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Inactivation of Genes Encoding Superoxide Dismutase Modifies Yeast Response to S-Nitrosoglutathione-Induced Stress

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Abstract—Antioxidant enzymes can modify cell response to nitrosative stress induced, for example, by nitric oxide or compounds decomposing with its formation. Therefore, we investigated the effects of S-nitrosoglutathione (GSNO) on cell survival, activity of antioxidant enzymes, and concentrations of reduced and oxidized glutathione in parental and isogenic strains defective in Cu,Zn- or Mn-superoxide dismutases (Cu,Zn-SOD and Mn-SOD, respectively), or in both of them. Stress was induced by incubation of the yeast with 1-20 mM GSNO. The strains used demonstrated different sensitivity to GSNO. A Cu,Zn-SOD-defective strain survived the stress better than the parental strain, while the double mutant was the most sensitive to GSNO. The ˙NO-donor at low concentrations (1-5 mM) increased SOD activity, but its high concentrations (10 and 20 mM) decreased it. The activity of catalase in all strains was enhanced by GSNO. Inhibition of protein synthesis by cycloheximide did not prevent the activation of SOD, but it prevented the activation of catalase. These facts suggest that SOD was activated at a posttranslational level and catalase activity was enhanced via *de novo* synthesis. A GSNO-induced increase in oxidized glutathione level in the studied yeast strains might account for cell killing by GSNO due to the development of oxidative/nitrosative stress.

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The yeast *Saccharomyces cerevisiae* do not possess nitric oxide synthase, which may explain the limited number of publications on metabolism of nitric oxide and related compounds, reactive nitrogen species (RNS), in this microorganism. However, this model subject may be useful to investigate the stress induced by RNS. The absence of endogenous ˙NO sources provides the possibility to exclude processes related to its generation. Very few works on the effects of RNS on the yeasts *S. cerevisiae* and *Schizosaccharomyces pombe* have been published [1-3]. But it was found that yeasts are a convenient model to investigate effects of oxidative and nitrosative stresses, particularly to evaluate the role of different molecular mechanisms in cell protection and signal transduction under the stresses induced by these species. It is important in this sort of research to differentiate nitrosative stress, which is developed by treatment with low concentrations of RNS, from their toxic effects induced by high RNS concentrations. Nitrosative stress can result in nitrosylation and nitrification of cellular components and frequently is associated with regulatory processes. The effects of high RNS concentrations are usually related with oxidative modification of cellular constituents, resulting in toxic effects up to cell death. Only one mechanism of specific defense against nitrosative stress has been described in yeast. Yeast hemoglobin has been claimed to be responsible for cell protection under this stress [4, 5]. It should be noted that metabolic and many other processes are rather similar in yeast and in mammals, which makes it possible to extrapolate the data obtained on yeast to multicellular eukaryotes.

The goal of this work was to investigate the response of yeast to stress induced by S-nitrosoglutathione (GSNO), a widely used ˙NO donor [6, 7]. The treatment with GSNO of yeast cells of wild strain and its derivatives defective in Cu,Zn- or Mn-SOD, as well as the two SOD (superoxide dismutase) types, demonstrated that low concentrations of the ˙NO-donor slightly reduced cell

Abbreviations: GR, glutathione reductase; GSH, reduced glutathione; GSNO, S-nitrosoglutathione; GSSG, oxidized glutathione; ˙NO, nitric oxide; RNS, reactive nitrogen species; SOD, superoxide dismutase.

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survival, while high ones were rather toxic. The measurement of activities of SOD and catalase and concentrations of reduced and oxidized glutathione demonstrated the induction of oxidative/nitrosative stress on incubation of the yeasts with GSNO.

MATERIALS AND METHODS

Materials. Phenylmethylsulfonyl fluoride (PMSF), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), sulfosalicylic acid, 2-vinylpyridine, reduced and oxidized glutathione, N,N,N′,N′-tetramethyl ethylenediamine (TEMED), and quercetin were obtained from Sigma-Aldrich Chemie GmbH (Germany); NADPH was from Reanal (Hungary); peptone and yeast extract were from Fluka (Germany). All the other chemicals were of analytical grade. S-Nitrosoglutathione was either from Sigma-Aldrich or synthesized with the procedure described by Sahoo et al. [3].

