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Communication

Reactive oxygen species as second messengers? Induction of the expression of yeast catalase T gene by heat and hyperosmotic stress does not require oxygen✪★

Zdzisława Krawiec[1], Tomasz Biliński[2]✉, Christoph Schüller[3] and Helmut Ruis[3]

[1]*Zamość College of Agriculture, Szczebrzeska 102, 22-400 Zamość, Poland;*
[2]*Pedagogical University of Rzeszów, T. Rejtana 16, 35-310 Rzeszów, Poland;*
[3]*Institute of Biochemistry and Molecular Cell Biology, University of Vienna and Ludwig Boltzmann-Forschungsstelle für Biochemie, Dr Bohr-Gasse 9, A-1030 Wien, Austria*

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It is shown that oxygen is not absolutely needed for stress-induced synthesis of catalase T in the yeast *Saccharomyces cerevisiae*. Yeast cells develop heat resistance after exposure to elevated temperatures in anoxia. The levels of catalase activity and thermotolerance are comparable to those in aerobically stressed cells. While these results obviously do not exclude a stress signaling role of reactive oxygen species in some systems, as postulated by other authors, they suggest that the question of the obligatory requirement for reactive oxygen species in other stress signaling systems should be rigorously re-investigated.

A growing number of reports suggest that, besides nitric oxide, reactive oxygen species (ROS) may be considered as key signaling molecules in living cells. The involvement of ROS as second messengers in the induction of numerous cellular processes has recently been postulated [1]. ROS could be involved in the induction of systemic acquired resistance in plants [2], in the activation of the NF-κB complex responsible for transcription of

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✉Corresponding author: Tomasz Biliński, tel./fax (48 17) 853 3504; fax. (48 17) 852 2044; e-mail: bilinski@univ.rzeszow.pl

Abbreviations: HSE, heat-shock element; ROS, reactive oxygen species; STRE, stress response element.

genes connected with the immune response of animal cells [3], in the signaling pathway dependent on platelet-derived growth factor [4] and in prevention of apoptosis [5]. These results conflict with the work of Jacobson & Raff [6] and Shimizu *et al.* [7], who showed that at low tension oxygen is not involved in apoptosis. Recently it was proposed that superoxide or another ROS acts as a mediator of Ras-induced cell cycle progression [8]. Reactive oxygen species may therefore participate in some of the most fundamental cellular processes. However, the mechanism of ROS action has not been clarified. Questions concerning a more general involvement of such species in cellular signaling are important in the context of claims derived from the results obtained with complex systems. It appears likely that ROS have no signaling role, at least in strictly anaerobic bacteria. However, it is not certain at present whether their second messenger role is limited to strictly aerobic eukaryotic organisms and how universal it may be in aerobes.

It has been found [9] that the level of ROS increases in heat-treated yeast cells. The same study shows that anaerobic growth results in a 500–2000-fold increase in thermotolerance, which was immediately abolished upon oxygen exposure. This suggests direct involvement of oxygen species in heat-induced deleterious processes leading to cell death. These findings and the protective roles of antioxidant enzymes during thermal stress [9] strongly suggest that the induction of aerobic thermotolerance could be mediated by ROS also in yeast cells. A regulatory role for ROS under conditions of thermal stress appears probable in the light of reports suggesting augmented production of ROS at elevated temperatures [10] and pointing to the induction of heat shock proteins by ROS *via* the heat shock element [11, 12]. Although the heat shock-induced synthesis of the cytoplasmic catalase T of the yeast *Saccharomyces cerevisiae* is not mediated *via* HSE but *via* the stress response element (STRE) [13], its physiological function suggests that it could also be mediated by ROS, particularly if one considers that the primary events connected with stress signaling *via* HSE or STRE are still poorly understood.

In the experiments described in this paper we investigated whether the expression of a catalase gene of a facultative anaerobe can be induced by stress factors such as heat or high osmolarity in the absence of oxygen.

