OXIDATIVE STRESS REGULATION IN THE YEAST
Ogataea polymorpha PRODUCER OF HUMAN α-SYNUCLEIN

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In this study we analyzed how exogenous glucose levels affect enzymatic and non-enzymatic antioxidant defense systems and markers of oxidative stress in cells of the methylotrophic yeast Ogataea polymorpha producing recombinant human α-synuclein, implicated in pathogenesis of neurodegenerative Parkinson's disease (PD). We found that glucose depletion up-induced activity of antioxidant enzymes superoxide dismutase, and catalase, and increased content of reduced and oxidized glutathione in the cells cultivated in the medium with 0,1% glucose, as compared to physiological growth condition (1% glucose-containing medium). In addition, low glucose concentration in the medium upregulated content of proteins carbonyl groups and of products of lipid peroxidation. Notably, the shift in the equilibrium toward pro-oxidant changes was similar for recombinant α-synuclein producer and parental wild-type strain. Thus, glucose limitation leads to the overproduction of reactive oxygen species in the methylotrophic yeast cells independently of the recombinant human α-synuclein production.

K e y w o r d s: yeast Ogataea polymorpha, Parkinson's disease, α-synuclein, oxidative stress.

Parkinson's disease (PD) is currently incurable chronic neurodegenerative disorder characterized by the loss of structure and functions and selective death of dopaminergic neurons in the *substantia nigra* of intermediate brain. As a consequence of neurons loss, ataxia (problems with coordination) and dementia (mental disorder) progressively develop [1].

One of the presumable causes of PD is so-called synucleinopathy, an incorrect folding, aggregation and accumulation of abnormal inclusions of protein α-synuclein, associated with so-called Lewy bodies. Such accumulation may occur due to the enhanced expression of the α-synuclein gene *SNCA,* point mutations in the gene that affect α-synuclein tertiary structure and lead to its abnormal polymerization, or decreased housekeeping autophagy [2]. Initially believed to be causing neurodegeneration, Lewy bodies nowadays are instead considered as protective in PD by sequestering more toxic oligomeric forms of α-synuclein [3].

Data gathered on a number of *in vitro* and *in vivo* PD models suggest that neurodegenerative process is triggered by certain etiological factors. Increased formation of reactive oxygen species (ROS) and resulting oxidative stress were implicated in different stages of PD and activation of cell death-related molecular pathways in dopaminergic neurons. Excess of oxidized DNA, lipids, and proteins in the brain tissues of PD patients with both familial and sporadic PD forms was reported, which can damage various cellular structures and eventually lead to cell death [4]. It is also considered that ROS-triggered oxidation is one of the mechanisms responsible for the formation of more toxic cross-linked α-synuclein oligomers [5]. And, vice-versa, neurons with α-synuclein gene multiplication were reported to have a higher basal level of oxidative stress [6].

Moreover, when monomer, oligomer, or fibril forms of exogenous α-synuclein were added to the culture of neuronal cells, oligomers triggered oxidative stress relatively more profoundly, and led to increase in lipid peroxidation and reduction in GSH level [7].

It also becomes more widely recognized that neurodegenerative diseases such as Alzheimer's disease (AD) and PD are linked to metabolic disorders, and this connection may be causatively two-sided [8]. Often, intracellular glucose deficiency or its excess cause metabolic disorders leading to obesity, diabetes type 2 and neurodegenerative diseases [9]. Thus, there is a need in further research to elucidate involvement of metabolic changes, including effects of glucose metabolism and oxidative stress in PD [10].

Eukaryotic microorganisms, nematodes, fruit flies, rats, and primates are the model organisms that have been most often used in studies on the molecular processes involved in neurodegeneration and for the design of new therapeutic approaches. Since the establishment of the first yeast model of an amyloid neurodegenerative disease [11], these unicellular eukaryotes have become valuable tools in such studies [12]. They are characterized by highly conserved basal cellular mechanisms, short life cycle, known genomes and well developed recombinant DNA techniques, what facilitated high-throughput genetic and drug screens for potential PD-related mechanisms and corresponding therapeutic compounds. As a proof of model reliability, many findings initially obtained in yeasts were later confirmed in mammalian systems [13].

Yeast is among the simplest systems, yet it is one of the best characterized eukaryotic organisms that provides a relevant biological context for the study cellular pathologies associated with PD. The first yeast model for PD was based on the human α-synuclein heterologous expression in *Saccharomyces cerevisiae* wild type cells, resulting in an α-synuclein dose-dependent growth defect, and in the accumulation of intracellular α-synuclein inclusions. α-Synuclein overexpression affected protea-

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Abbreviations: CAT – catalase, GFP – green fluorescent protein, Glc – glucose, GSH – reduced glutathione, PD – Parkinson's disease, ROS – reactive oxygen species, SOD – superoxide dismutase, TBA – thiobarbituric acid, DTNB – 5,5-dithio-bis-(2-nitrobenzoic) acid, TBHP – tert-butyl hydroperoxide, DCFH-DA – 2,7-dichlorohydrofluorescein diacetate.

somal activity, vesicular trafficking, and disturbed lipid metabolism, leading to the lipid droplets accumulation. Since then, various groups used this model to dissect the molecular mechanisms underlying α-synuclein toxicity.

As a potential PD model, the methylotrophic yeast *Ogataea* (*Hansenula*) *polymorpha* shares all useful characteristics with the traditional model of bakers' yeast *Saccharomyces cerevisiae*, but also exhibits physiological growth temperature of 37°C, high tolerance to ROS and efficient antioxidant systems, and regulation of respiratory metabolism in response to excess of sugars independent of aerobic fermentation [14]. We earlier constructed in a recombinant strain of *O. polymorpha* constitutively expressing human α-synuclein conjugated with GFP (green fluorescent protein) [15, 16].

We also observed that under physiological conditions in the medium with 1% glucose constitutive expression of α-synuclein increased cell death, but this effect was evident at the late exponential growth phase and was translated only to a minor growth retardation relative to parental control cells [16].

The aim of this study was to analyze whether recombinant α-synuclein affects prooxidant-antioxidant balance in *O. polymorpha* cells, also under conditions of glucose excess or limitation, and whether different glucose levels affect α-synuclein aggregation. Our results indicate that wild-type α-synuclein does not exert a profound effect on these parameters, but *O. polymorpha* still can be further developed into an informative yeast model of PD and possibly of other metabolic disorders.