Strains and growth condition. *Saccharomyces cerevisiae* strains used in this study were YPH250 (*MATa trp1-Δ1 his3-Δ200 leu2-Δ1 lys2-801 ade2-101 ura3-52*) and its isogenic mutants. The *SOD1* gene was disrupted using the pUC-sod1Δ::URA3 plasmid which was donated by Dr. Gralla (University of California at Los Angeles) [8]. To disrupt *SOD2*, the pUCsod2Δ::TRP1 plasmid was constructed as follows: the *SOD2* gene was amplified using the primers SOD2-F1 (5′-TGGCTGAGATTCTC-CTTGATGGCGAAGCAA-3′) and SOD2-R1 (5′-TCGGGAATAAAGGCTGAGCTCATTTTCGTA-3′). The SacI site was designed in the SOD2-R1 primer (underlined). The amplified fragment was digested with BamHI and SacI, and the resultant fragment was cloned into the BamHI-SacI site of pUC19. The resultant plasmid (pUCSOD2) was digested with StyI followed by Klenow treatment, and the StyI-StyI fragment in *SOD2* was replaced with the *TRP1* gene, which had been treated with Klenow fragment. The resultant plasmid (pUCsod2Δ::TRP1) was digested with BamHI and SacI, and the *sod2Δ::TRP1* fragment was introduced to the *SOD2* loci of wild-type and *sod1Δ::URA3* strains of YPH250. Disruption of *SOD1* and *SOD2* was confirmed by PCR.

Yeast culture was grown to stationary phase (72 h) at 28°C on an orbital shaker (175 rpm) in a liquid YPD medium (1% w/v yeast extract, 2% w/v bacto-peptone, 2% w/v glucose). For experiments, cells were prepared from overnight culture grown in YPD medium. The resulting cultures were counted and cells were inoculated into the main culture to concentration about $0.3 \cdot 10^6$ cell/ml.

Cell treatment and survival measurement. Yeast cells of the used strains were treated with GSNO at concentrations of 1, 2.5, 5, 10, and 20 mM for 1 h directly in growth media. For protein synthesis inhibition prior to GSNO treatment cells were pretreated with protein synthesis inhibitor cycloheximide (250 μg/ml) for 30 min. Yeast cell survival was determined by counting colony forming units (CFU). Cell suspensions treated by GSNO for 1 h were diluted in sterile distilled water and 75 μl of the resulting suspensions were plated onto YPD-agar in Petri dishes (d = 10 cm) and incubated for 3 days at 28°C.

Preparation of cell free extracts. Cell free extracts were prepared by vortexing cells with glass beads (0.4-0.5 mm) followed by centrifugation as described earlier [9, 10] and were kept on ice for immediate use.

Assay of enzyme activities. The activity of SOD was assayed at 406 nm as the inhibition of quercetin oxidation by superoxide anion as described previously [9, 10]. One unit of SOD activity was defined as the amount of soluble protein of supernatant that inhibited the maximal rate of quercetin oxidation by 50%. The measurement of glutathione reductase (GR) activity is described elsewhere [9, 10]. NADPH consumption was registered at 340 nm and the extinction coefficient for the reduced form of this coenzyme of 6220 $M^{-1} \cdot cm^{-1}$ was used. Dismutation of hydrogen peroxide by catalase was assayed as described earlier [9, 10]. Hydrogen peroxide consumption was measured at 240 nm using extinction coefficient for hydrogen peroxide of 39.4 $M^{-1} \cdot cm^{-1}$. The reactions were started by addition of cell free extract. One unit of catalase or GR activity was defined as the amount of supernatant protein that utilizes or produces 1 μmol of substrate or product per minute. All activities were measured at 25°C and expressed per milligram of soluble protein in supernatant.

Measurement of total and oxidized glutathione. The levels of total and oxidized glutathione were measured by a method with DTNB and glutathione reductase with small modifications [11]. Treated cells were pelleted by centrifugation (5000*g* for 5 min), washed with two volumes of 50 mM phosphate buffer (pH 7.5), and repelleted by centrifugation. The resulting pellets were suspended in 1 ml 2.3% sulfosalicylic acid, mixed with 0.25 ml of glass beads (0.4-0.5 mm), and disrupted by a vortex mixer for three cycles (1 min of mixing and 2 min of chilling on ice). Cell debris was removed by centrifugation at 16,000*g* for 15 min, and the resulting supernatants were used for determination of total glutathione. For determination of oxidized glutathione, samples were mixed with 2-vinylpyridine (19 : 1) and incubated at room temperature for 1 h to remove reduced glutathione. The medium for determination was as follows: 50 mM potassium phosphate buffer, 1 U/ml of glutathione reductase, 0.2 mM NADPH, 0.6 mM DTNB, and 10-150 μl of sample. Final volume was adjusted to 1.25 ml. The reduction of DTNB was measured at 412 nm. The calibration graph was used to calculate the level of glutathione in the sample. The level of reduced glutathione was calculated as the difference between total and oxidized glutathione. The concentration of glutathione was calculated as nmol per optical density (more correctly, light scattering) of yeast culture at 600 nm ($nmol/OD_{600}$).

