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Limited Effectiveness of Antioxidants in the Protection of Yeast Defective in Antioxidant Proteins

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Efficacy of several antioxidants in the protection of the yeast *Saccharomyces cerevisiae* mutants deficient in CuZn-SOD and deficient in glutaredoxin 5 to growth restriction induced by oxidants was studied. Ascorbate and glutathione protected the *Δsod1* and *Δgrx5* mutants against the effects of *t*-butyl hydroperoxide and cumene hydroperoxide, *Δsod1* mutants against oxytetracycline and *Δgrx5* mutants against menadione and 2,2′-azobis-(2-amidinopropane). However, Tempol, Trolox and melatonin were much less effective, showing prooxidative effects and, at high concentrations, hampering the growth of the mutants in the absence of exogenous oxidants. These results point to a complication of cellular effects of antioxidants by their prooxidative effects and to the usefulness of cellular tests to evaluate the biological effectiveness of antioxidants.

Keywords: Yeast; *Saccharomyces cerevisiae*; Superoxide dismutase; Glutaredoxin 5; Ascorbate; Glutathione

INTRODUCTION

Inhibition of oxidative biological damage by antioxidants in *in vitro* systems can be anticipated in most cases, knowing the chemical properties of the oxidant and the antioxidant. However, the situation becomes more complicated when studying the effects of antioxidants in more complex systems like cells and organisms where adverse effects of antioxidants or products of their reactions and metabolism may decide on their biological efficacy.

We have found that ascorbate is able to protect *Saccharomyces cerevisiae* lacking CuZn-superoxide dismutase (SOD1) against toxicity of pure oxygen atmosphere, shortening of replicative life span[1] and auxotrophy for lysine and methionine (Zyracka *et al.*, submitted). Ascorbate restored also growth of *Δsod1* mutant on a hypertonic medium, alleviating the effect of hypertonicity mediated by oxidative stress. In studies of the protection of the SOD1-deficient yeast against the effects of hypertonicity, we compared a range of antioxidants finding that only some of them were effective while other did not act positively or even enhanced the hampering effect of the hypertonic medium.[2] I. a., nitroxide radicals (Tempo and Tempol) proved ineffective although their antioxidant action is well documented not only *in vitro* systems but also in mammalian cell systems and whole-body studies on experimental animals.[3,4] This limited potency of antioxidants at the cellular level is puzzling and brings about a question whether it is peculiar to the object studied, i.e. yeast, or dependent on the type of oxidative agent. In this study, we compared the protection of *Δsod1* mutants of *S. cerevisiae* devoid of SOD1 and of glutaredoxin 5 against different oxidants in order to check whether: (i) the effectiveness of antioxidants is dependent on the oxidative agent employed (ii) the different antioxidants show similar protection of strains differing in the defect of the antioxidant defense system.

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MATERIAL AND METHODS

Reagents

Trolox was from Aldrich, cumene hydroperoxide from Fluka and 2,2′-azobis-(2-amidinopropane) (AAPH) from Polysciences. All other reagents were from Sigma and were of analytical grade.

Yeast Strains and Growth Conditions

The following yeast strains were used: wild-type SP-4 (MATα leu1 arg4)[5] and its isogenic CuZnSOD disruptant MS-2 (MATa leu1 arg4 *sod1* :: *natMX*) obtained by one-step gene replacement,[2] wild-type W303-1A (MATa *trp1-1 ade2-1/leu2-3,112 ura3-1 his3-11,15*) and its isogenic glutaredoxin 5 disruptant MML100 (MATa *trp1 ade2 leu2 ura3 his 3 grx5* :: *kan MX4*). Two latter strains were kindly provided by Dr Enrique Herrero (Lleida).