Materials and Methods

Strains, media and microbial techniques. *O. polymorpha* strains used in this work were prototrophic wild-type strain NCYC495pr (*leu1-1 ScLEU2*) and its derivative producer of the N-terminally GFP-tagged human α-synuclein NCYC495/SNCA (*leu1-1 ScLEU2 APH P_{MET25}-yEGFP3-SNCA-T_{CYC1}*) [15]. Yeast cells were cultivated on standard liquid or solid media at 37°C: rich medium YPD (1% yeast extract, 2% bacto-peptone, 1% glucose) and synthetic YNB medium (0.17% yeast nitrogen base without amino acids and ammonium sulfate (YNB, Difco), 0.5% ammonium sulfate). Solid media contained agar at 2% concentration. Concentration of carbon source (glucose) was 0.1, 1, 2.5 and 10% (w·v^{-1}), unless indicated otherwise. Cell density was determined by absorbance at 600 nm. Cells of both

strains for biochemical experiments were pre-incubated in the media with different glucose concentrations for 15 h, what corresponds to the late exponential growth phase irrespective of the present in the medium glucose level.

Superoxide dismutase activity assay. The activity of yeast superoxide dismutase (SOD, EC 1.15.1.1) was measured by the method based on the reducing nitroblue tetrazolium (NBT) using a superoxide radical as described previously [17] with modification. The absorbance of the reaction mixture was read at $\lambda = 540$ nm against the blank. The results were expressed as% of inhibition of NBT reduction per 1 mg of protein. Protein concentration was determined with Folin reagent.

Catalase activity assay. Catalase (CAT, EC 1.11.1.6) activity was measured by following the color intensity of the complex formed by H_2O_2 with molybdenum salts. The 1 ml of reaction mixture consisted of 0.05 M Tris-HCl buffer (pH 7.8), 0.03% solution of H_2O_2, and the test sample (yeast cell-free extracts) with final protein concentration 50 μg/ml in reaction mixture. The reaction was stopped by adding 0.5 ml of 4% solution of ammonium molybdate after 10 min of incubation. Measurements were carried out by spectrophotometry at a wavelength of $\lambda = 410$ nm. Units of CAT activity was calculated as nmoles of $H_2O_2 \cdot min^{-1} \cdot mg^{-1}$ of protein.

Total and reduced glutathione assay. Total intracellular glutathione (GSH+GSSG) content was quantified by a modifying standard assay based on the reduction of 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) in the presence of glutathione reductase and NADPH as described in [18]. Concentration of reduced glutathione (GSH) was measured without glutathione reductase addition to reaction mixture. Concentration of GSH was expressed in nmoles per 1 mg of protein in cell-free extract.

Lipid peroxidation assay. Malon dialdehyde (MDA) in yeast cell-free extracts was assayed via formation of a colored complex with thiobarbituric acid (TBA) at high temperature, and concentration of MDA-TBA complex was expressed in nmoles per 1 mg of protein. Extinction coefficient of MDA-TBA abduct at 532 nm is 155,000 $M^{-1} \cdot cm^{-1}$ [19]. The 1 ml of reaction mixture consisted of 10 mM phosphate buffer (pH 7.4), 1 mM potassium permanganate, and the tested sample (yeast cell-free extracts) with final protein concentration 1 mg/ml in reaction mixture. First, 0.125 ml of 10 mM ferrous sulfate was added to start the reaction. Then, 0.25 ml of 20% trichloro-acetic acid was added to stop the reaction. 0.25 ml of 1 M HCl and 0.5 ml of 0.7% TBA was added to 1 ml of previous reaction mixture after centrifugation.

Quantification of oxidative protein modifications. The level of oxidative modifications of proteins (OMPs) was assayed via spectrophotometric detection of aldehyde and ketone groups of aliphatic amino acid residues reacting with the 2,4-dinitrophenylhydrazine reagent to form protein-conjugated 2,4-dinitrophenylhydrazones with a characteristic absorption spectrum at $\lambda = 370$ nm (OMP_{370}, represented by neutral carbonyl groups) and at $\lambda = 430$ nm (OMP_{430}, represented by basic carbonyl groups) [20]. The reaction mixture consisted of 0.85% sodium chloride, 0.1 M 2,4-dinitrophenylhydrazine, 2 M hydrochloric acid, 10% trichloroacetic acid, 8 M urea solution, and the tested sample. The level of OMP_{370} was expressed using molar absorption coefficient of 22,000 $M^{-1} \cdot cm^{-1}$, and level of OMP_{430} using molar absorption coefficient of 16,800 $M^{-1} \cdot cm^{-1}$.

Western blot analysis. Recombinant human α-synuclein was monitored by Western blot analysis using mouse anti-α-synuclein specific antibodies (Thermo Fisher Scientific). Protein extracts were separated by electrophoresis under reducing conditions on SDS-PAGE minigels and electroblotted onto PVDF membrane (Amersham Pharmacia Biotech). The immunoreactive bounds were visualized by chemiluminescence (ECL, Amersham Pharmacia Biotech).

N-terminally GFP-tagged α-synuclein detection by fluorescence microscopy. For fluorescence microscopy cultures of the yeast cells were synchronized in YPD medium, then cells were washed three times in distilled water and transferred into YNB with different glucose concentration (with $OD_{600} = 0.1$) to induce *GFP-SNCA* expression. After 15 h of cultivation cells were collected by centrifugation and placed on ice until observation. Images were captured on fluorescence microscope (Axio Imager A1; Carl Zeiss MicroImaging, Jena, Germany) coupled to a monochrome digital camera (Axio Cam MRm; Carl Zeiss MicroImaging) and processed using the AxioVision 4.5 (Carl Zeiss MicroImaging) and Adobe Photoshop CS5 software (Adobe Systems, Mountain View, CA). The mean fluorescence intensity per cell was calculated as sum of intensity of fluorescence signals of the cells in the field of view (in conventional units) divided on the number of the cells in the same field of view under light microscopy. The average number of cells in the field

of view was 25-30. At least five fields of view were used for analysis.