Protein concentration and statistical analysis. Protein concentration was determined by the Coomassie brilliant blue G-250 dye-binding method [12] with bovine serum albumin as a standard. Experimental data are expressed as means ± S.E.M., and statistical testing used ANOVA followed by Dunnett's test.

RESULTS AND DISCUSSION

The reduction of nitrate and nitrite anions catalyzed by yeast flavohemoglobin under anaerobic conditions can result in intracellular ˙NO generation in spite of the absence of NO-synthase in this organism. Decomposition of nitrosothiols or catalase-catalyzed transformation of hydroxylamine in the presence of hydrogen peroxide can also result in the formation of ˙NO in yeast cells [13]. Taking this into account, it is interesting to investigate the influence of ˙NO and related compounds on yeast for the evaluation of the role of metabolism of different RNS in biological systems. Nitric oxide can interact with many compounds, particularly with reactive oxygen species. This can affect cell response to treatment with GSNO, and therefore we suggested that the modification of processes of ˙NO generation and degradation might influence yeast cell reaction under conditions used by us. Therefore, we investigated GSNO effects on surviving of wild type and isogenic derivatives defective in superoxide dismutase genes, as well as the activities of antioxidant enzymes and levels of oxidized and reduced glutathione.

Survival. Figure 1 shows that incubation of yeast with GSNO induced concentration-dependent decrease in survival of wild type yeast with maximum effect at 20 mM (78% of control value). Inactivation of the *SOD2* gene slightly increased yeast resistance to GSNO. However, cells defective in two SODs were much more sensitive to treatment with GSNO, and at 20 mM only 30% of the cells survived. High sensitivity of the double mutant in SOD relative to that of wild type corresponds well with the data of Jakubowski and colleagues [1], who studied the effects of GSNO on yeast culture growth by the turbidimetric method.

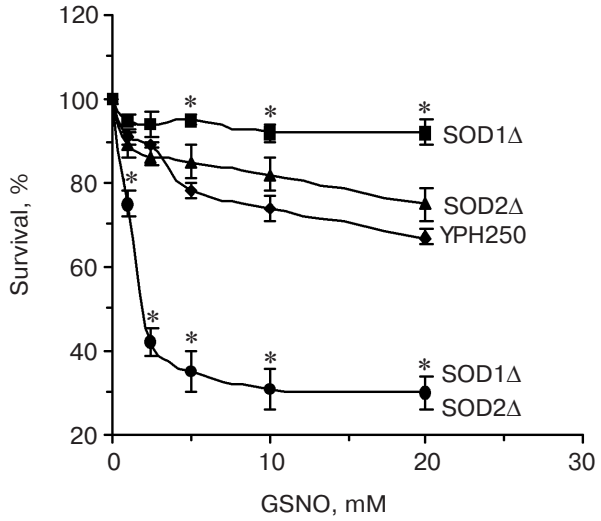

Fig. 1. Survival of wild type and defective in superoxide dismutase genes yeast strains under treatment with S-nitrosoglutathione.

It is believed that defect of two SODs results in weak yeast growth under aerobic conditions because of high intracellular concentration of superoxide anion [14]. Nitric oxide quickly interacts with superoxide anion with formation of peroxynitrite. The latter is extremely toxic for the cell, injuring cellular components and causing cell death. The transformation of ˙NO into NO^- can be catalyzed by SOD via a one-electron mechanism, which may realize the protective function of SOD and result in decreased ˙NO concentration. However, the mechanisms of cell protection in Cu,Zn-SOD deficient strain remain unknown.

