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POSSIBLE PATHWAYS INVOLVED IN ACTIVATION OF CATALASE AND SUPEROXIDE DISMUTASE WITH SODIUM NITROPRUSSIDE IN YEAST *Saccharomyces cerevisiae*

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The effect of nitric oxide (•NO) on biological systems depends very much on many circumstances. Nitric oxide can activate redox sensitive pathways that in many cases results in an increase of antioxidant potential of the cell. However, the direct effects of nitric oxide on the activity of principal antioxidant enzymes such as catalase and superoxide dismutase (SOD) have not been studied. In the present work we exploited the yeast model to elucidate a possibility of regulation of the mentioned activity by NO-donor sodium nitroprusside (SNP). We demonstrated that nitric oxide spontaneously generated at SNP decomposition increased the activity of catalase and SOD 1.3 times. Using inhibitors of mRNA (actinomycin D) and protein (cycloheximide) synthesis, the strain deficient in Yap1p, a master regulator coordinating yeast adaptive response to oxidative stress, we have found that these enzymes are up-regulated via synthesis of new molecules at transcription and translation levels. This response is mediated by Yap1p. Despite the increase of SOD activity in yeast cells possibly includes the activation of the present apoprotein by Ccs1p, the ways of nitric oxide regulation of Ccs1p activity are still unclear.

K e y w o r d s: yeast, nitric oxide, sodium nitroprusside, catalase, superoxide dismutase.

Since the discovery of important physiological function of nitric oxide (•NO), the processes of its generation, detoxification and interaction with intracellular targets are extensively studied [1, 2]. The modulatory role of antioxidant enzymes such as catalase and superoxide dismutase (SOD) in the reactions, which are •NO and related species involved in, was studied *in vitro* and the important role of these enzymes under cell survival toward nitrosative stress was described [3, 4]. Two different patterns of antioxidant enzyme involvement in metabolism of reactive nitrogen species (RNS) can be mentioned. The first one includes direct reactions of catalase and both Cu, Zn- and Mn-containing SOD with RNS. This may result in inactivation of the enzymes and detoxification of RNS. The second pattern includes the prevention of secondary reactions between RNS or metal-nitrosyl complexes and hydrogen peroxide or superoxide anion. Therefore, it is critically important to reveal how catalase and SOD can be involved in metabolism of RNS on the one hand, and on the other one – how the activity of these enzymes is regulated by nitric oxide and its derivative. Therefore, this work aimed to elucidate possible mechanisms involved in budding yeast response to nitric oxide exposure.

Materials and Methods

All chemicals were obtained from Sigma-Aldrich Chemie GmbH (Germany), excepting peptone and yeast extract which were from Fluka (Germany).

The yeast *Saccharomyces cerevisiae* of the YPH250 strain (*MATa trp1-Δ1*, *his3-Δ200*, *lys2-801*, *leu2-Δ1*, *ade2-101*, *ura3-52*) was kindly provided by prof. Yoshiharu Inoue (Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto, Japan). Cells were grown to stationary phase (72 h) at 28 °C in an orbital shaker (175 rpm) in a liquid YPD medium (1% w/v yeast extract, 2% w/v peptone, and 2% w/v glucose). Cells were prepared from an overnight culture grown in YPD medium and were used for all experiments. Cultures were inoculated with ~ 0.3×10^6 cells/ml.

Cell treatment and survival measurement. Yeast cells of strains tested were treated with sodium nitroprusside (SNP) at concentrations of 1, 5 and 10 mM for 1 h directly in growth media. To eliminate the role of oxygen the yeast cells were also treated them under anaerobic conditions. All possible effects caused by stable products of SNP disproportion were studied by using donor solutions kept for 24-48 h at room temperature for full decomposition. For inhibition of protein or mRNA