ROS detection by fluorescence microscopy. ROS detection by fluorescence microscopy was performed as described previously [21] with minor modifications. Cell suspension was prepared in 1 ml of YNB without carbon source. In parallel, a control sample containing tert-butyl hydroperoxide (TBHP) was prepared. For this, 10 µl of 100 mM TBHP was added to the suspension. 10 µl of water was added to the experimental sample. The samples were incubated at 37°C for one hour. After incubation, the cells were washed twice in liquid YNB without carbon source, resuspended in 1 ml dH_2O and 10 µl of 2 mM 2,7-dichlorohydrofluorescein diacetate (DCFH-DA) was added to the resulting suspensions. Then cells were incubated for 30 min in the dark at 37°C. Images were captured on fluorescence microscope Axio Imager A1 as described above.

Statistical analysis. All data are presented as mean values ± standard deviation (SD). *P*-value of 0.05 was considered statistically significant. Microsoft Excel 2007 software was used for all statistical calculations. Designations were as follows: *$P < 0.05$ compared to NCYC495 pr (wild-type strain) grown on medium with 1% glucose (physiological condition); #$P < 0.05$ compared to NCYC495/*SNCA* (model strain) grown on the same medium. Two-factor ANOVA in Excel without replication was used to study the effect of glucose concentration and recombinant α-synuclein on the parameters of antioxidant defenses and the content of oxidative modification products of proteins and lipids in the cells of the α-synuclein-producing strain.

Results and Discussion

ROS generation in O. polymorpha cells at different concentrations of glucose in growth media. ROS are produced in the yeast cell during mitochondrial electron transfer or redox reactions and are physiological components of cellular homeostasis, whereas increase in ROS due to the failure of antioxidant defenses damages cell biomolecules and compromises cell viability [22].

In cells of *S. cerevisiae*, high glycolytic flux was found to be associated with increased ROS levels, whereas increase in mitochondrial respiratory capacity under reduced glucose decreased ROS generation [23]. Partial uncoupling of mitochondrial respiration also led to an increase in reactive species generation [24]. Thus, mitochondrial respiratory ca-

pacity was suggested as a key factor affecting ROS production in yeasts.

We exploited ability of *O. polymorpha* cells to transport glucose by facilitated diffusion [25] to model conditions for ROS overproduction. For this, cells of the wild-type strain (NCYC495 pr) were pre-grown during 15 hours on mineral medium with different concentrations (0.1, 1.0, 2.5 and 10%) of glucose as a carbon source and 2,7-DCFH-DA was utilized for detection of oxidative species as described in Materials and Methods (Fig. 1, *A*).

We unexpectedly found that glucose increase above 1.0% (which is assumed to represent "physiological conditions") in culture medium did not lead to elevation of cellular ROS content, whereas glucose deprivation upon cultivation on 0.1% glucose for 15 h did so (Fig. 1, *A*, *B*). Mean fluorescence intensity per cell digitized by GelPro software confirmed generation of ROS excess in *O. polymorpha* cells cultivated at low glucose. This result is in contrast to the negative effect of glucose restriction on ROS production in *S. cerevisiae* [23]. We further exploited the established phenomenon to address cumulative effects of ROS and recombinant human α-synuclein on physiology of the recombinant yeast strain (NCYC495/*SNCA*).

Effect of different concentrations of extracellular glucose on α-synuclein properties. α-Synuclein is a small protein of 140 amino acids, classified as a "natively unfolded" protein. It is exclusive to and conserved in vertebrates. At very high concentrations *in vitro*, α-synuclein forms β-stranded fibrils resembling those found in neurodegenerative plaques [26]. It is believed that various environmental factors including oxidative stress change the propensity of α-synuclein monomer to form oligomers and fibrils [22]. In this respect, the main limitation of the *S. cerevisiae* PD models is that despite forming α-synuclein cytoplasmic inclusions [27], also found in human neurons [28], they are not comprised of insoluble α-synuclein amyloid fibrils as found in Lewy bodies [29]. Rather, yeast inclusions consist of clusters of vesicles harboring α-synuclein monomers and of α-synuclein aggregates formed by its oligomeric species [30].

We used previously constructed recombinant strain NCYC495/*SNCA* of *O. polymorpha* [15] to analyze the possible effects of different glucose concentrations or ROS generation on properties of cytosolic human α-synuclein. Fluorescence microscopy revealed that generation of oxidative species
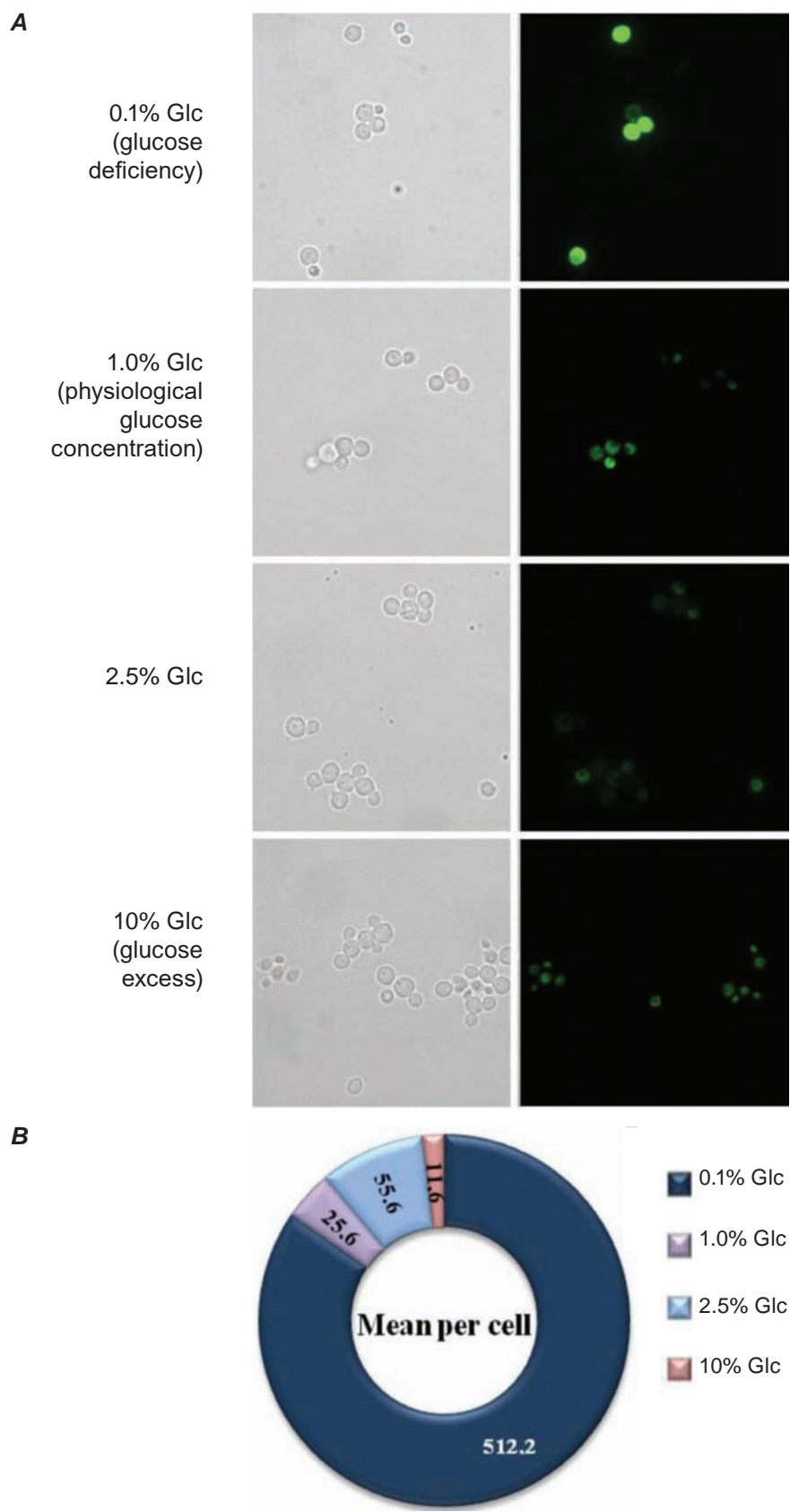
*Fig. 1. In vivo ROS detection in cells of O. polymorpha wild-type strain. (**A**) Qualitative DCFH-DA assay of ROS in O. polymorpha wild-type cells. (**B**) Mean fluorescence intensity per cell digitized by GelPro software. ROS detection was performed with tert-butyl hydroperoxide (TBHP) as described in Materials and Methods*