Activities of SOD, catalase, and glutathione reductase. Treatment of wild type YPH250 cell with 1-5 mM GSNO significantly increased SOD activity, while incubation with 10 and 20 mM of the donor had virtually no effect (Fig. 2a). Recently we described a similar type of concentration dependence when studying the effects of treatment by hydrogen peroxide on SOD activity in *S. cerevisiae* YPH250 strain [15]. These changes in SOD activity under conditions of oxidative stress were explained as being a result of interaction of two processes—increased synthesis of new molecules and SOD inactivation via oxidation. Although direct inactivation of SOD by ˙NO has not been described, Cu,Zn-SOD was found to be inactivated by peroxynitrite—a product of interaction between ˙NO and superoxide anion [16]. To clarify the possible mechanisms leading to enhanced SOD activity in YPH250 strain on incubation with GSNO, we preincubated yeast cells with cycloheximide, an inhibitor of protein synthesis in eukaryotes. In this case, we also found concentration-dependent SOD activation by GSNO (Fig. 2b), which demonstrates that the synthesis of the enzyme *de novo* was not involved in upregulation. However, the concentration dependence was transformed. A similar activation pattern of SOD was found earlier under treatment of yeast with another ˙NO-donor, sodium nitroprusside, when we blocked protein synthesis by cycloheximide [17].

One very important question arises during investigation of effects of ˙NO-donors: can the involvement of stable products of donor decomposition be excluded? To address this question, we incubated stock GSNO solutions at room temperature for 24 h, which is known to be enough to decompose them. Incubation of yeast cells with decomposed GSNO solutions did not result in SOD activation. Therefore, we concluded that the stable products of GSNO decomposition are not responsible for SOD activation.

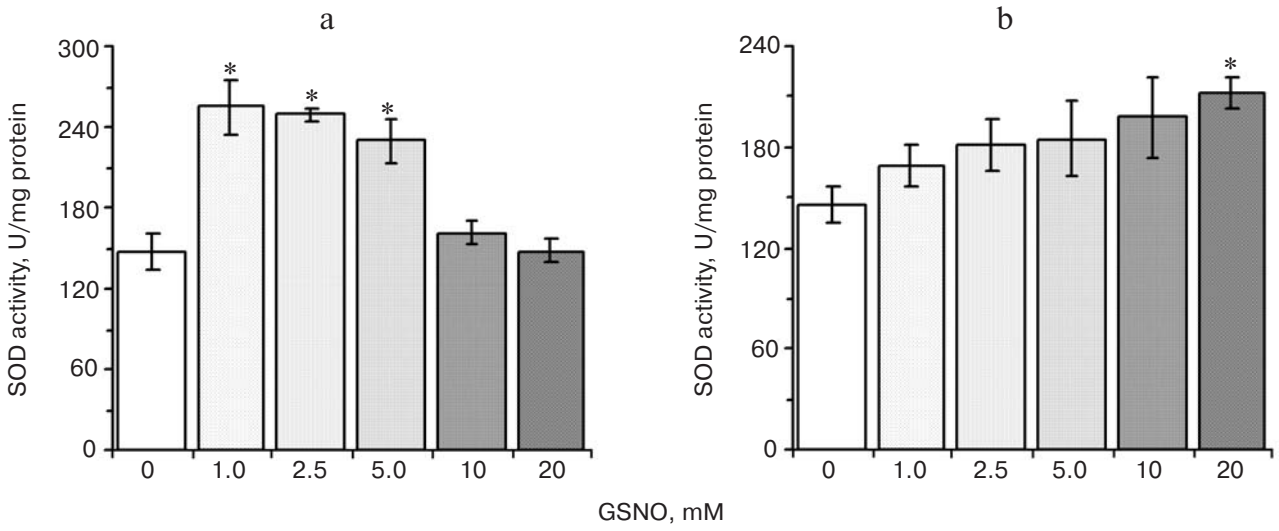

Fig. 2. Activity of superoxide dismutase in YPH250 yeast cells treated with S-nitrosoglutathione. a) Yeast cells were treated with different GSNO concentrations; b) similarly to (a), but before incubation with GSNO the cells were pretreated for 30 min with cycloheximide (250 μg/ml), an inhibitor of protein synthesis in eukaryotes.

To identify which type of SOD, namely SOD1 (Cu,Zn-containing) or SOD2 (Mn-containing) was activated on incubation of the yeast with GSNO, we used isogenic to YPH250 strains defective in specific genes. Unexpectedly, in cells of *SOD1Δ* strain we found only weak and not reliable dose-dependent tendency to increase, whereas in *SOD2Δ* strain no activation was found at all (not shown). These results demonstrate certain limitations of the approach used to answer the questions addressed.

