solid YPD medium (in the absence of hypertonic stress) was also studied. No enhancement of colony growth by any antioxidant tested was seen. No growth inhibition was seen for most of the antioxidants except for Tempo (moderately hampering colony growth at 0.5 mM and strongly inhibiting it at 1 mM concentration), Tiron (slightly inhibitory at 1 mM), tyrosine (slightly inhibitory at 2 mM), Trolox (moderately hampering growth at 1 mM) and melatonin (moderately inhibiting growth at 0.5 mM concentration (not shown)). Therefore, also the interplay of protection and toxicity does not explain the whole lack of effects of most antioxidants tested. *Nota bene*, the toxic effects of high concentrations of these compounds indicates that they enter the cells.

It has been pointed out that antioxidants added to the medium may alter the redox potential of the

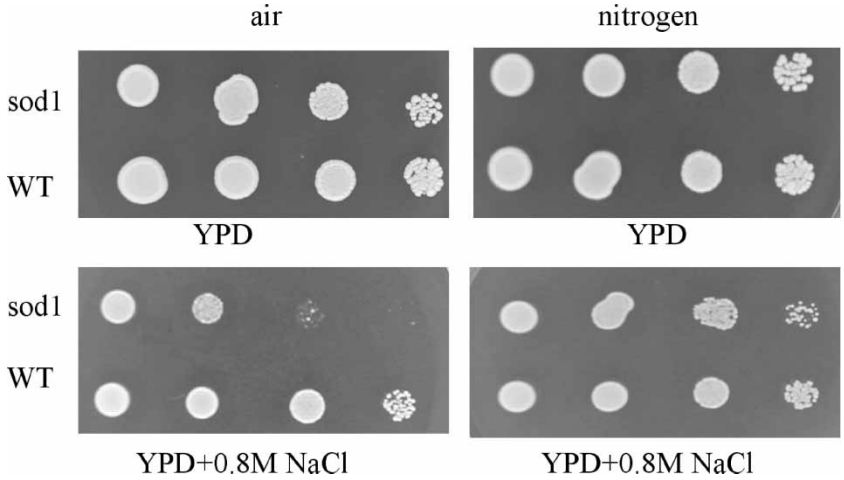

Figure 6. Effect of anaerobic atmosphere on the sensitivity of Δ*sod1* yeast cells to the hypertonic medium. Cells from logarithmic phase were plated at amounts of about: 10^5, 10^4, 10^3 and 10^2. Colony growth was recorded after 48 h.