MATERIALS AND METHODS

The following strains of *Saccharomyces cerevisiae* were used: SP-4 – Matα *leu1 arg4*, SP-20 – Mat**a** *leu1 ade1* [14], cz1 – Matα *leu1 arg4 cgr4* [14], GG-18 – Matα *leu2 ura3 his3 trp1 ade8 cta1*-2 URA3::CTT1-18/7x-LEU2-*lacZ* [19] and DCA4-7ApCTA1-*lacZ* – Mat**a** *arg4 his4 leu2 ura3 ctt1-1 cig1* /pCTA1-*lacZ*/ ::URA3 [15] (kindly supplied by M. Skoneczny). The first three strains have isogenic background except for the nutritional markers.

Anaerobic experiments. A number of controls had to be carried out in the course of the experiments described in this paper to establish reliably that strict anoxia was maintained in our anaerobic cultures:

- Closed systems were used [14], and all additions to cultures were made with the help of side arms.
- All solutions placed in the side arms were degassed and a small amount of dithionite crystals was added to the solutions to remove oxygen.
- In preliminary experiments cycloheximide was added from the side arm to the cultures to prevent protein synthesis induced by oxygen, just prior to the opening of the anaerobic vessels. This precaution appeared to be unnecessary and in further experiments cycloheximide was added within less than 5 s after the vessels were opened, or when samples were taken.

- The cultures were grown in twin vessels [14]. One of them was stressed and the other served as an unstressed control.
- When osmotic shock experiments were performed in single vessels, control samples were withdrawn from the culture through a bottom stopcock before addition of NaCl solution.
- In initial experiments one of the twin bottles was used as an oxygen trap by placing in it high amounts of dithionite solution and filter paper to increase the surface of sorption. This precaution turned out to be unnecessary.

Besides these control experiments, we applied the following criteria of anoxia proposed by Skoneczny [15]:

- Resazurin and methylene blue were used to monitor the redox potential of cultures. Resazurin was used to establish the redox potential at the beginning of the experiment, because it is apparently metabolized by growing yeast cells, which does not happen with methylene blue. Purified nitrogen containing no more than 50 ppb impurities was flushed through the inoculated media for at least two hours, and fresh 1% dithionite solution was then introduced into the medium with the use of a glass syringe through the stopcock until decolorization of the redox indicators occurred.
- A control tester strain DCA4-7ApCTALacZ bearing a *cig1* mutation, in which catalase A synthesis is partly relieved from glucose repression [16], was used. This strain expresses a gene coding for peroxisomal Acyl-CoA oxidase and a *CTA1* promoter-driven reporter gene under anaerobic conditions [15]. The absence of catalase A activity, which was interpreted to result from severe limitation of heme availability for catalase A formation under anaerobic conditions, gives functional evidence for the establishment of strict anaerobicity. (In standard strains these enzymes are not synthesized in the presence of glucose [15, 17, 18].) Cultures were placed on a rotatory New Brunswick G-10 shaker and incubated at 22°C until they reached late exponential phase of growth. Osmotic shock was accomplished by pouring concentrated NaCl solution from the side arm into the culture to reach the final concentration of 0.3 M. The cells were incubated for 1 h. Heat shock was administered by placing the vessel in a vigorously shaken 37°C water bath for 1 h.

Aerobic cultures were grown in similar vessels, but closed with cotton stoppers assuring access of oxygen. Other aerobic procedures were identical to anaerobic ones.

Complete medium (1% Yeast Extract, 1% Bacto-Peptone, 2% glucose) was used. Anaerobic medium was supplemented with 0.5% Tween 80 and 0.0025% ergosterol.

β-Galactosidase and catalase activities were determined according to published methods [20, 21].

Dithionite, resazurin, Tween 80, ergosterol, and methylene blue were obtained from Sigma Co. Oxiclear was obtained from Aldrich Co. Difco Yeast Extract, Bacto Peptone and Agar were used to prepare the media. The remaining chemicals were of laboratory grade.