Incubation of YPH250 yeast cells with GSNO increased the activity of catalase (Fig. 3a). This activation was blocked by preincubation with cycloheximide and, moreover, a small decrease in the catalase activity was found (not shown). This suggests the involvement of protein synthesis in enhancement of catalase activity. Incubation of yeast cells with decomposed GSNO also did not affect catalase activity (not shown). In *SOD1Δ* strain, we did not find catalase activity changes (not shown), whereas in *SOD2Δ* strain even reduction of the activity was disclosed (Fig. 3b). Unexpectedly, the double SOD mutant behaved similarly to wild type YPH250—a small but reliable concentration-dependent increase in catalase activity was seen (Fig. 3c). A reduction of hydrogen peroxide consumption was found at stimulation of hepatocytes by cytokines and incubation with synthetic nitric oxide donors [18, 19]. It seems the inactivation of *SOD* genes might substantially modify cellular response to ˙NO-induced stress.

The activity of glutathione reductase (GR) varied substantially between the strains used. In wild type YPH250 cells, it was 23.6 ± 1.1 mU/mg protein and virtually corresponded to that in the double mutant (24.6 ± 3.1 mU/mg protein) and *SOD2Δ* strain (23.2 ± 0.8 mU/mg protein). However, in cells of *SOD1Δ* strain it was only ~9 mU/mg protein. None of the GSNO concentrations used affected GR activities, which does not correspond to the data on inactivation of the enzyme by peroxynitrite in *Sch. pombe* [3].

Content of oxidized and reduced glutathione. Total concentration, levels of reduced and oxidized forms, as well as the ratio of concentrations of oxidized and reduced forms are frequently used as markers of intensity of free radical processes and general indices of redox status of the cell [3, 11, 20]. The concentrations of reduced glutathione were substantially different in parental strain YPH250 and its derivatives defective in *SOD* genes. In strains YPH250, *SOD1Δ*, *SOD2Δ*, and *SOD1ΔSOD2Δ*, they consisted of 27.5 ± 3.4, 15.9 ± 1.6, 16.1 ± 7.1, and 11.0 ± 0.3 nmol/OD_{600}, respectively. In accordance with widely accepted opinion (see data of Sahoo and colleagues on *Sch. pombe* [3]), this can reflect the development of oxidative stress in the strains defective for the *SOD* genes. This opinion is supported by data on *SOD* knockout yeast strains from different laboratories [1, 9, 10, 21]. The concentrations of oxidized glutathione (GSSG) were different in the strains used. The lowest GSSG level was found in the cells of wild strain YPH250 (0.40 ± 0.06 nmol/OD_{600}), while *SOD1Δ* strain demonstrated the highest value (1.13 ± 0.01 nmol/OD_{600}). The GSSG concentration in the double SOD mutant was 0.77 ± 0.03 nmol/OD_{600}, which is close to the wild strain, and *SOD2Δ* strain demonstrated an intermediate position – 0.88 ± 0.08 nmol/OD_{600}. The ratio of oxidized and reduced glutathione forms ([GSSG]/[GSH]) is most frequently used as a marker of oxidative and nitrosative stress. This ratio was 1.48 ± 0.15% in the parental strain