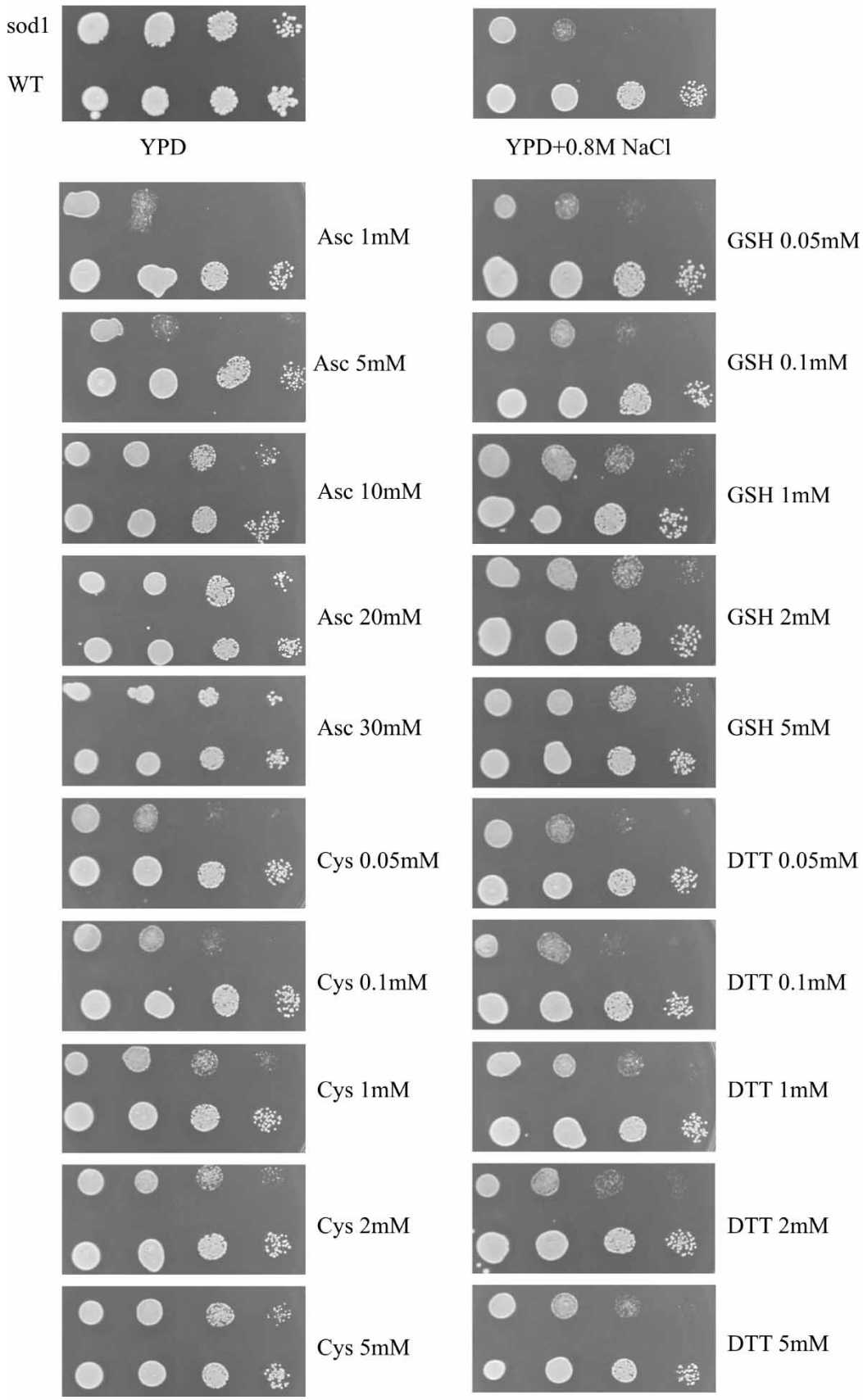

Figure 7. Effect of selected antioxidants on the sensitivity of Δ*sod1* yeast cells to the hypertonic medium. Cells from logarithmic phase were plated at amounts of about: 10^5, 10^4, 10^3 and 10^2. Colony growth was recorded after 48 h.

Table I. Effect of various antioxidants on the growth of Δ*sod1* mutant on the hypertonic solid YPD medium containing 0.8 M NaCl.

Antioxidant	Effects (mM)					Antioxidant	Effects (mM)		
Ascorbate	1	5	10	20	30	Serotonin	0.05	0.1	0.5
	−	−	+++	+++	+++		−	−	−
Cysteine	0.05	0.1	1	2	5	Quercetin	0.05	0.1	0.5
	+	+	++	++	+++		−	−	−
Deferoxamine mesylate	0.05	0.1	1			Tempo	0.1	0.5	1
	−	−	−				++	−	−
Dithiothreitol (DTT)	0.05	0.1	1	2	5	Tempol	0.05	0.1	1
	+	+	++	++	++		−	−	−
Reduced glutathione (GSH)	0.05	0.1	1	2	5	Tiron	0.1	0.5	1
	+	+	++	+++	+++		−	−	−
Melatonin	0.05	0.1	0.5			Trolox	0.1	0.5	1
	−	−	−				−	−	−
Pantothenic acid	1					Tyrosine	0.1	0.5	2
	−						−	−	−
Phenylbutylnitrone (PBN)	0.1	0.5	1			Uric acid	0.05	0.1	1
	−	−	−				−	−	−

(−) No improvement of growth; (+) (++) (+++) Low, medium and full restoration of growth, respectively.

medium and simulate anoxic conditions [17,18] which was protective. This effect cannot be completely excluded. High concentrations of oxidized GSSG (5 and 10 mM) which increase the redox potential of the medium hampered the growth of the mutant in hypertonic medium, and potassium ferricyanide (increasing the redox potential of the medium and not entering into the cells) were more harmful to the cells grown in the hypertonic medium than ferrocyanide (decreasing the redox potential of the medium; not shown). However, these effects were small and complicated by toxicity of both iron complexes used.

