Increased stress parameter synthesis in the yeast *Saccharomyces cerevisiae* after treatment with 4-hydroxy-2-nonenal[1]

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Abstract The metabolism of glutathione (GSH), a marker of oxidative stress and trehalose, a rather general physiological stress marker, was examined in exponentially growing *Saccharomyces cerevisiae* cells after treatment with 4-hydroxynonenal (HNE). GSH was entirely depleted within a 2 h incubation with 250 μM HNE. After removal of the aldehyde it was replenished by de novo synthesis leading to an overshooting GSH level, which later decreased to the basal level. In addition, trehalose was elevated 4-fold in HNE-treated yeast cells compared to control cells. We conclude that increased GSH levels upon HNE treatment are a general phenomenon of eukaryotic cells to ensure protection and survival during further harsh conditions. Furthermore, we have discovered a new indication for the stress marker trehalose in *S. cerevisiae*.

Key words: Glutathione; Trehalose; Lipid peroxidation; 4-Hydroxy-2-nonenal; (*Saccharomyces cerevisiae*)

1. Introduction

The tripeptide glutathione (GSH; L-gamma-glutamyl-L-cysteinyl-glycine) is found in virtually all cells [1] and is the major non-protein thiol compound in yeast and mammalian cells [2]. It has a wide-spread range of actions [3–6]. Among them, the detoxification of reactive metabolites [7] either by the reaction catalyzed by GSH-S-transferase or by a spontaneous conjugation, e.g. for the adaptive response to oxidative damage in *Saccharomyces* cerevisiae [8], is one of the most important functions.

Several studies have shown that fluctuations in the GSH content occur if cells are treated by heat, ethanol [9], aldehydes [10], fungicides [11] and oxidized low-density lipoprotein (oLDL) [12]. Even yeast cells taken from the logarithmic phase differ from those taken from the stationary phase [13]. Aldehyde and oLDL treatment show a biphasic pattern concerning GSH levels. In a first step GSH is depleted and in a subsequent step an overshooting GSH increase can be observed [12,14,15]. These experiments, which have been conducted with mammalian cells, show furthermore that the induction of GSH synthesis by the major lipid peroxidation product 4-hydroxy-2-nonenal (HNE), increases the resistance to the cytotoxicity of this aldehyde.

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[1]Dedicated to my son Matthew, W.W.

Induction of thermotolerance of all living cells is a rapid molecular response to adverse environmental conditions designated as the heat shock response. Acquired thermotolerance is closely linked to the synthesis of heat shock proteins (HSP) [16,17]. Nevertheless, it becomes more and more evident that thermotolerance is provided by more than one protection mechanism [18–21]. It was reported [22] that trehalose (α-D-glucopyranosyl α-D-glucopyranoside), a nonreducing disaccharide, is an efficient stabilizer of proteins against thermal denaturation at physiological concentrations, superior to other micromolecules. It preserves the properties of a hydrated membrane by substituting water and binding to the polar head groups of phospholipids [23]. Moreover, genetic methods revealed trehalose as a contributor to thermotolerance induction [24].

The protective mechanism is dependent on a specific trehalose carrier which enables the sugar to protect the cell membrane by translocating trehalose from the cytosol to the extracellular environment [25]. It was reported that agents like ethanol, copper sulfate and hydrogen peroxide, which induce stress response lead to trehalose accumulation [26].

In this study we have investigated the effects of HNE on these two particular stress response mechanisms in yeast, namely the formation of GSH and trehalose.

2. Materials and methods

2.1. Strain

The wild-type *S. cerevisiae* strain SP-4 (α, leu[1] arg[4]) was used which has been routinely cultivated by Prof. Bilinski at the Zamosc College of Agriculture in Poland. Cell concentrations were determined with a haemocytometer (Bürker-Türk).

2.2. HNE preparation

HNE was synthesized as described [27] and stored as 4-hydroxy-2-nonenal-diethylacetal (≈6.6 mg/ml) at −20°C. To prepare an aqueous HNE solution a volume equal to about 21 μmol was transferred into a vial and the chloroform was removed by gassing with nitrogen. The oily residue was dissolved in 5 ml of 10^{-3} M HCl and was stirred for 60 min. The exact concentration was estimated from the UV-spectrum of an aliquot diluted 200-fold with distilled water based on the molar absorptivity coefficient = 13 750 l mol^{-1} cm^{-1} at 223 nm. The solution was then brought to the desired final concentration by dilution with PBS buffer (pH 7.4).

2.3. Medium and cultivation

YEPG: 2% glucose, 1% peptone, 1% yeast extract from DIFCO; all others from MERCK, Darmstadt, Germany.