in low-glucose medium did not cause apparent aggregation of GFP-α-synuclein in the model yeast cells (Fig. 2, *A*). Digitization of Western blot data (Fig. 2, *B*) using ImageJ software revealed that cells grown at different glucose concentrations exhibited similar GFP-α-synuclein protein levels, only slightly decreasing for appr. 26% under growth on the medium with 10% glucose as carbon source (Fig. 2, *C*). We speculated that this modest difference may be due to a different proficiency of heterologous protein degradation, e.g. via autophagy.

It was also observed that cells shifted to 2.5% glucose-containing medium often contained a temporary single α-synuclein aggregate, which disappeared in the course of cultivation of the yeast culture. Thus, although glucose limitation in *O. polymorpha* apparently leads to increase in ROS production, it did not cause observable aggregation of α-synuclein in the cells of the recombinant strain.

Comparative analysis of the content of oxidized proteins and lipids. We next addressed the question how the presumed oxidative stress affects intracellular proteins and lipids in the studied *O. polymorpha* strains. Oxidative modifications of the biomolecules such as protein carbonyls, nitrated proteins, malondialdehyde, acrolein, isoprostanes and some others can be used as markers of oxidative and nitrosative stresses [31]. Notably, the mentioned posttranslational modifications are also characteristic for neurons of PD patients.

We observed only subtle difference in the abundance of carbonyl groups content in the wild-type cells grown in low glucose medium and under physiological conditions with 1% glucose. Increase in glucose concentration to 2.5% or 10% led to the decrease in this parameter. However, in the recombinant α-synuclein producer, both glucose limitation (0.1%) or its excess (10%) led to an increase in the level of basic and neutral carbonyl groups (Fig. 3, *A*, *B*). Notably, the content of protein carbonyl groups in cells of both strains under different glucose concentrations (except of the α-synuclein producing strain NCYC495/*SNCA* adapted to 10% glucose) did not exceed that in the wild-type cells cultured at physiological conditions with 1% glucose.

A possible explanation for a relative increase in protein carbonyl groups in NCYC495/*SNCA* at elevated glucose is that they result from secondary reactions of the nucleophilic side chains of Cys, His, and Lys residues with reactive carbonyl derivates (ketoamines, ketoaldehydes, deoxyosones) generated

in the reducing sugars reaction [32]. Another option may be glycation and glycoxidation reactions with lysine residues, with the eventual formation of the advanced glycation end products (AGEs), such as carboxymethyllysine and pentosidine. Lysine constitutes 11% of the α-synuclein amino acids and overproduction of this recombinant protein may therefore hypothetically increase the level of carbonyl groups as compared to the control wild-type strain.

The protonated form of the superoxide anion and the hydroxyl radical are responsible for the process of autocatalytic lipid peroxidation [33]. This process results in conversion of unsaturated lipids into polar lipid hydroperoxides, which can alter membrane fluidity and render certain membrane proteins non-functional [34]. Consequently, we next addressed the question whether changes in lipid peroxidation occur in the tested strains under varying culture conditions. It was found that only under conditions of glucose limitation and concomitantly increased ROS cellular content of thiobarbituric acid (TBA)-reactive products (see Materials and Methods) significantly increased in the wild-type and the recombinant strain, indicating the accumulation of lipid peroxidation products (Fig. 3, *C*). Therefore, it is glucose limitation, but not α-synuclein synthesis, that apparently evokes lipid peroxidation processes.

Effect of exogenous glucose on antioxidative defense systems in O. polymorpha. A strong causative link has been previously found between free radicals and neurodegenerative diseases [35]. Free radicals, including ROS, if increased above homeostasis limits, are removed by cellular antioxidant defenses that involve enzymatic (superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx)), or non-enzymatic components, such as glutathione (GSH).

To get insight whether recombinant human α-synuclein alters prooxidant–antioxidant balance in *O. polymorpha*, we incubated yeast strains at different glucose concentrations and analyzed activities of SOD and CAT, and content of GSH (Fig. 4).