Yeast was grown either on liquid YPD medium (1% Difco Yeast Extract, 1% Yeast Bacto-Peptone (Difco) and 2% glucose), or on solid YPD medium, containing 2% agar. Liquid cultures (150 μl) were run in a Heidolph Inkubator 1000 at 1200 rpm at 28°C and their growth was monitored turbidimetically at 600 nm in an Anthos 2010 type 17550 microplate reader. An example of a growth curve obtained in such experiments is shown in Fig. 1. In solid media tests, several dilutions (5×10^8, 5×10^7, 5×10^6 and 5×10^5 cells/ml) of yeast culture in a volume of 5 μl, were inoculated on Petri dishes containing solid YPD medium. Cultures were incubated at 28°C and inspected after 48 h. 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.

All experiments were repeated at least three times yielding consisting results.

RESULTS

Sensitivity of Yeast Mutants to Oxidants

Sensitivity of the yeast strains tested to several oxidants are summarized in Table I.

Hydrogen peroxide (2 and 3 mM) hampered the growth of the *Δgrx5* mutant much more than of its parent strain, not affecting the growth of the *Δsod1* strain on the solid medium.

t-Butyl hydroperoxide (*t*-BOOH) did not affect the growth of the yeast at concentrations of 100 and 200 μM. *t*-BOOH, 500 μM and 1 mM, was more inhibitory to *Δsod1* and *Δgrx5* strains than to their isogenic wild-type counterparts while affecting the growth of wild-type strains at a concentration of 1 mM on the solid medium. *t*-BOOH, 200 μM, did not affect the growth of the wild-type strain in the liquid medium and hampered the growth of the *Δsod1* and *Δgrx5* mutants.

On the solid medium, cumene hydroperoxide (CumOOH) did not affect the growth of the yeast at concentrations of 100 and 200 μM. CumOOH, 500 μM, was more inhibitory to *Δsod1* and *Δgrx5* strains than to their isogenic wild-type counterparts and completely inhibited the growth of all strains at a concentration of 1 mM. In the liquid medium, 200 μM CumOOH hampered the growth of the wild-type strains and completely prevented the growth of the *Δsod1* and *Δgrx5* mutants.

AAPH, 100 mM, hampered the growth of the *Δsod1* mutant and its wild-type counterpart to a similar extent and hampered the growth of the *Δgrx5* strain not affecting its parent wild-type strain in the liquid medium.

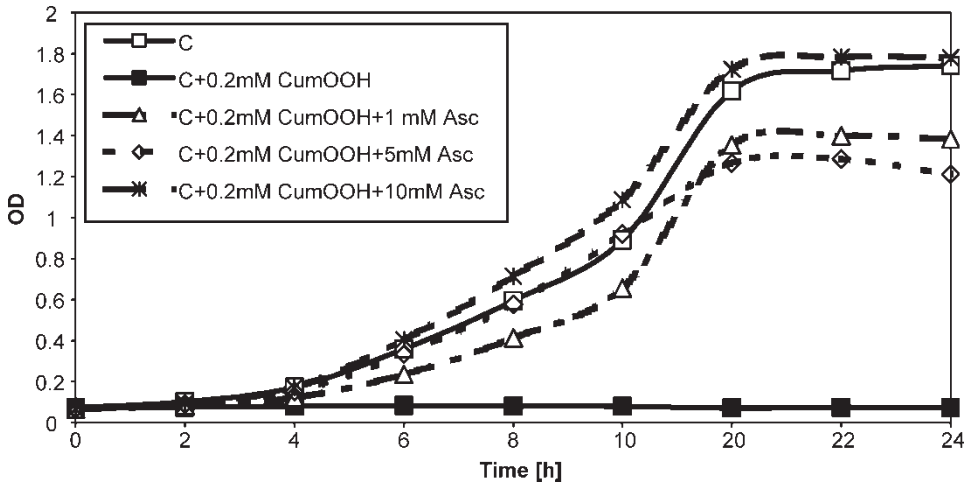

FIGURE 1 Effect of ascorbate on the growth of *Δsod1* mutants in the liquid medium.