Stock cultures have been stored on solid agar at 4°C until use. The preculture was done in a 300 ml Erlenmeyer flask with 100 ml YEPG medium which was inoculated with a stock culture. The flasks were

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shaken continuously on an orbital shaker at 150 rpm and 25°C over night.

2.4. Treatment with HNE

In a 300 ml Erlenmeyer flask 25 ml of YEPG medium was mixed with 25 ml of HNE solution of different molarity or PBS buffer (control). After addition of 10^8 cells, derived from the preculture, they were shaken continuously at 150 rpm and 25°C. Three flasks were prepared for each dose of the aldehyde and control, respectively. After the incubation procedure cells were counted with a Bürker-Türk chamber and 10^8 cells from each flask were washed twice with 50 ml of PBS buffer each (8 min incubation at 25°C, centrifugation) and finally resuspended in 1 ml of HCl (0.1 N).

2.5. Cell disruption

For preparation of cell extracts about 260 mg of glass beads (0.4 mm; Roth) were added to the cell suspension (1 ml) in an Eppendorf tube (1.5 ml) and disruption was done by vortexing (3×1 min). The tubes were cooled in an ice bath after each minute of vortexing. Cell debris were removed by a 5 min centrifugation in an Eppendorf centrifuge. The supernatant was used for monobromobimane (mBBr) derivatization and protein determination.

2.6. Preparation of mBBr derivatives of GSH

Aliquots (0.4 ml) of the supernatant were filled up to 0.6 ml with 0.1 M HCl and 0.4 ml of 200 mM HEPES (pH 12.4) were added. After vortexing 20 μl of 2–3 mM mBBr (Calbiochem, Mannheim, Germany) were added to 330 μl of this mixture and the reaction allowed to proceed for 15 min under dim light at room temperature. The reaction was stopped by the addition of 0.25 ml of 10% acetic acid.

Aliquots (5 μl) of a standard solution (1 mM GSH, Boehringer Mannheim, Germany) plus 595 μl of 0.1 M HCl were subjected to the same derivatization procedure. Monobromobimane derivatives were either stored at −20°C or used immediately for the reverse-phase HPLC analysis.

2.7. High-performance liquid chromatography of reduced GSH

Separations were carried out on a Beckman gradient system equipped with a 421 A Controller, a Model 110 A and a Model 110 B Solvent Delivery Module, a BIO-RAD Model AS-100 HRLC Autoinjector, a Shimadzu RF-535 Fluorescence HPLC Monitor, and a Hewlett Packard 3396 Series II Integrator.

For the separation a Spherisorb ODS-2, 5 μm column (4.6×250 mm) equipped with a guard column (4.6×40 mm) and a flow rate of 1 ml/min was used. Solvent A was a mixture of 950 ml of ultra-pure water (Millipore), 50 ml of methanol p.a. and 2.5 ml of acetic acid titrated to pH 3.9 with 10 N NaOH; solvent B was a mixture of 900 ml of methanol p.a., 100 ml of ultra-pure water, and 2.5 ml of acetic acid titrated to pH 3.9 with 1 N NaOH.

The elution profile was as follows: 0–30 min, 0–70% B, linear gradient; 30–31 min, 70% B, isocratic; 31–31.2 min, 70–100% B, linear gradient; 31.2–36.5 min, 100% B, isocratic (column regeneration); 36.5–37 min, 100–0% B, linear gradient; 37–46 min, 0% B, isocratic.

Identification of GSH was done via retention time (15.2 min) of a 1 mM GSH solution, which was used as standard. The intra-assay coefficient of variation was 1.3%.

2.8. Induction of trehalose accumulation and extraction

Accumulation of trehalose was induced by exposing 10^9 cells to a 250 μM HNE solution for 2 h as described above.

After the incubation procedure 10^9 cells were collected by centrifugation, washed twice with PBS buffer and resuspended in 12 ml of distilled water. After a short sonication and the addition of 28 ml of ethanol the mixture was brought to boiling for 1 h. Thereafter the precipitate was removed by filtration and the trehalose in the filtrate was brought to dryness by a rotary evaporator and was subsequently resuspended in 2 ml of distilled water. This suspension was filtered through a 0.2 μm filter to prevent solid cell material from the HPLC column. The filtrate was then used for HPLC analysis.

2.9. High-performance liquid chromatography of trehalose

For the separation a Beckman 112 Solvent Delivery Module, a Bio-Rad HPLC column heater, a RI-Detector ERC-7512, a Linear Integrator and a Bio-Rad column HPX 87P 5 μm (4.6×250 mm) were used at 90°C. The eluent at a flow rate of 0.6 ml/min was distilled water, which has been degassed by boiling.