The highest SOD activity was detected in cells of both α-synuclein producer and control strain grown on glucose-depleted medium (0.1%), and constituted about 12% of the inhibition of NBT reduction per mg of cell protein (Fig. 4, *A*). This data correlated with the concomitantly increased ROS content in the cells of both strains (Fig. 1, *B*) and indicated activation of enzymatic antioxidant sys-

B

Glc, %	0.1	1.0	2.5	10
α-Synuclein				

C

Glc, %	Band area
0.1	5106
1.0	4335
2.5	4292
10	3667
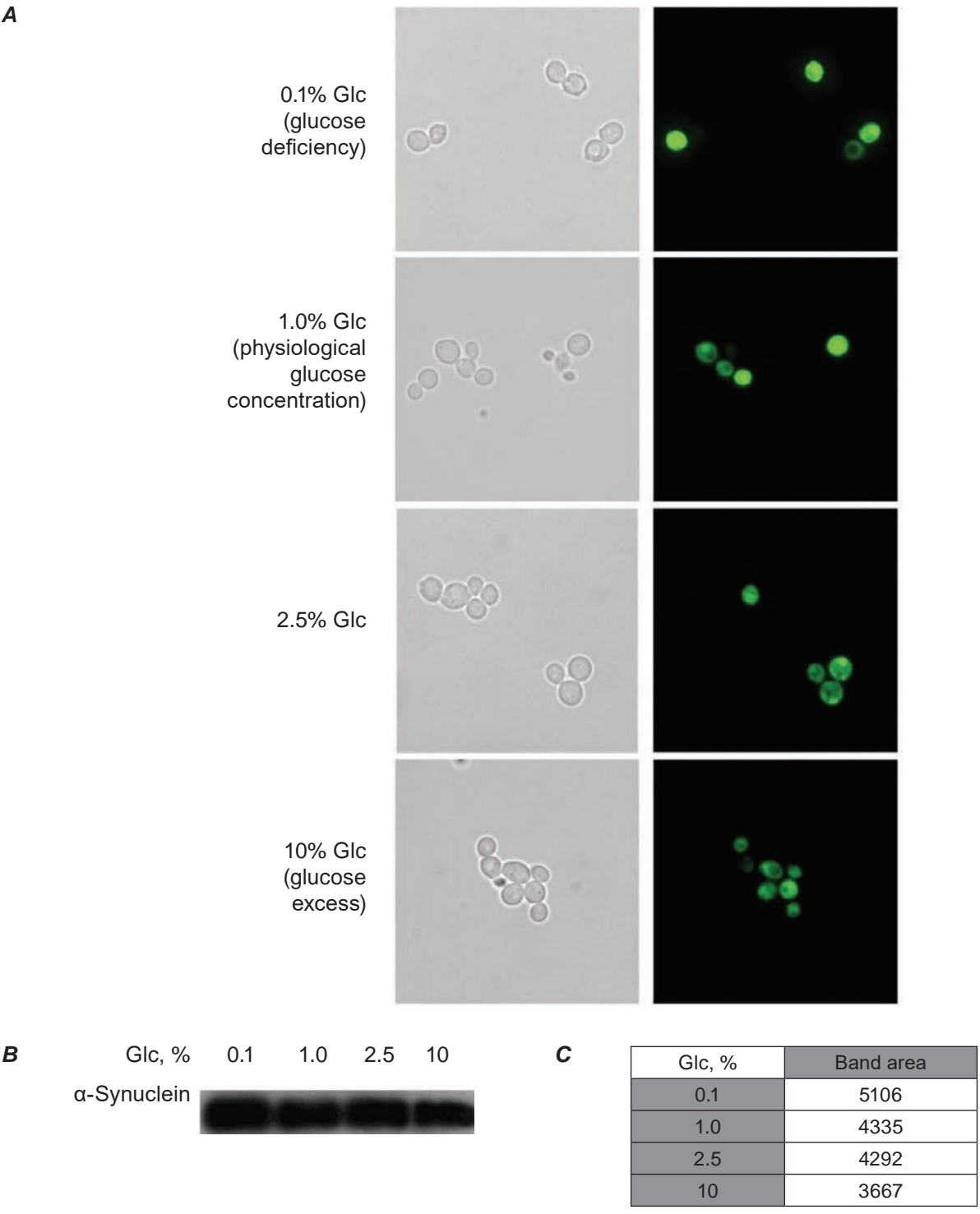
*Fig. 2. Visualisation of GFP-α-synuclein fusion protein in cells of O. polymorpha recombinant strain NCYC495/SNCA. (**A**) Phase contrast and fluorescence microscopy images of cells grown in media with different glucose concentrations. (**B**) Western blot detection of GFP-α-synuclein in cell-free extracts. Anti-GFP antobodies were utilized for chymeric protein visualisation as described in Materials and Methods. 30 μg of total cell protein were loaded per line. (**C**) Digitization of Western blot data using ImageJ software. Results are presented in relative units. Cells of O. polymorpha wild-type strain and α-synuclein producer were preincubated in mineral YNB medium with different concentrations of glucose and prepared for the analyses as described in Materials and Methods*

tem. Interestingly, SOD activity in the wild-type cells gradually decreased with increasing glucose concentration, whereas in cells of the recombinant α-synuclein producer, it exhibited an opposite regulation (Fig. 4, *A*).

Similarly, to SOD, CAT activity was also highest in the cells of both strains cultured under glucose deprivation and ROS induction, whereas in the cells grown on media with elevated glucose concentrations, activity of this enzyme was significantly lower and did not exceed values of the wild-type cells cultured under physiological conditions of 1% glucose (Fig. 4, *B*). It is known that SOD converts superoxide anion into hydrogen peroxide, which in turn is a substrate for CAT. Therefore, it is reasonable that these two enzymes function in a tandem, and increase in SOD activity is accompanied by the increased CAT activity (Fig. 4, *A*, *B*). Of note, CAT activity in α-synuclein producer at glucose-deprived conditions was comparably higher relative to the wild-type cells.

It should be also noted that changes in intracellular GSH levels in both strains overall resembled that in activities of the analyzed enzymes, SOD in particular. Level of GSH was increased on 35% in the wild type strain and on 61% in the model strain NCYC495/*SNCA* grown on glucose-deficient medium. The GSH content became higher in α-synuclein producer with increasing glucose concentration, whereas it decreased, similarly to SOD, in the wild-type strain (Fig. 4, *A*, *C*).

Analysis of content of oxidized glutathione (GSSG) revealed that its level was increased proportionally to the content of GSH (Fig. 5) in *O. polymorpha*. Cells cultivated on glucose-deficient medium had increased content of reduced and also oxidized glutathione compared to cells grown under physiological conditions.

The content of GSSG in wild-type cells grown on medium with 0.1% glucose was 83%, while the GSH content was 17%, compared with higher glucose-grown cells, in which content of GSSG was 73-74%, and GSH was 26-27%. Thus, in the case of glucose deficiency in the wild-type cells, not only the level of reduced glutathione increased, but also the percentage of oxidized glutathione increased, which indicates the upregulation of oxidative processes in these cells.