TABLE I Sensitivity of the yeast strains tested to oxidants

Medium		Solid				Liquid			
Oxidant	Concentrations	SP-4	*Δsod1*	W303-1A	*Δgrx5*	SP-4	*Δsod1*	W303-1A	*Δgrx5*
H_2O_2	1 mM	+++	+++	+++	+++				
	2 mM	+++	+++	++	−				
	3 mM	+++	+++	++	−				
t-BOOH	100 μM	+++	+++	+++	+++				
	200 μM					+++	++	+++	++
	500 μM	+++	++	+++	+				
	1 mM	++	+	±	−	−	−	−	−
	2 mM	−	−	−	−				
	5 mM	−	−	−	−	−	−	−	−
CumOOH	200 μM	+++	+++	+++	+++	++	−	+	−
	500 μM	++	+	++	+				
	1–5 mM	−	−	−	−	−	−	−	−
AAPH	100 mM					++	++	+++	++
NaOCl	100 μM					+++	+++	+++	+
	200 μM					+++	+++	+++	+
Chloramine	100–500 μM					+++	+++	+++	+++
	1 mM					+++	++	+++	+++
	2 mM					+++	+	+++	++
	5 mM					−	−	−	−
Menadione	50 μM	+++	++	+++	+				
	100 μM	+++	+	++	+				
	300 μM	−	−	−	−				
	500 μM	−	−	−	−				
Juglone	100–200 μM	+++	+++	+++	+++	−	−	−	−
	500 μM	+++	++	+++	++	−	−	−	−
	1 mM	++	+	+	±	−	−	−	−
Oxytetra-cyclin	0.1–0.8 mg/ml	+++	++	+++	+++	+++	+++	+++	+++
	1.0 mg/ml	+++	+	+++	+++	++	++	+++	+++

+++, Growth not inhibited (as in control samples, not treated with an oxidant); ++, partial inhibition; +, strong inhibition; −, complete inhibition of growth.

Sodium hypochlorite, 100 and 200 μM, did not affect the growth of the SP-4, *Δsod1* and W303-1A strains in the liquid medium while inhibiting the growth of the *Δgrx5* strain.

In the liquid medium, chloramine did not affect the growth of the wild-type strains at concentrations of 0.1–2 mM and completely inhibited the growth of all the strains tested at 5 mM. Chloramine, 1 and 2 mM, hampered the growth of the *Δsod1* mutant and 2 mM chloramine the growth of the *Δgrx5* mutant.

Both *Δsod1* and *Δgrx5* strains were more sensitive to 50–100 μM menadione on the solid medium; 300 μM menadione completely inhibited the growth of all the strains tested. A similar naphthoquinone, juglone, did not affect the growth of the yeast on the solid medium at concentrations of 100 and 200 μM. Juglone, 300 μM, hampered the growth of *Δsod1* and *Δgrx5* mutants while not affecting the wild-type strains; 1 mM juglone affected also the growth of the wild-type strains but was much more inhibitory to the *Δsod1* and *Δgrx5* mutants. The yeast was more sensitive to juglone in the liquid medium; 100–500 μM juglone completely inhibited the growth of all the strains tested.

Oxytetracycline has been reported to inhibit growth of *Δsod1* yeast at a concentration of below 20 μg/ml.[6,7] We were not able to reproduce this result; in our hands, the *Δsod1* disruptant did not show enhanced sensitivity to oxytetracycline up to a concentration of 800 μg/ml and only the concentration of 1 mg/ml hampered the growth of the mutant on the solid medium, having no effect on the *Δgrx5* strain. We were unable to observe any increased sensitivity of the mutants to 2 μg/ml–1 mg/ml oxytetracycline in the liquid medium. Highest concentrations of the antibiotic slightly inhibited the growth of all strains to a similar extent.

None of the mutants showed increased resistance to 0.01–2 mM alloxan (not shown).

Protection by Antioxidants

Several antioxidants were tested for their ability to protect against growth inhibition of yeast cells

caused by some of the oxidants, more easy to handle in routine tests (Tables II and III).

Ascorbate proved to be the most effective antioxidant among the compounds tested. It completely restored the growth of *Δsod1* and *Δgrx5* mutants in the presence of 1 mM *t*-BOOH and 500 μM CumOOH (at concentrations of 1–30 mM on the solid and in the liquid medium) and in the presence of 1 mg/ml oxytetracycline (at the concentration of 30 mM) on the solid medium. It protected *Δgrx5* mutants against the toxicity of 100 μM menadione (starting from the concentration of 5 mM) and 100 mM AAPH (at all concentrations tested).