Trehalose was identified via retention time (11.7 min) of a 1 mM standard solution (Merck, Germany). The intra-assay coefficient of variation was 1.28%.

2.10. Protein determination

Soluble protein concentration were determined with the Pierce Coomassie protein assay reagent (PIERCE).

3. Results

3.1. Effect of HNE on the GSH content of SP-4 cells

A long-term incubation with 100 μM HNE for 2 h before measurement of the GSH content caused a slight GSH decrease as can be seen from Table 1fter treatment cells were washed twice with PBS buffer and resuspended in fresh YEPG medium. At 4 h after the washing step, cells showed a 206% rise in GSH level which decreased 20 h later to the control level where it remained constant for the following 22 h of observation.

Cells exposed to a 250 μM HNE concentration, which represents approximately the IC_{50} value of this strain [28], was accompanied by the total loss of GSH after 2 h of incubation (Table 1). Subsequent to the transfer of the cells to fresh medium GSH levels began to recover and attained 75% of control cells within the following 4 h, and this percentage remained constant for another 20 h. Further 22 h later the shaking cultures were turbid indicating growth activity and the GSH content exceeded the control level by 65%.

To analyse short-term effects of HNE upon GSH contents, yeast cells were incubated for 10 min at 25°C with a concentration of 250 μM HNE (Fig. 1). The aldehyde pulse resulted in a severe decrease by 95% in GSH content. Following this treatment GSH gradually recovered and had approximately doubled after 3 h. Over the next 2 h GSH began to approach control values.

3.2. Effect of HNE on the trehalose content

To test whether HNE can induce trehalose accumulation in yeast, we treated cells with a concentration of 250 μM of this aldehyde for a period of 2 h and analysed the trehalose content of SP-4 cells immediately after extraction. In HNE-treated cells the disaccharide level increased up to 4-fold compared to control cells as shown in Table 2.

Table 1
GSH content of HNE-treated and control cells

Time	GSH nmol/mg protein	
(h)	Control	HNE treated (100 μM)
2	34.2 ± 5.1	30.8 ± 1.2
6	28.8 ± 4.1	59.5 ± 5.9
26	37.8 ± 2.4	39.3 ± 0.8
48	38.8 ± 3.9	39.8 ± 2.5
	Control	HNE treated (250 μM)
2	45.5 ± 1.9	0.0
6	50.8 ± 6.1	37.4 ± 2.6
26	158.5 ± 4.4	115.3 ± 10.7
48	168.8[a]	278.2 ± 21.7

Results of glutathione determinations are shown as means ± SD for separate determinations of three shaking flasks prepared under identical conditions in parallel and are expressed as nmol GSH/mg protein in the cell lysate.
[a]GSH determination was done in duplicate.