RESULTS

The inducibility of the expression of the *CTT1* gene coding for cytosolic catalase T by various stress factors [22], as well as the formation of active enzyme in anaerobically grown cz1 strain bearing the *cgr4* mutation [14], suggested that stress conditions induce the synthesis of this enzyme in the absence of oxygen. Table 1 summarizes experiments with the tester strain DCA4-7ApCTA*lacZ*, two wild type strains SP-4 and SP-20, and the cz1 strain bearing the *cgr4* mutation. The results show that catalase T synthesis is induced by heat shock and hyperosmotic stress both under aerobic and anaerobic conditions. The lack of catalase A activity in anaerobically grown anoxia tester strain DCA4-7ApCTA1-*lacZ* confirms that experimental conditions

Table 1. Aerobic and anaerobic synthesis of catalases A and T under stress conditions

Strain and stress type	Catalase activity (units)*	
	Aerobic conditions	Anaerobic conditions
DCA4-7ApCTALacZ (anoxia tester strain)	1.2	0.0
SP-4 (standard strain)		
Control	0.4	0.0
Heat shock	7.3	5.5
Osmotic shock	9.7	6.3
SP-20 (standard strain)		
Control	0.7	0.0
Heat shock	6.5	4.8
cz 1 (bearing *cgr4* mutation)		
Control	1.2	1.2
Heat shock	4.8	8.9

*Only catalase T is present in cultures grown on glucose except in strain DCA4-7ApCTALacZ deficient in catalase T in which catalase A synthesis is released from glucose repression.

prevented the leakage of oxygen into the system.

The gene *CTT1* encoding catalase T is under the control of a heme control element [23] and a negative element [24] in addition to the STRE sequence. It seemed important to answer the question whether also in anoxia STRE is responsible for the induction of the synthesis of catalase T. Table 2 presents results of experiments with strain GG18 in which expression of a reporter gene under a promoter activated exclusively *via* STRE sequences [19] could be studied. These experiments demonstrate that the induction of expression of a STRE-dependent reporter gene construct as well as of the wild type *CTT1* gene takes place in cells after stress treatment under anoxia.

The possible role of oxygen in acquiring aerobic thermotolerance by yeast cells was tested in order to verify the initial assumption that ROS could play a regulatory role in this more general stress response. Figure 1 suggests that the ability of cells to develop aerobic thermotolerance [25] after mild heat shock is independent of the presence of oxygen.

Table 2. β-Galactosidase and catalase T activities in strain GG-18 in the presence and absence of oxygen

Experimental conditions		Catalase activity (units)	β-Galactosidase activity (units)
Aerobic	Control	0.8	6.5
Aerobic	Osmotic shock	8.9	12.9
Anaerobic	Control	0.0	2.9
Anaerobic	Heat shock	5.6	7.1
Anaerobic	Osmotic shock	7.8	8.2

DISCUSSION

It was the aim of this investigation to re-examine the role of oxygen in the expression of the *CTT1* gene and in the synthesis of enzymatically active catalase T of the yeast

Saccharomyces cerevisiae. The synthesis of catalase T has previously been described as strictly dependent on the availability of molecular oxygen in wild type yeast cells [26, 27], but at the time of these investigations it had not been discovered yet that catalase T can be induced by stress [13, 22]. Heme plays an important role in the transcription of the *CTT1* gene [23] and is required as the prosthetic

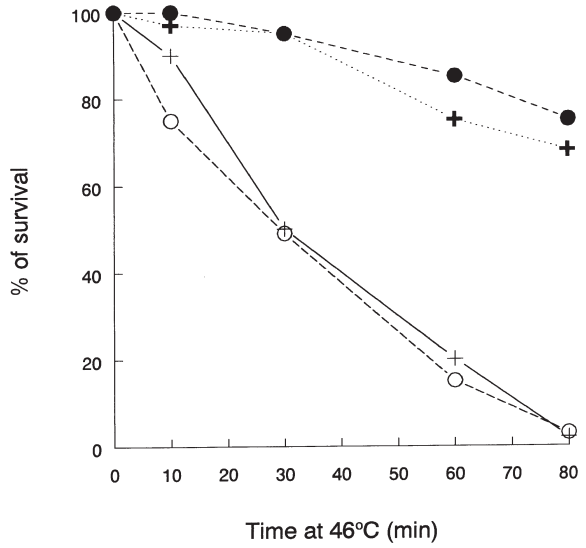

Figure 1. Thermotolerance of yeast cells heat shocked under aerobic and anaerobic conditions.