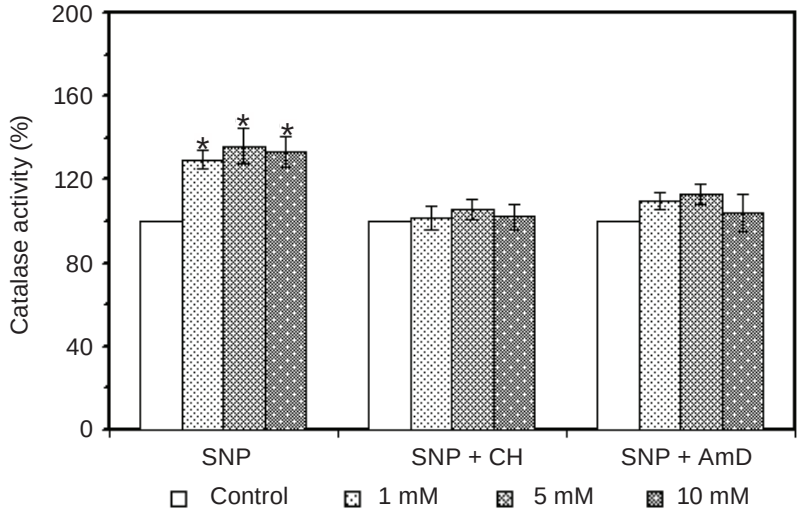

*Fig. 1. Catalase activity in wild-type (YPH250) cells after 1 h treatment with 1, 5 and 10 mM sodium nitroprusside (SNP); pretreated for 30 min with 250 μg/ml cycloheximide prior SNP exposure (SNP+CH); pretreated for 30 min with 10 μg/ml actinomycin D prior SNP exposure (SNP+AmD). Data are shown as means ± SEM (n = 6). *Significantly different from the control (untreated) cells with P < 0.05*

synthesis the yeast cells prior to SNP treatment were pretreated with cycloheximide (250 μg/ml) [5] or actinomycin D (10 μg/ml), respectively, for 30 min [6].

Preparation of cell extracts and assay of enzyme activity. Extracts were prepared by vortexing yeast cells with glass beads (0.5 mm), as described previously [7]. They were kept on ice for immediate use. The activity of SOD was assayed at 406 nm as the inhibition of quercetin oxidation by superoxide anion as described previously [7]. 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 activity of catalase was measured as described earlier [7]. Hydrogen peroxide decomposition by catalase was measured at 240 nm using an extinction coefficient for hydrogen peroxide of 39.4 $M^{-1}cm^{-1}$. The reaction was started by addition of cell-free extracts. One unit of catalase activity was the amount of the enzyme decomposing 1 μmol of hydrogen peroxide per minute. All enzyme activities were measured at 25 °C and expressed per 1 mg soluble protein in the supernatant.

Protein concentration and statistical analysis. Protein concentration was determined by the Coomassie brilliant blue G-250 dye-binding method [8] with bovine serum albumin as a standard. Experimental data are expressed as means ± S.E.M., and statistical testing used the ANOVA followed Dunnett's test.

Results and Discussion

Catalase activity

Yeast cell treatment with 1 mM SNP increased the catalase activity by 29% (Fig. 1). At the same time, 5 and 10 mM SNP enhanced the catalase activity by 36 and 33%, respectively. Pretreatment of yeast cells prior to SNP treatment by actinomycin D and cycloheximide completely blocked the increase in enzyme activity.

The yeast treatment by SNP under anaerobic conditions did not affect catalase activity (Table 1). The activation of catalase was not observed also after cell treatment with stable products of SNP decomposition (Table 1).

Sodium nitroprusside did not affect the catalase activity in yeast cells defective by Yap1p transcription factor (O. Lushchak et al., unpublished data).

Superoxide dismutase activity

Superoxide dismutase (SOD) decreases the concentration of superoxide anion and in this way prevents the oxidation of cellular components. Nitrosative stress generated by SNP increased SOD activity (Fig. 2). The SOD activity consisted of 131, 139 and 127% in yeast cells treated with SNP at concentrations of 1, 5 and 10 mM, respectively, as compared with respective control. Surprisingly, the activation of SOD was found at inhibition of protein synthesis by yeast cell preincubation with

Table 1. Catalase activity (U/mg protein) in yeast cells treated with sodium nitroprusside (SNP) under anaerobic conditions or treated by stable products of SNP decomposition (n = 6)