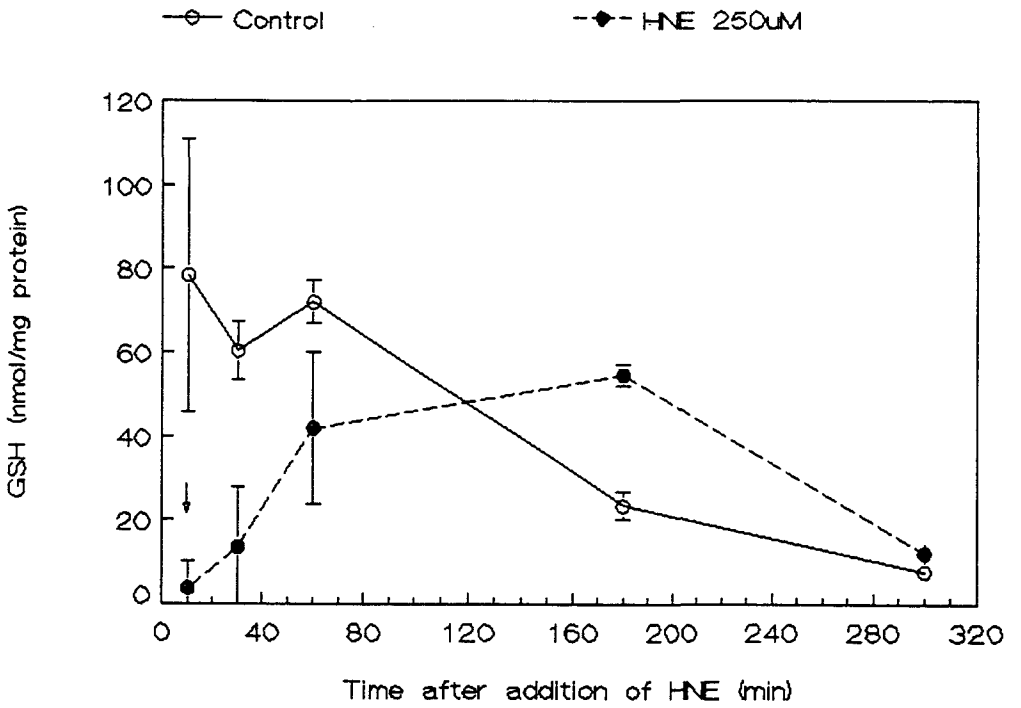

Fig. 1. Time course of the GSH content of SP-4 cells after a short-term treatment with 250 μM HNE. 10^8 Yeast cells were incubated with 250 μM HNE for 10 min. Subsequently, cells were washed twice with PBS buffer, resuspended in fresh YEPD medium and shaken again at 25°C. At the time periods shown, cells were homogenized and GSH determined. Results are shown as means of three shaking cultures prepared under identical conditions and are expressed as nmol GSH/mg protein. The arrow indicates the removal of the aldehyde.

4. Discussion

HNE is a potent inducer of the SOS response in *Salmonella typhimurium* [29] and modulates the intracellular GSH content in mammalian cells [12,14,15].

Until now, reports concerning effects of lipid peroxidation products on *S. cerevisiae* are still missing. One of the reasons might be that *S. cerevisiae* lacks polyunsaturated fatty acids (PUFA) [30], the primary targets of oxygen radical attack in the course of lipid peroxidation.

Yeasts other than *S. cerevisiae* are able to produce PUFAs [31] e.g. members of *Mucorales* and even *S. cerevisiae* introduces linolenic acid into cellular lipids during anaerobic growth [32–34]. Thus, the possibility exists that lipid peroxidation could occur in these cells when they are exposed to oxidative stress. As a consequence it can be assumed that *S. cerevisiae* is able to counteract against lipid peroxidation products, e.g. by binding to GSH. This eukaryotic organism is therefore an alternative model in stress research to mammalian cells and can be favoured, because tests can be performed much more easily with fewer contamination problems and above all much faster. Moreover, it should be noted, that *S. cerevisiae* resembles tumour cells with respect to their low PUFA availability, which is the most rate-limiting factor of lipid peroxidation, as well as to their rigid cell wall.

In the present study we have used the *S. cerevisiae* wild-type strain SP-4 as a representative of an eukaryotic non-mammalian organism to determine, whether yeast cells have the capacities to maintain their GSH levels in the presence of HNE. This can be achieved either by de novo synthesis from amino acids, by liberation of GSH from GSH–protein conjugates or by the reduction of GSSG. Our results indicate that the response of *S. cerevisiae* cells to HNE treatment is very similar to that found in mammalian cells. A strong initial depletion of GSH during a short incubation with HNE was followed by a rapid replenishment after removal of the aldehyde. The molecular response to this treatment culminated in an overshooting GSH peak, which gradually returned to the control level. The time set of this effect was dependent on the concentration of HNE and the incubation time. Poot et al. [14] have dem-

Table 2
Trehalose levels of SP-4 cells after a long-term HNE treatment

	Concentration of HNE (μM)	Trehalose (μg/10^9 cells)	Percentage (%)
I	0	200.6	100
	250	735.4	367
II	0	168.0	100
	250	728.0	433
Mean	0	184.3	100
	250	731.7	397

10^9 Yeast cells were incubated for 2 h with HNE, washed twice and boiled for 1 h to extract the disaccharide. Following this procedure the trehalose content of the cell lysate was determined by HPLC. Data are shown as μg trehalose per cells and as the corresponding percentages. The results of two independent experiments which were performed in duplicate are shown.