Strain SP-4 was grown at 22°C in aerobic and anaerobic conditions in the complete medium containing 10% glucose as a carbon source, as described in Table 1. Anaerobic vessels with exponential cultures were transferred to 37°C for 60 min and heated to 46°C immediately after opening the vessels and intense aeration. Cultures were maintained at 46°C with shaking, for up to 80 min. Control samples were taken before shock treatment and were also exposed to high temperature. After various time points samples were taken to establish thermotolerance. The number of colonies formed by samples taken at time point 0 was taken as 100%. ● , Anoxia, control; ● , Anoxia, heat shock; ● , Aerobic, control; ● , Aerobic, heat shock.

group of active catalase. It is generally accepted that in eukaryotic cells heme synthesis requires the presence of molecular oxygen as an electron acceptor [28, 29].

Our earlier reports [14, 30] on anaerobic synthesis of catalase T in yeast strains bearing *cgr4* and *cas1* mutations suggest that some other electron acceptors might replace oxygen in heme synthesis under anaerobic conditions. Alternatively, heme necessary for catalase formation in these strains and under conditions described in this paper might be derived from redistribution of a pre-existing heme pool.

The results of this investigation show that the induction of formation of catalase T under strict anaerobicity occurs when cells are stressed by heat shock or by increases in external osmolarity. In this context it is important to emphasize that strict anoxia was indeed maintained in our experiments as demonstrated by the use of controls and by criteria proposed by Skoneczny [15]. A rigorous test of the inducibility of *CTT1*, a gene controlled *via* STRE [22], by stress factors in the absence of oxygen seemed crucial to us since it provided a rigorous test of the question whether ROS formed as a consequence of stress under aerobic conditions play an obligatory signaling role in this system. This is apparently not the case, at least when Msn2 and Msn4, the transcription factors binding to STREs [31], are activated by stress, as this process can be activated under anaerobic conditions. It is important to emphasize that catalase T formed in all strains used was the product of the *CTT1* wild type gene including its complete promoter containing a heme control element [23] and a negative element in addition to STRE. In contrast, β-galactosidase formed in strain GG18 was the product of a reporter gene driven exclusively by STREs. It is not surprising therefore that expression of this reporter leads to basal expression under anaerobic non-stress conditions. Induction of expression of the reporter gene by stresses under anoxia demonstrates that this induction occurs *via* the STRE binding transcription factors Msn2 or Msn4.

In this context it is important to emphasize that in all strains tested the STRE-driven reporter gene as well as the *CTT1* gene were induced by heat shock and by osmotic shock un-

der strict anoxia to a similar extent as under aerobic conditions. While these results obviously do not exclude a stress-signaling role of ROS in some other systems, they show that the presence of oxygen is not essential for catalase T gene expression when this is induced by stress factors *via* STRE.

The results presented in this paper suggest that the presence of oxygen is not needed for developing thermotolerance by heat shocked yeast cells. Thermotolerance is a complex phenomenon, which could not be dependent only on the expression of genes possessing the STRE sequence in their promoters [25]. Thus, an increase of the formation of ROS, observed during thermal stress, cannot be considered as a causative factor of stress response leading to the development of thermotolerance. It is noteworthy that even some results concerning cells of higher eukaryotes [6, 7] are also in conflict with current opinions concerning stress-signaling roles for ROS.

Stress induced anaerobic synthesis of catalase T is even more surprising, because catalases are typical proteins whose function is strictly connected with the presence of oxygen. It has been recently found [32] that anaerobic catalase levels in another simple eukaryote, *Schizosaccharomyces pombe*, resistant to oxidative stress, are higher than in the presence of oxygen. These independent findings show that in two distantly related unicellular eukaryotes basic regulatory mechanisms, known to prevent catalase synthesis in the absence of oxygen under "physiological conditions", could be bypassed under stress conditions.

The role of oxygen or its reactive products in gene expression in eukaryotes is therefore much less clear than in prokaryotic systems [33, 34]. The question of the obligatory requirement of ROS in other eukaryotic stress signaling systems may therefore deserve a rigorous re-investigation.

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