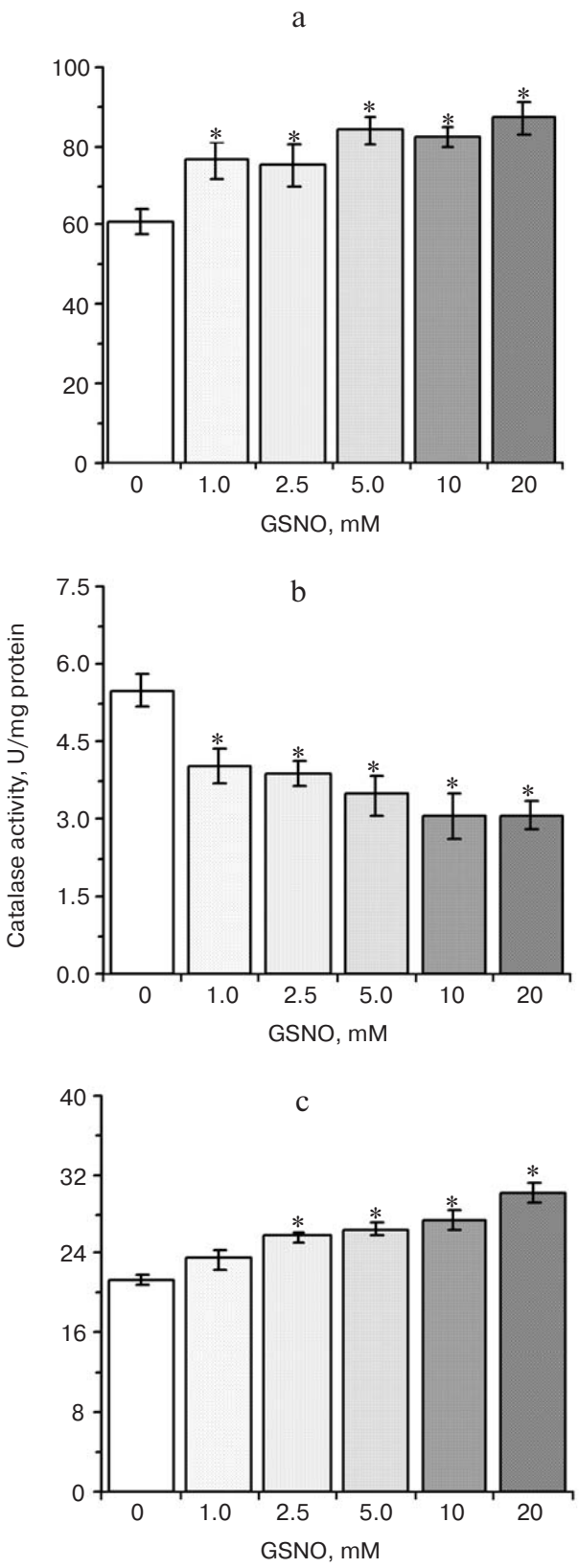

Fig. 3. Activity of catalase in yeast cells treated by S-nitrosoglutathione: a) wild type YPH250; b) defective in *SOD2* (*SOD2Δ*); c) defective in the two *SOD* genes (*SOD1ΔSOD2Δ*).

cells (Fig. 4a), which is rather common in biological systems [20]. The strains *SOD2Δ* and *SOD1ΔSOD2Δ* demonstrated ratios 5.55 ± 0.25 and 6.90 ± 0.50%, respectively (Fig. 4). The highest [GSSG]/[GSH] ratio, 7.26 ± 0.65%, was found in cells of *SOD1Δ* strain. This complicated pattern of [GSSG]/[GSH] probably reflects the presence of multilevel regulatory systems responsible for the response of yeast cells on the inactivation of the *SOD* genes. The highest sensitivity of the double SOD mutant on incubation with GSNO could be connected with low initial GSH concentration and/or its high consumption under this treatment.

Incubation of yeast cells with GSNO did not affect the concentrations of reduced glutathione in wild strain or single SOD mutants. However, in the double SOD mutant the incubation with ˙NO-donor at concentrations 5, 10, and 20 mM reduced GSH concentrations by 23, 30, and 33%, respectively, demonstrating the intensification of glutathione oxidation under nitrosative stress.

Incubation of yeast cells with GSNO increased the [GSSG]/[GSH] ratio in all strains used, reflecting the development of oxidative/nitrosative stress (Fig. 4, a-d). Maximal GSNO effect was found in parental YPH250 strain. For example, at 20 mM ˙NO-donor this ratio in YPH250 strain increased 1.8-fold (Fig. 4a), while in the double SOD mutant strain it increased 1.5-fold (Fig. 4d). It should be noted that all GSNO concentrations used increased the [GSSG]/[GSH] ratio. In cells of *SOD1Δ* strain only the incubation with 2.5-20 mM increased the ratio, whereas in *SOD2Δ* strain only 20 mM GSNO enhanced the ratio 1.2-fold.

The interaction between glutathione and ˙NO resulting in formation of RS-N-OH adduct, which further can be transformed into S-nitrosoglutathione, might be the first steps of glutathione oxidation under ˙NO-induced stress. Subsequent interaction of a formed GSNO molecule with a second GSH molecule results in the formation of disulfide glutathione (GSSG) and release of ˙NO. Oxidized glutathione can be formed as a product of reduction by peroxynitrite of oxidized cellular constituents [22]. In conclusion, it can be said that the described consequences of incubation of yeast cells with GSNO on total concentration and ratio of oxidized and reduced glutathione reflect the development of oxidative/nitrosative stress.