Similarly, to the wild type, model strain NCYC495/*SNCA* had an increased level of GSH and GSSG under growth on glucose-deficient medium.

The ratio between GSH and GSSG was the same in the cells cultivated at the medium with 0.1, 1, and 2.5% glucose. Content of GSH in NCYC495/*SNCA* cells was 14-16% and GSSG – 84-86% at these conditions. But glucose excess (10%) in the growth medium caused increased on 10% level of GSSG. Thus model strain differed from the wild type strain in its reaction on glucose concentration in the medium.

To find out which of the factors (glucose concentration or recombinant α-synuclein) evoked stronger impact on the prooxidant-antioxidant equilibrium in the cells of NCYC495/*SNCA* strain, we used two-factor ANOVA analysis as described in Materials and Methods. This statistical method confirmed that it is glucose concentration that has a dominant effect on the lipid oxidation, SOD and CAT activity and GSH content in the recombinant strain (Fig. 6).

Effects of α-synuclein overexpression in the most famous model eukaryotic microorganism yeast *S. cerevisiae* consist of impairment of proteasome activity, accumulation of cytoplasmic lipid droplets, ER stress, activation of the heat-shock response, mitochondrial dysfunction, shorter chronological life span and induction of autophagy and mitophagy, impairment of endocytosis, ROS production and, finally, induction of apoptosis. Among the signs of an aging organism are the suppression of key cellular processes, such as glucose metabolism, autophagic and proteasomal degradation, accumulation of cellular slag etc. PD is a disease associated with aging and has all key signs inherent in the old organism. Glucose hypometabolism is one of the hall marks of PD neurons.

In this work we analyzed the effects of recombinant α-synuclein production and exogenous glucose concentrations on prooxidant-antioxidant balance in the cells of the model methylotrophic yeast *O. polymorpha*. Both factors, hypoglycemia and ROS imbalance have been implicated in PD pathogenesis but the mechanisms involved remain controversial.