Glutathione (GSH) was almost as effective as ascorbate though it failed to protect the *Δsod1* strain against oxytetracycline and to protect both strains against hydroperoxide toxicity in the liquid medium. GSH was the only antioxidant able to confer some protection (at the highest concentration used, viz. 5 mM) against juglone.

Three other antioxidants studied were much less effective, conferring some protection to the *Δsod1* strain against *t* BOOH on the solid medium and to the *Δgrx5* strain against AAPH in the liquid medium. On the solid medium, Trolox was a weak protectant of the *Δsod1* mutants against *t* BOOH toxicity on the solid medium and protected *Δgrx5* mutants against *t* BOOH, and less effectively, against CumOOH. Melatonin failed to show protective action in any of the test systems applied.

Higher concentrations of some antioxidants proved toxic to the yeast in the absence of any oxidant. Trolox, 500 μM and 1 mM, and melatonin, 100 and 500 μM, hampered the growth of *Δsod1* mutants on the solid medium and in the liquid medium; 1 mM Tempol inhibited the growth of *Δsod1* mutants in the liquid medium. Trolox, 500 μM, and melatonin, 500 μM, inhibited also the growth of the *Δgrx5* mutants. In some cases these antioxidants actually aggravated the effects of oxidant agents (Tables II and III, Fig. 2). On the solid medium, Tempol enhanced growth inhibition of *Δsod1* mutants caused by *t*-BOOH, and of *Δgrx5* mutants caused by CumOOH and menadione, 500 μM and 1 mM Trolox augmented growth inhibition of *Δsod1* mutants induced by juglone and of *Δgrx5* mutants induced by menadione, and melatonin (especially at the highest concentration used) enhanced growth inhibition caused by all the oxidants tested. In the liquid medium this effect also occurred but sometimes was impossible to measure if the oxidant caused practically complete inhibition of growth.

DISCUSSION

Our interest in the studies of the effect of antioxidants on yeast cells stems from the idea of the use of yeast for detection and quantification of antioxidants. We proposed a simple test based on

TABLE II Protection of the *Δsod1* mutants by antioxidants

Medium		Oxidant							
		Solid					Liquid		
Antioxidant	Concentration	1 mM *t* BOOH	0.5 mM CumOOH	OTC 1 mg/ml	1 mM Juglone	C	0.5 mM *t* BOOH	0.2 mM CumOOH	No oxidant
Ascorbate	1 mM	###	###	0	0		##	##	
	5 mM	###	###	0	0		###	##	
	10, 20 mM	###	###	0	0		###	###	
	30 mM	###	###	###	0		###	###	
GSH	10 μM	##	0	0	0				
	50 μM	##	0	0	0		0	0	
	100 μM	##	0	0	0		0	0	
	1 mM	###	###	0	0		0	0	
	2 mM	###	###	0	0		0	0	
	5 mM	###	###	0	#		0	0	
Tempol	50 μM	#	−	0	0		−	0	
	100 μM	##	−	0	0		−	0	
	1 mM	#	−	0	−	−	−	0	−
Trolox	100 μM	0	0	0	0		0	0	
	500 μM	0	#	0	−	−	0	0	−
	1 mM	0	#	0	−	−	0	0	−
Melatonin	50 μM	0	−	0	0		0	0	
	100 μM	0	−	0	0	−	−	0	−
	500 μM	−	−	−	−	−	−	0	−

0, no effect of an antioxidant; #, some protective effect; ##, medium protective effect; ###, restoration of growth to the level in the absence of an oxidant; −, inhibition of growth by the antioxidant; C, control (no oxidant).