A next potentially relevant parameter is the redox potential of the antioxidants for one-electron reduction. It is obvious from Table III that compounds of high redox potential are not protective. Ascorbate, which protects against osmotic stress, has a low standard one-electron redox potential. Thiols, which also protect, have high redox potential for one-electron oxidation. However, it has been pointed out that thiol free radical readily associate with another thiol molecules forming a complex of extremely low potential (glutathione-glutathionyl radical, etc.) [19,20]. If to assume that the main reaction pathway of thiol radicals formed in reaction with superoxide would be further reduction to a dithiol, one could conclude that only antioxidants of low redox potentials for one-electron redox reaction protect against increased superoxide production under hyperosmotic conditions. What may be a mechanism of such action? Apparently, this type of selectivity of antioxidant action could be expected if there are critical targets in yeast cells of very low redox potential which could be easily oxidized by superoxide but also by secondary radicals of antioxidants formed in the course of superoxide scavenging. Fe–S clusters of cellular proteins critical for cell metabolism may be

Table II. Rate constants of various antioxidants for the reaction with O_2^- and their protective effect Δ*sod1* yeast against osmotic stress.

Antioxidant	Rate constant k [$M^{-1}s^{-1}$]	Reference	Protection against osmotic stress
Tyrosine	< 10	[24]	−
Melatonin	1.25×10^3	[25]	−
Cysteine	$> 5 \times 10^4$	[24]	+
Tempo	7×10^4	[26]	−
Deferoxamine mesylate	$< 2 \times 10^5$	[24]	−
Trolox	2.0×10^5	[24]	−
Ascorbate	2.7×10^5	[24]	+
Glutathione	6.7×10^5	[24]	+
PBN	$< 1 \times 10^6$	[24]	−
DTT	1.0×10^6	[24]	+
Tiron	1.0×10^7	[24]	−

Table III. Standard one-electron redox potentials of various antioxidants and protection of Δ*sod1* by the antioxidants against osmotic stress.

Antioxidant	One-electron redox potential E_o' [V]	Reference	Protection against osmotic stress
$RSSG^{\cdot -}/RSSG$	−1.5	[27]	+
$GS^{\cdot}/GS^-$	1.1	[28]	
Dithiothreitol$^{\cdot +}$/ Dithiothreitol	1.702	[29]	
$Ascorbate^{\cdot}/Ascorbate^-$	−0.174	[30]	+
Uric acid$^{\cdot +}$/ Uric acid	0.260	[29]	−
Serotonin$^{\cdot +}$/ Serotonin	0.364	[31]	−
Trolox$^{\cdot +}$/Trolox	0.480	[32]	−
Quercetin$^{\cdot +}$/ Quercetin	0.60	[33]	−
Melatonin$^{\cdot +}$/ Melatonin	0.660	[31]	−
Tempo oxoammonium cation/Tempo	0.722	[34]	−
Cysteine$^{\cdot +}$/Cysteine	0.730	[29]	+
Tempol oxoammonium cation/ Tempol	0.810	[34]	−
Tyrosine$^{\cdot +}$/Tyrosine	0.890	[35]	−

such targets. The standard redox potentials of Fe–S redox clusters in proteins are indeed low, ranging from −645 to 0 mV [21,22]. In *E. coli*, superoxide dismutase *sodAsodB* mutants show auxotrophy due to inactivation of enzymes containing Fe–S clusters involved in the biosynthesis of branched amino acids [23]. It can be expected that a similar damage limits the growth of *S. cerevisiae* deficient in cytosolic superoxide dismutase and may become a critical growth-limiting factor when the flux of superoxide is increased under hypertonic conditions.

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