Using strains of *O. polymorpha* it was observed that extracellular glucose levels affected the most intracellular ROS production and the key markers of oxidative stress, such as activities of antioxidant enzymes SOD and CAT, intracellular reduced glutathione levels and the extent of oxidative modification of proteins and lipids. All these reporters were increased in cells of both recombinant and the wild-type strains grown under glucose limitation, lipids being a primary target. The mechanism involved
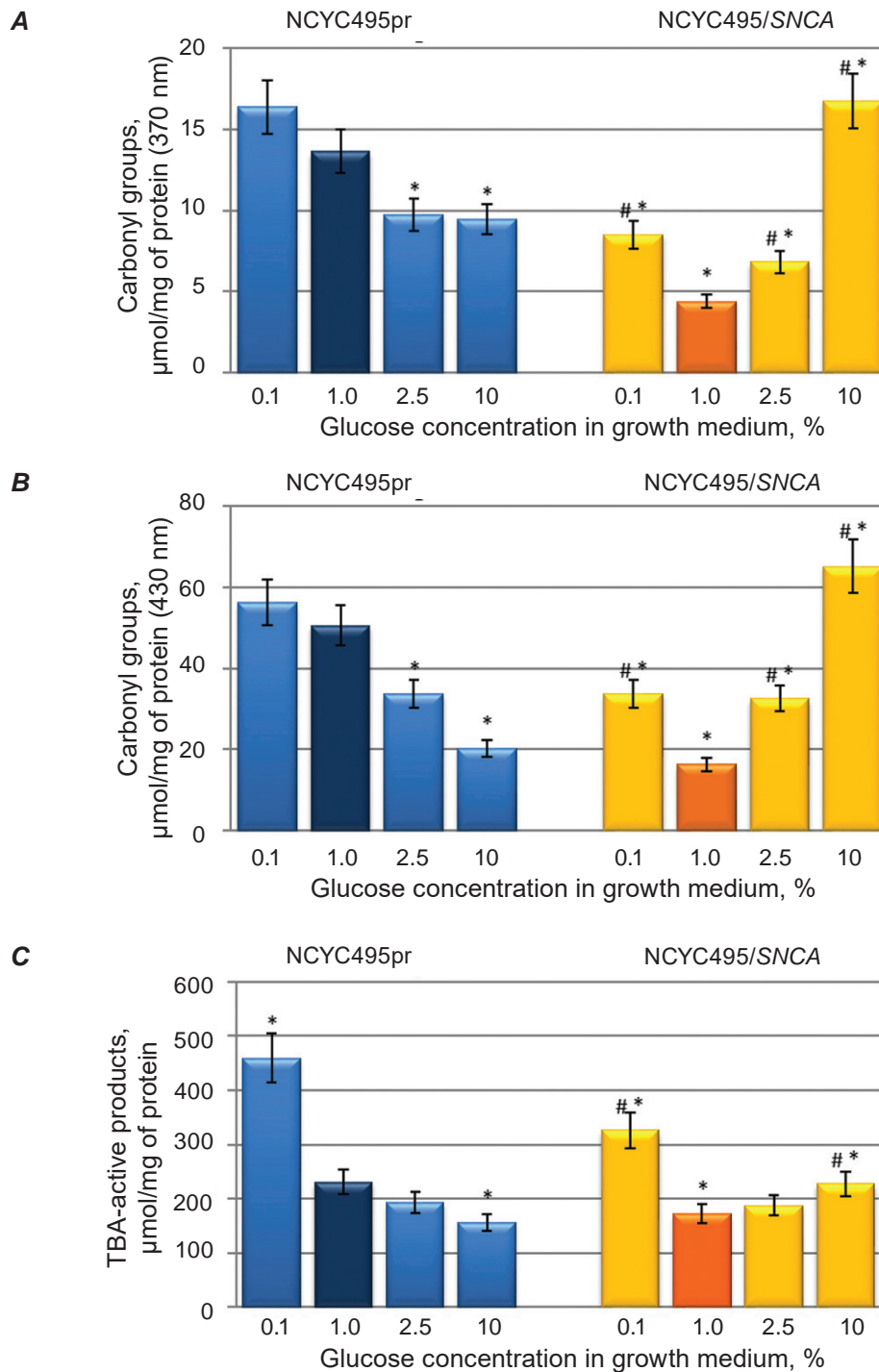
*Fig. 3. Content of the oxidized proteins and lipids in O. polymorpha cells adapted to different concentrations of glucose. (**A**) Content of neutral carbonyl groups (λ = 370 nm) of proteins in the wild-type (NCYC495 pr) and GFP-α-synuclein-producing (NCYC495/SNCA) strains. (**B**) Content of basic carbonyl groups (λ = 430 nm) of proteins (**C**) Content of TBA-active products. Assays were conducted as described in Materials and methods. Yeast cells grown on the medium with 1% glucose (dark shade) represent cells cultivated under physiological conditions. (*,#P < 0.05, *difference is significant as compared to NCYC495 pr grown in the medium with 1% glucose, #difference is significant as compared to NCYC495/SNCA grown in medium with 1% glucose)*
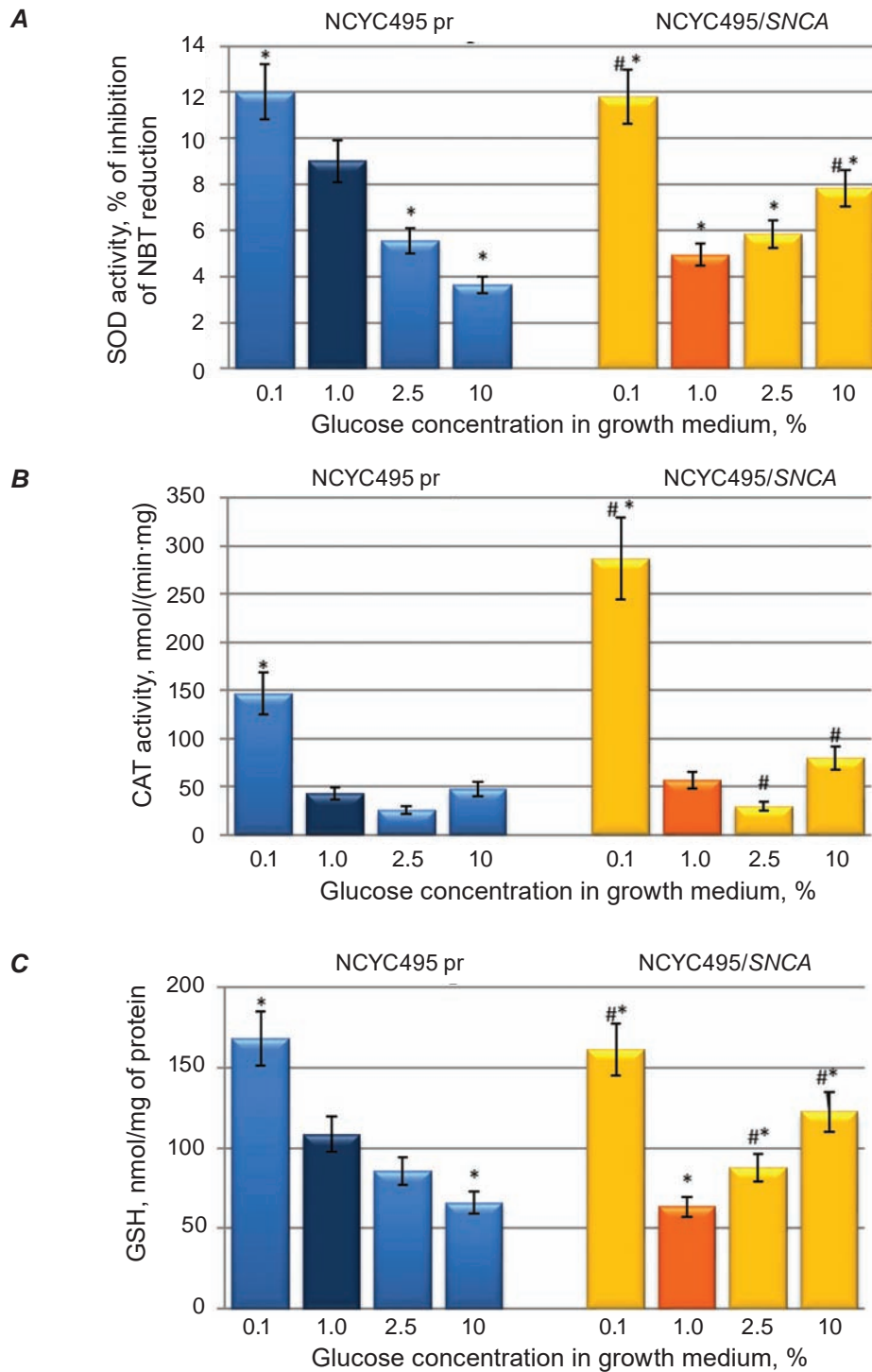
Fig. 4. Analysis of the components of the antioxidant defense system in O. polymorpha. In the cells of the wild-type (NCYC495 pr) and α-synuclein-producing (NCYC495/SNCA) strains pre-grown at indicated concentrations of glucose the following parameters were assayed as described in Materials and Methods (A) Activity of superoxide dismutase (SOD). (B) Activity of catalase (CAT) and (C) Content of reduced glutathione (GSH). Yeast cells grown in the medium with 1% glucose were considered as representing physiological conditions. (*,#P < 0.05, *difference is significant as compared to NCYC495 pr grown in the medium with 1% glucose, #difference is significant as compared to NCYC495/SNCA grown in the medium with 1% glucose)
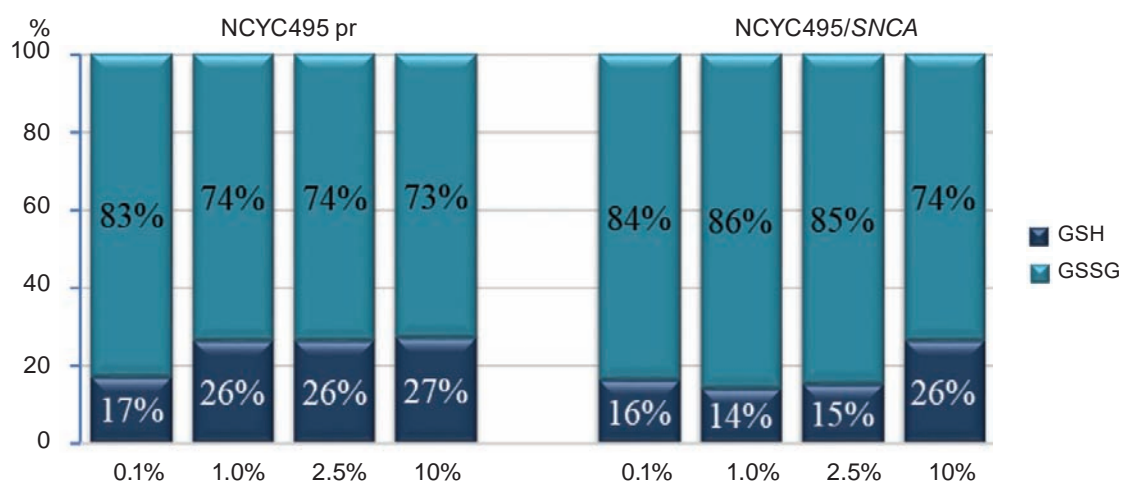
Fig. 5. Percents of intracellular reduced (GSH) and oxidized (GSSG) glutathione in O. polymorpha cells adapted to different concentrations of glucose. Yeast cells grown in the medium with 1% glucose were considered as representing physiological conditions
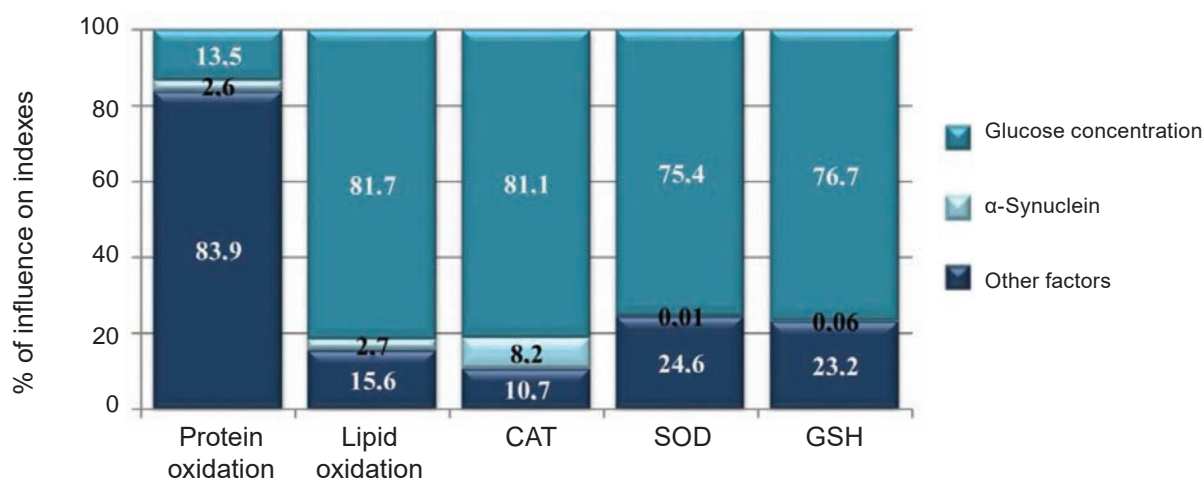