Contentration of SNP	Anaerobic conditions	Decomposition time	
		24 h	48 h
Control	52.6 ± 5.3	52.3 ± 4.3	52.3 ± 6.0
1 mM	52.6 ± 5.1	48.0 ± 5.4	53.8 ± 6.7
5 mM	52.5 ± 3.6	57.8 ± 10.8	58.0 ± 5.1
10 mM	49.8 ± 3.2	56.3 ± 3.0	56.9 ± 3.7

cycloheximide: the activity was found to be by 37, 83 and 45% higher, respectively, after treatment with SNP at concentrations of 1, 5 and 10 mM (Fig. 2). However, the pretreatment with actinomycin D completely prevented SOD activation under SNP-induced stress response (Fig. 2).

The increase in SOD activity was not found in yeast cells exposed to SNP under anaerobic conditions (Table 2). The activity of the enzyme was about 200 U/mg of protein.

In order to exclude possible effects caused by stable products of SNP decomposition namely, free iron and cyanide, yeast cells were treated with solutions decomposed for 24 and 48 h SNP. None of used solutions affected SOD activity (Table 2).

The increase of enzyme activity can result from many different processes – from regulation of specific gene transcription via activation of apoenzyme and posttranslational modification to regulation of protein degradation. In the present work we studied mechanisms involved in an increase of antioxidant enzyme activity such as superoxide dismutase and catalase under cell exposure to sodium nitroprusside (SNP).

The protein Yap1 regulates the expression of SOD and catalase genes in response to oxidative stress [9]. The involvement of Yap1p in this reaction is mediated by glutathione peroxidase 3 (GPx3), which serves as a primary sensor of hydrogen peroxide and is subjected to oxidation of certain thiol group of GPx3, which further can be reduced by Yap1p. The oxidized form of Yap1 regulates transcription of many genes and SOD and catalase ones are among them. As it was shown in our previous work, the increase of SOD and catalase activity was not observed in YAP1 deficient yeast cells treated with SNP. Thus, an analysis of yeast cells possessing fluorescent Yap1p fusion with GFP showed that Yap1 protein was accumulated into the nucleus in response to SNP treatment [O. Lushchak et al., unpublished data]. The mechanism of Yap1p oxidation by SNP was not still proposed. As it can be seen from Fig. 3, Yap1p can be directly or indirectly activated by nitric oxide generated spon-

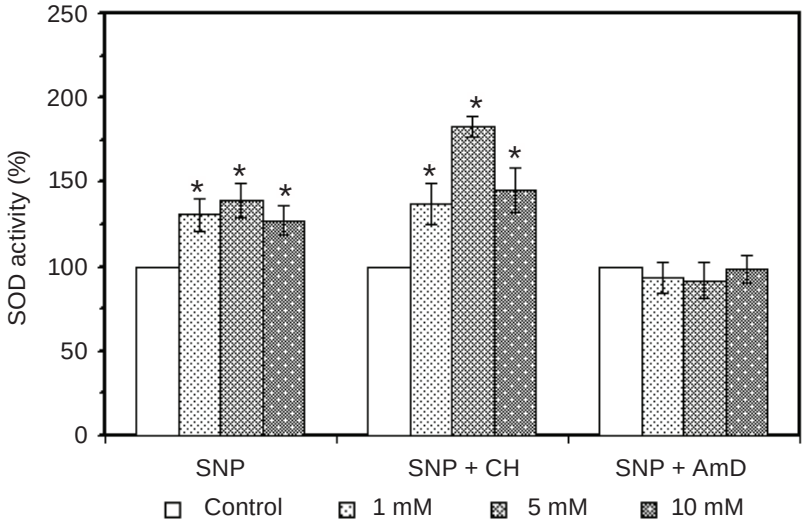

*Fig. 2. SOD activity in wild-type (YPH250) cells after 1 h treatment with 1, 5 and 10 mM SNP. The other information as in Fig. 1. *Significantly different from the control (untreated) cells with $P < 0.05$*