To exclude the involvement of externally added glutathione entering the *S. cerevisiae* cells under the conditions used by us, we used a yeast strain defective in *GSH1* (inactivation of this gene results in inability to produce γ-glutamylcysteine synthase, a key enzyme of glutathione synthesis). These yeast cells were treated with GSNO, and the concentration of GSH entering the cells was investigated. We were not able to measure glutathione entering the cells, although it is well known that yeast cell membrane possesses glutathione transporter. It seems that under the conditions used GSH did not enter the yeast cells.

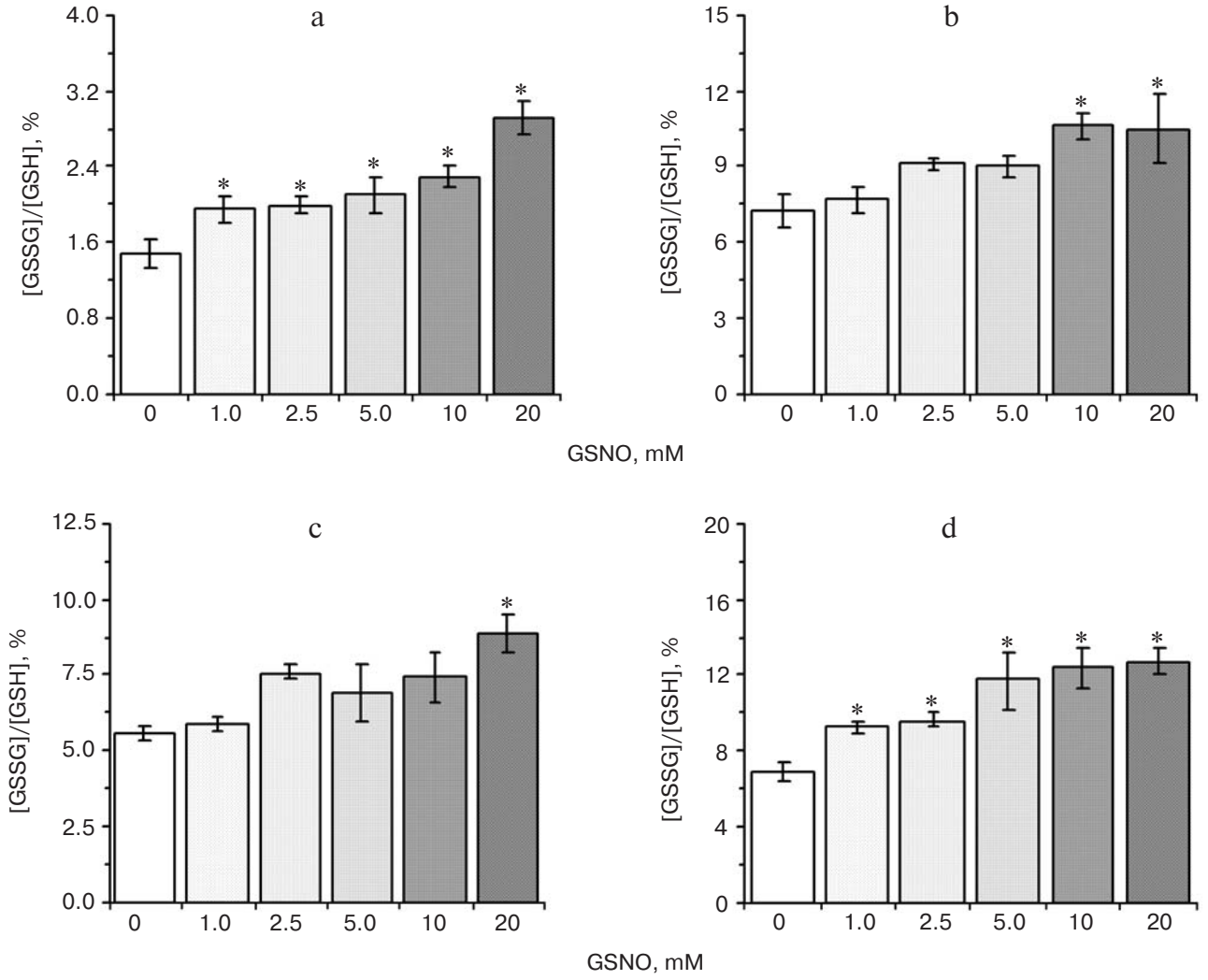

Fig. 4. Effect of S-nitrosoglutathione on the [GSSG]/[GSH] ratio in yeast cells: a) wild type strain YPH250; b) defective in *SOD1* (*SOD1Δ*); c) defective in *SOD2* (*SOD2Δ*); d) defective in the two *SOD* genes (*SOD1ΔSOD2Δ*).