TABLE III Protection of the *Δgrx5* mutants by antioxidants

Medium		Oxidant								
		Solid					Liquid			
Antioxidant	Concentration	0.5 mM *t* BOOH	0.5 mM CumOOH	100 μM menadione	1 mM Juglone	C	0.5 mM *t* BOOH	0.2 mM CumOOH	100 mM AAPH	C
Ascorbate	1 mM	# # #	# #	0	0		# #	# # #	# #	
	5 mM	# # #	# #	#	0		# # #	# # #	# # #	
	10 mM	# # #	# #	# #	0		# # #	# # #	# # #	
	20 mM	# # #	# # #	# # #	0		# # #	# # #	# # #	
	30 mM	# # #	# # #	# # #	0		# # #	# # #	# # #	
GSH	50 μM	0	0	0	0		0	0	0	
	100 μM	0	0	#	0		0	0	#	
	1 mM	# # #	# # #	# # #	0		0	0	# #	
	2 mM	# # #	# # #	# # #	0		0	0	# # #	
	5 mM	# # #	# # #	# # #	#		0	0	# # #	
Tempol	50 μM	0	−	−	0		−	0	#	
	100 μM	0	−	−	0		−	0	#	
	1 mM	0	−	−	0		−	0	#	
Trolox	100 μM	0	0	0	0		0	0	0	
	500 μM	# #	#	−	0		0	0	0	−
	1 mM	# #	#	−	0	−	0	0	0	−
Melatonin	50 μM	0	−	0	0		−	0	0	
	100 μM	0	−	0	0		−	0	0	
	500 μM	−	−	−	−	−	−	0	0	−

0, no effect of an antioxidant; #, some protective effect; ##, medium protective effect; ###, restoration of growth to the level in the absence of an oxidant; −, inhibition of growth by the antioxidant; C, control (no oxidant).

the abolition of lysine and methionine auxotrophy of *Δsod1* mutants of *S. cerevisiae* by antioxidants (Zyracka *et al.*, submitted). The advantage of the test consists in its simplicity and low cost. In the simplest form of a test on solid medium, only the medium and Petri dishes are necessary for evaluation of rescue the yeast by antioxidants. Moreover, the test includes the possible adverse effects of antioxidants and/or their metabolites at the cellular level. However, in studies of the restoration of auxotrophy, the cells responded positively only to some antioxidants. Similarly, only a fraction of antioxidants was able to protect cells against the effects of hyperosmotic medium mediated by oxidative stress.[2] This study was aimed at an evaluation of protection of the yeast to other agents involving oxidative stress and inclusion of another yeast strain, deficient in glutaredoxin 5, in the comparison.

The results on the sensitivity of the mutants tested to various oxidants partly support and partly extend literature data. The increased sensitivity of both mutants to menadione, and of the *Δgrx5* mutant to hydrogen peroxide has been reported previously.[8–10] The *Δgrx5* mutant, in contrast to the *Δsod1* mutant, was hypersensitive to hypochlorite (Table I). Augmented sensitivity of the *Δgrx5* mutant to the source of peroxyl radical and to hypochlorite, and of both mutants studied to

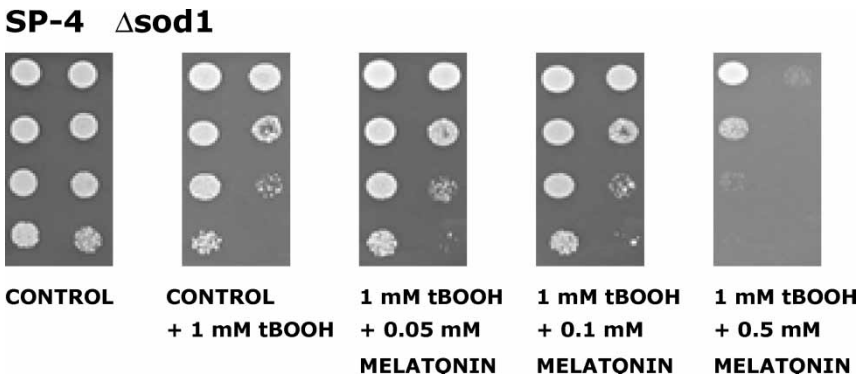

FIGURE 2 Effect of melatonin on the growth of the *Δsod1* mutant in the presence of *t* BOOH.

organic peroxides, chloramine and juglone has not been reported.