Table 2. Superoxide dismutase activity (U/mg protein) in yeast cells exposured under sodium nitroprusside (SNP) in anaerobic conditions or treated by stable products of SNP decomposition (n = 6)

Contentration of SNP	Anaerobic conditions	Decomposition time	
		24 h	48 h
Control	197 ± 9	151 ± 16	153 ± 6
1 mM	205 ± 20	167 ± 9	147 ± 12
5 mM	223 ± 10	164 ± 10	162 ± 20
10 mM	192 ± 12	170 ± 14	162 ± 9

taneously from SNP. In the presence of oxygen, SNP can serve as an electron donor to reduce oxygen to superoxide anion. Electron transport chain of mitochondria is the main endogenous source of intracellular superoxide anion [10]. To our mind, the possible mechanism involved in Yap1p oxidation includes the formation of peroxinitrite via reaction between nitric oxide and superoxide anion. Because under anaerobic conditions SNP did not increase catalase or SOD activity, we suggest that oxygen mediates SNP effects on yeast cells. This idea is clearly supported by the data from Table 1 and Table 2, where no activation of SOD and catalase was found under anaerobic conditions.

Previous studies with catalase defective yeast strains showed that SNP affected the increase of peroxisomal catalase A, while cytosolic catalase T did not response [11]. In the present study, the catalase activity in the yeast cells pretreated with mRNA and protein synthesis inhibitors actinomycin D and cycloheximide, respectively, was not changed (Fig. 1). These data suggest that an increase in catalase activity is a result of *de novo* synthesis of new enzyme molecules. Moreover, catalase activity was not affected in anaerobic conditions (Table 1). Whereas, the heme synthesis in *S. cerevisiae* is independent of the presence of oxygen [12], the absence of catalase activation under anaerobic conditions is a possible consequence of a decrease of the process of heme incorporation in apoenzyme.

If it is clear, that SNP induces synthesis of new catalase molecules, the situation with SOD is more complicated. Yeast cell pretreatment with actinomycin A prevented the increase of SOD activity (Fig. 2), while the increase of SOD activity was observed in the yeast cells with protein synthesis inhibited by cycloheximide (Fig. 2). The formation of catalytically active molecules of many enzymes with prosthetic metal-containing groups is con-

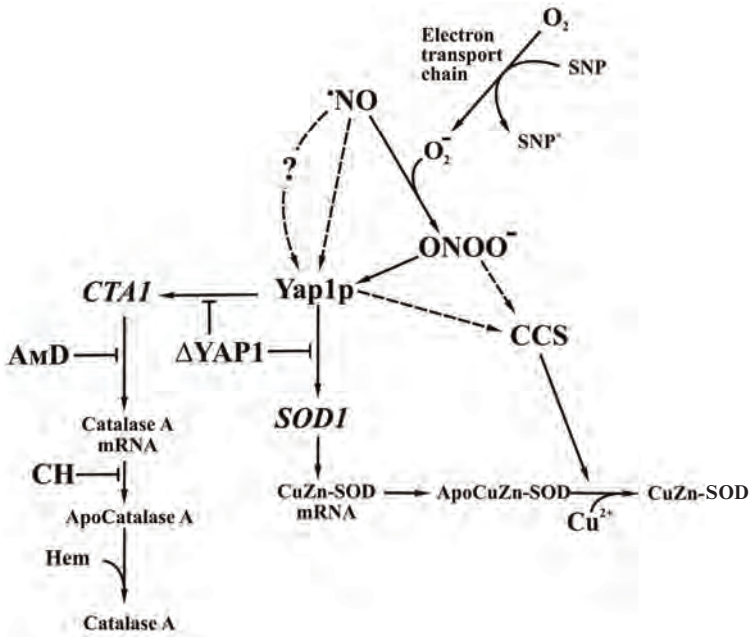

Fig. 3. Possible pathways involved in nitric oxide-mediated activation of catalase and SOD

trolled by specific chaperones. Chaperon protein Ccs1 incorporates copper ion into the active site of Cu,Zn-SOD [13]. The idea that yeast cells contain some amount of catalytically inactive apoprotein (apoCu,Zn-SOD) has been discussed by us earlier [14]. It cannot be excluded, that Ccs1p becomes more active after modification at SNP treatment. Ccs1p can be either oxidized/nitrosylated by peroxinitrite formed by reaction of nitric oxide and superoxide anion, or modified by interaction with Yap1p. At this moment it is impossible to select between these two possibilities.