We conclude that incubation of the yeast cells with S-nitrosoglutathione results in development of mild oxidative/nitrosative stress and induces cell response at gene transcription and posttranslational levels. Our data correspond well with those of Horan and colleagues [2], who were able to demonstrate the upregulation of 15 genes induced by ˙NO-donors, and which are known to be involved in the response to oxidative stress as well. Moreover, they found that yeast cell response to nitrosative stress, at least partly, is coordinated by transcription regulators Hap1p, Yap1p, and Msn2/4p. In preliminary experiments, we also found that Yap1 is probably involved in adaptive response of yeast cells to GSNO-induced stress (Lushchak et al., in preparation). This specific regulator of yeast response to oxidative stress may coordinate processes resulting in an increase in activities of antioxidant enzymes—catalase and superoxide dismutases—in response to GSNO-induced stress.

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REFERENCES

1. Jakubowski, W., Bilinski, T., and Bartosz, G. (1999) *Biochim. Biophys. Acta*, **1472**, 395-398.
2. Horan, S., Bourges, I., and Meunier, B. (2006) *Yeast*, **23**, 519-535.
3. Sahoo, R., Dutta, T., Das, A., Ray, S. S., Sengupta, R., and Ghosh, S. (2006) *Free Rad. Biol. Med.*, **40**, 625-631.

4. Cassanova, N., O'Brien, K. M., Stahl, B. T., McClure, T., and Poyton, R. O. (2005) *J. Biol. Chem*., **280**, 7645-7653.
5. Liu, L., Zeng, M., Hausladen, A., Heitman, J., and Stamler, J. S. (2000) *Proc. Natl. Acad. Sci. USA*, **97**, 4672-4676.
6. Noble, D. R., Swift, H. R., and Williams, D. L. H. (1999) *Chem. Commun.*, 2317-2318.
7. Liu, S. X., Xuan, B. O., Chen, Z., Varma, R. S., and Chiou, G. C. (1997) *J. Ocul. Pharmacol. Ther.*, **13**, 105-114.
8. Gralla, E. B., and Valentine, J. S. (1991) *J. Bacteriol.*, **173**, 5918-5920.
9. Lushchak, V., Semchyshyn, H., Lushchak, O., and Mandryk, S. (2005) *Biochem. Biophys. Res. Commun*., **338**, 1739-1744.
10. Lushchak, V., Semchyshyn, H., Mandryk, S., and Lushchak, O. (2005) *Arch. Biochem. Biophys.*, **441**, 35-40.
11. Akerboom, T. P. M., and Sies, H. (1981) *Meth. Enzymol.*, **77**, 373-382.
12. Bradford, M. M. (1976) *Anal. Biochem*., **72**, 289-292.
13. Klink, M., Swierzko, A., and Sulowska, Z. (2001) *Apmis*, **109**, 493-499.
14. Gralla, E. B. (1997) in *Oxidative Stress and the Molecular Biology of Antioxidant Defenses* (Scandalios, J. G., ed.) Cold Spring Harbor Press, N. Y., pp. 495-525.
15. Bayliak, M. M., Semchyshyn, H. M., and Lushchak, V. I. (2006) *Biochemistry* (Moscow), **71**, 1013-1020.
16. Alvarez, B., and Radi, R. (2003) *Amino Acids*, **25**, 295-311.
17. Lushchak, O. V., and Lushchak, V. I. (2008) *Redox Report*, **13**, 144-152.
18. Kim, Y.-M., Bergonia, H. A., Muller, C., Pitt, B. R., Watkins, W. D., and Lancaster, J. R. (1995) *Adv. Pharmacol.*, **34**, 277-291.
19. Wink, D. A., Hanbauer, I., Grisham, M. B., Laval, F., Nims, R. W., Laval, J., Cook, J., Pacelli, R., Liebmann, J., Krishna, M., Ford, R. C., and Mitchell, J. B. (1996) *Curr. Top. Cell. Regul.*, **34**, 159-187.
20. Halliwell, B., and Gutterige, J. M. C. (1989) *Free Radicals in Biology and Medicine*, Clarendon Press, Oxford.
21. Longo, V. D., Gralla, E. B., and Valentine, J. S. (1996) *J. Biol. Chem*., **271**, 12275-12280.
22. Radi, R., Beckman, J. S., Bush, K. M., and Freeman, B. A. (1991) *J. Biol. Chem.*, **266**, 4244-4250.