Fig. 6. Two-factor ANOVA analysis of the effects of glucose concentration and α-synuclein expression on oxidative stress reporters in cells of O. polymorpha. Statistical ANNOVA without replication analysis was performed with the data shown in Fig. 4 for the α-synuclein producer as described in Materials and Methods

is not known and will be addressed in a separate study. Simultaneously, over synthesis of human α-synuclein had rather limited separate effect on the tested parameters, and affected only a subset of the oxidative stress reporters. We also did not observe visible changes in α-synuclein localization and aggregation under conditions of ROS induction.

The concentration of glucose in the growth medium did not significantly affect the level of synthesis of heterologous human α-synuclein in yeast cells. During the first 40 h of cultivation, the level of intracellular alpha-synuclein was approximately the same (Fig. 3, *A*, *B*) in the model strain cells cul-

tured on media with different concentrations of glucose as a carbon source. Expression of *SNCA* gene under the constitutive promoter of the *MET25* gene provided a constant level of this protein in the yeast cells for a long period of its cultivation. In our previous study we analyzed growth of yeast strains on the medium with different concentrations of glucose and observed only slight difference in growth kinetics of *O. polymorpha* between the wild-type strain (NCYC495 pr, WT) and recombinant strain (NCYC495/*SNCA*). Of note, for our biochemical studies we used cells pre-incubated at different exogenous glucose concentrations for 15 h, what more

or less corresponds to the late exponential growth in all cases independent on extracellular glucose level.

O. polymorpha appeared to be intrinsically resistant to a combination of the stress conditions (glucose deficiency and α-synuclein overproduction). Our data nevertheless warrant further research to identify conditions leading to recombinant α-synuclein aggregation in *O. polymorpha* cells, such as cultivation on alternative carbon sources, expression of α-synuclein mutant forms prone to aggregation, application of alternative auxiliary stress factors, or others.

РЕГУЛЯЦІЯ ОКСИДАТИВНОГО СТРЕСУ У ДРІЖДЖІВ *Ogataea polymorpha* – ПРОДУЦЕНТА α-СИНУКЛЕЇНУ ЛЮДИНИ

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У роботі досліджено вплив рівня позаклітинної глюкози на ензиматичну та неензиматичну ланки системи антиоксидантного захисту, а також маркери оксидативного стресу в клітинах метилотрофних дріжджів *Ogataea polymorpha*, які продукують рекомбінантний протеїн α-синуклеїн людини, задіяний у патогенезі нейродегенеративної хвороби Паркінсона. Встановлено, що дефіцит глюкози зумовлював підвищення активності ензимів антиоксидантного захисту супероксиддисмутази та каталази, вмісту відновленого й окисленого глутатіону в клітинах, культивованих на середовищі з 0,1% глюкози, у порівнянні з фізіологічними умовами (середовище з 1% глюкози). Окрім цього, низька концентрація глюкози в середовищі спричиняла підвищення вмісту карбонільних груп протеїнів і продуктів пероксидного окислення ліпідів. Слід зазначити, що зміщення рівноваги у бік прооксидантних змін було схожим в обох досліджуваних штамів: продуцента людського α-синуклеїну та вихідного батьківського штаму. Таким чином, лімітування глюкози в культуральному середовищі призводило до надпродукції активних форм кисню в клітинах метилотрофних дріжджів незалежно від синтезу рекомбінантного α-синуклеїну людини.

К л ю ч о в і   с л о в а: *дріжджі Ogataea polymorpha*, хвороба Паркінсона, α-синуклеїн, окислювальний стрес.

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