From the present work we can conclude that an increase in catalase and SOD activity in yeast cells, treated with SNP is a result of different pathways involved. An increase of peroxisomal catalase activity is a result of *de novo* synthesis that is regulated by Yap1p. The increase of SOD activity in yeast cells at protein synthesis inhibited should be additionally studied both on the part interaction of Yap1p and Ccs1p and regulation of Ccs1p activity.

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МОЖЛИВІ ШЛЯХИ АКТИВАЦІЇ НІТРОПРУСИДОМ НАТРІЮ КАТАЛАЗИ І СУПЕРОКСИДДИСМУТАЗИ У ДРІЖДЖІВ *Saccharomyces cerevisiae*

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Вплив оксиду азоту (•NO) у біологічному середовищі залежить від багатьох факторів. Оксид азоту може активувати шляхи, чутливі до зміни редокс статусу, що у свою чергу може призводити до підвищення антиоксидантного потенціалу клітини. Проте вплив оксиду азоту на активність основних антиоксидантних ензимів – каталази і супероксиддисмутази (СОД) – досі залишається невідомим. У роботі на дріжджовій моделі досліджено можливі шляхи регуляції активності антиоксидантних ензимів оксидом азоту, генерованого нітропрусидом натрію (НПН). Показано, що •NO, генерований під час спонтанного розкладання НПН, підвищує активність каталази і СОД в 1,3 раза. Використання інгібіторів синтезу мРНК (актиноміцин D) і протеїну (циклогексимід), а також клітин дріжджів, дефектних за геном протеїну Yap1, дало можливість показати, що активність ензимів зростає внаслідок інтенсифікації синтезу на рівні транскрипції і трансляції. До того ж цей ефект безпосередньо пов'язаний із функціонуванням протеїну Yap1. Хоча підвищення активності СОД імовірно передбачає активацію наявного апопротеїну за участю протеїну Ccs1, шляхи регуляції функціонування протеїну Ccs1 оксидом азоту залишаються невідомими.

Ключові слова: дріжджі, оксид азоту, нітропрусид натрію, каталаза, супероксиддисмутаза.

ВОЗМОЖНЫЕ ПУТИ АКТИВАЦИИ НИТРОПРУССИДОМ НАТРИЯ КАТАЛАЗЫ И СУПЕРОКСИДДИСМУТАЗЫ У ДРОЖЖЕЙ *Saccharomyces cerevisiae*

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Действие оксида азота (•NO) в биологической среде зависит от многих факторов. Оксид азота может активизировать пути, чувствительные к изменениям редокс статуса, что, в свою очередь, может привести к повышению антиоксидантного потенциала клетки. Однако действие оксида азота на активность основных антиоксидантных энзимов – каталазы и суперксиддисмутазы (СОД) – до сих пор остается неизвестным. В данной работе на дрожжевой модели изучены возможные пути регуляции активности антиоксидантных энзимов оксидом азота, генерированного нитропруссидом натрия (НПН). Нами показано, что оксид азота, генерированный при спонтанной диссоциации НПН, повышает активность каталазы и СОД в 1,3 раза. Использование ингибиторов синтеза мРНК (актиномицин D) и протеина (циклогексимид), а также клеток дрожжей, дефектных по гену протеина Yap1, дало возможность установить, что активность энзимов возрастает в результате интенсификации синтеза на уровне транскрипции и трансляции. К тому же этот эффект непосредственно связан с функционированием протеина Yap1. Хотя повышение активности СОД, скорее всего, не исключает активацию наявного апопротеина

при участии протеина Ccs1, пути регуляции функционирования протеина Ccs1 оксидом азота неизвестны.

Ключевые слова: дрожжи, оксид азота, нитропруссид натрия, каталаза, супероксиддисмутаза.

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