The sensitivity of the *Δsod1* mutant to the peroxides may be due to the formation of secondary reactive oxygen species formed in the metabolism of these compounds.[11] The *Δsod1* mutant was sensitive to oxytetracycline, in contrast to the *Δgrx5* mutant, but, unlike other authors,[6] we were unable to see this effect only at a very high concentration of the oxytetracycline. The discrepancies may be due to different genetic background of the strains tested.

The insensitivity of the yeast to alloxan, an agent whose diabetogenic action on the beta cells of the pancreas is ascribed to its redox cycling activity, is noteworthy.[12,13] Apparently, the drug is not reduced so efficiently in the yeast as it is in mammalian cells, especially the β-cells of the pancreas.

Results of this study point to a general similarity of the sensitivities of the *Δsod1* and *Δgrx5* mutants. The possible reason for this similarity may involve a common target for oxidative damage. Superoxide is not very reactive but is able to inactivate proteins containing Fe–S clusters which is the reason for auxotrophy of microorganisms deficient in superoxide dismutase.[14,15] Glutaredoxin 5 has been demonstrated to be a part of the mitochondrial machinery involved in the synthesis and assembly of iron–sulfur centers.[10] Thus, there may be a deficiency of functional critical Fe–S proteins may be damaged in both mutants.

The present results confirm our previous findings from other systems,[2,16] (Zyracka *et al.*, submitted) that that only some antioxidants are protective. The growth inhibition by juglone was the least susceptible to the action of antioxidants, only high concentrations of glutathione showing some effectiveness (Table II). Apparently, the main reaction of juglone is the binding to glutathione and thiol groups of proteins[17–19] and the only way to protect against this action is to provide glutathione excess.

If to exclude juglone from the comparisons, ascorbate and glutathione proved to be the most universal antioxidant, able to protect against all the oxidants tested. The results of tests on the solid and in the liquid medium are divergent in the case of the protective effects of glutathione against peroxides. The reason for this disparity may be due to the low activity of glutathione peroxidase in the yeast. In mammalian cells, glutathione peroxidase is the main mechanism of elimination of organic peroxides. Yeast *S. cerevisiae* does not have Se-glutathione peroxidases but their less reactive cysteine counterparts.[20] Additionally, the SP-4 strain has a very low glutathione peroxidase activity (Grzelak *et al.*, submitted). Therefore, the effect of glutathione may be less important during short-term incubation (test in the liquid medium) than in a 48-h test on the solid medium. This effect should be less important for ascorbate, believed to react non-enzymatically with oxidants.[21,22]

In contrast to ascorbate and glutathione, Tempol, Trolox and melatonin were less effective and even aggravated the effects of oxidants, especially at high concentrations, showing a prooxidative action. Their high concentrations hampered the growth of the strains deficient in antioxidant proteins which, again, may be a symptom of their prooxidative action. Similar effects on the limited effectiveness of antioxidants have been reported for the protection of *Δsod1* yeast against paraquat.[16]

We hypothesized that the main basis for the prooxidative action of some antioxidants is the reactivity, mainly the high redox potential of their secondary radicals formed in the reactions with the oxidants.[2] This feature may be of special importance if the Fe–S clusters of proteins constitute the critical target since their one-electron redox potential is low, ranging between −645 mV and 0 V.[23,24]

The prooxidative action of ascorbate has been broadly discussed.[25–27] However, it seems that similar property may be even more important for other antioxidants. Prooxidative action of nitroxides,[28,29] Trolox[30] and melatonin[31] has been reported from model systems. Moreover, possible toxic effects of metabolites of the antioxidants should be also taken into account.

The prooxidative effects of some antoxidants revealed in the cellular tests may contribute to the limited effectiveness of the *in vivo* applications of antioxidants[32–34] and be a complication of the use of yeast as a test system for antioxidants[1] (Zyracka *et al.*, submitted). On the other hand, the cellular system of testing antioxidants may allow for a preselection of antioxidant compounds which are of potential therapeutic value.

Acknowledgements

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