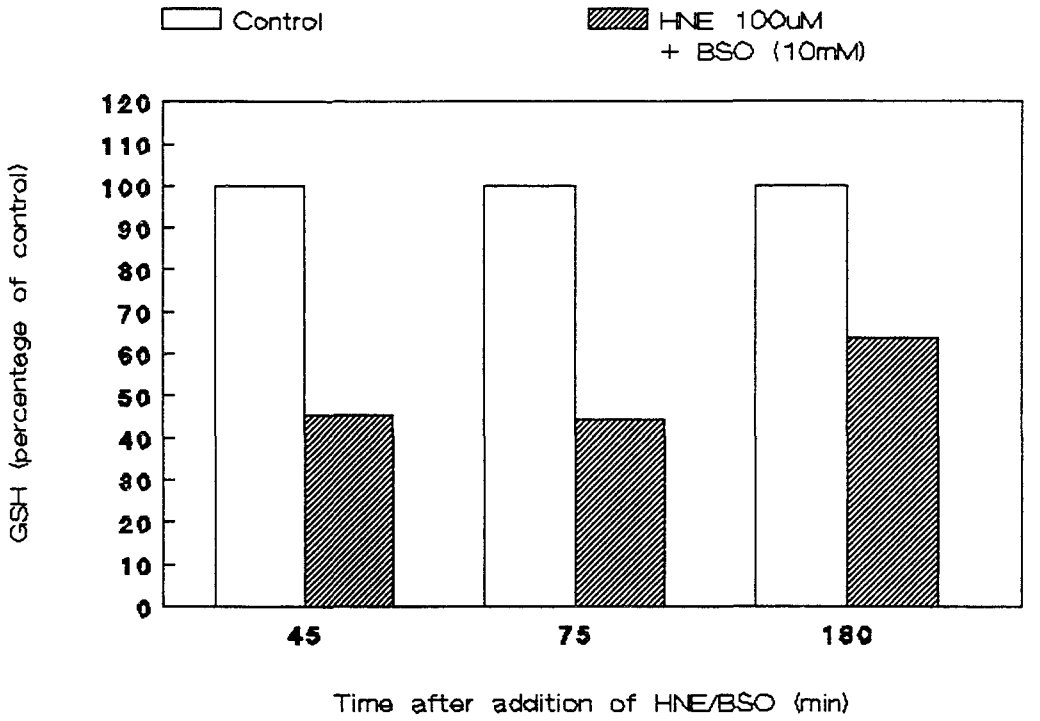

Fig. 2. Inhibition of GSH synthesis in presence of BSO (10 mM) after HNE (100 μM) treatment. SP-4 cells were treated with 100 μM HNE plus 10 mM BSO. The data are the result of single determinations at certain times (45, 75 and 180 min) and are expressed as percentage of control.

onstrated that the maintainance of the GSH level in fibroblasts results from de novo synthesis, because the incubation with HNE did not lead to GSSG formation nor to a rise in the flux through the pentose phosphate shunt, which replenishes NADPH for the reductive pathway. Furthermore, de novo synthesis was substantiated by the sensitivity of a GSH-synthetase deficient strain to HNE as well as by the dramatically lowered viability of monocytes and macrophages during treatment with Buthionine Sulfoximine (BSO), a specific inhibitor of GSH-synthetase, in presence of oxidized LDL [15].

In the presence of BSO plus HNE we could not observe elevated GSH levels in SP-4 cells either (see Fig. 2). Thus, our results support the hypothesis that HNE can induce the synthesis of GSH in eukaryotic cells as a stress response to achieve increased resistance to the cytotoxicity of this aldehyde and related aldehydes derived from the breakdown of lipid peroxides.

Future efforts should be directed to further investigation of the role of GSH and its protective role during lipid peroxidation with the aid of *S. cerevisiae* gsh^- mutant strains [35].

Our results emphasize furthermore the toxicity of HNE by its induction of trehalose accumulation. It was reported that trehalose plays an important role in survival of yeast exposed to several stress conditions such as heat, desiccation [20], starvation [36], freeze-thaw stress [19] and chemical treatment [26].

Our observation of an HNE-induced increase of trehalose is consistent with the finding that chemicals such as ethanol, copper sulfate or hydrogen peroxide increase the content of this sugar as well [26]. Attfield et al. [19] have reported a threshold level of trehalose to attain resistance to stress, which has been found at $> 5\%$ dry cell weight. The recent notion of thermotolerance is a protective cooperation between trehalose and some HSPs. The accumulation of trehalose is a 'fire brigade like action', which prevents protein inactivation. The second step i.e. blocking nonproductive protein–protein interactions and refolding of proteins to their native state, is mediated by HSPs [16,22].

This is furthermore substantiated by the fact that trehalose is a cellular protectant at non-lethal heat treatment, while it is less evident that it accounts, by itself, for the resistance to severe heat stress [37]. A spicy detail at the end of the story is that TPS1 and TPS2, which encode subunits of the trehalose-6-phosphate synthase/phosphatase complex, belong to the family of HSPs [24].

Summarizing, our results indicate that the eukaryote *S. cerevisiae* might be a potent alternative model for free radical research on mammalian cells and suggest further studies with well characterized mutant strains.

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