Neutral trehalase Nth1p of *Saccharomyces cerevisiae* encoded by the *NTH1* gene is a multiple stress responsive protein

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Abstract We have shown previously that expression of the *NTH1* gene is increased at heat stress (40°C) both at the mRNA and enzymatic activity levels. This increased expression was correlated to the requirement of the *NTH1* gene for recovery after heat shock at 50°C and the presence of stress responsive elements STRE (CCCCT) 3 times in its promoter region [S. Nwaka et al., *FEBS Lett.* 360 (1995) 286–290; S. Nwaka et al., *J. Biol. Chem.* 270 (1995) 10193–10198]. We show here that expression of the *NTH1* gene and its product, neutral trehalase (Nth1p), are also induced by other stressors such as H_2O_2, $CuSO_4$, $NaAsO_2$, and cycloheximide (CHX). Heat-induced expression of the *NTH1* gene is shown to be accompanied by accumulation of trehalose. In contrast, the chemical stressors which also induce the expression of *NTH1* did not lead to accumulation of trehalose under similar conditions. Our data suggest that: (1) heat- and chemical stress-induced expression of neutral trehalase is largely due to de novo protein synthesis, and (2) different mechanisms may control the heat- and chemical stress-induced expression of *NTH1* at the transcriptional level. Participation of neutral trehalase (Nth1p) in multiple stress response dependent and independent on trehalose is discussed.

Key words: Neutral trehalase Nth1p; *NTH1*; Multiple stress response; Trehalose; Gene expression; Cycloheximide; *Saccharomyces cerevisiae*

1. Introduction

Stress is a non-physiological state of a cell that may result from environmental insult, pathophysiological state and/or metabolic disturbance. In order to survive stress temporally, organisms synthesize heat-shock proteins (this is part of the so-called heat-shock response) which is conserved during evolution [1,2].

In the yeast *Saccharomyces cerevisiae*, two types of stress proteins are known: (1) classical heat-shock proteins which contain the heat-shock element (HSE) in their promoter. A transcriptional activator called heat-shock factor (HSF) interacts with HSE during stress to bring about increased expression of heat-shock proteins. Examples are hsp104 and hsp70; (2) stress proteins which contain the stress responsive element (STRE) in the promoter of their genes. This element is believed to be bound by a special kind of transcriptional activator(s) different from HSE to bring about increased expression; examples are proteins encoded by *CTT1*, *DDR2*, *TPS2* genes [3–6]. Some *hsp* genes contain both HSE and STRE, and stress-induced expression may be mediated by the two elements [7].

Exponentially growing cells of *S. cerevisiae* exposed to non-lethal heat stress (37–40°C) induce stress proteins and acquire tolerance to lethal heat-shock exposure (50–55°C). This phenomenon is called acquired thermotolerance. In contrast, stationary cells are tolerant to heat-shock exposure (50–55°C) without preceding heat stress — a phenomenon called intrinsic thermotolerance. While many factors appear to participate in thermotolerance, the heat-shock proteins have been shown to be particularly necessary. A similar heat protective function has also been described for trehalose because trehalose concentration increases in cells exposed to heat stress and some other stressors (reviewed in [8,9]).

Trehalose is hydrolyzed by trehalase and three trehalases have been described in *S. cerevisiae*: (1) a neutral trehalase encoded by the *NTH1* gene. This enzyme is located in the cytosol and is responsible for hydrolysis of cytosolic trehalose. It has a pH optimum for activity at 7.0 and is regulated by a cAMP-dependent phosphorylation process [10–14]; (2) a putative trehalase Nth2p encoded by the *NTH2* gene, which is a homolog of the *NTH1* gene [15]. The *NTH2* gene is expressed as demonstrated by mRNA; however, the putative Nth2p has no detectable trehalase activity [13]; (3) the acid trehalase encoded by the *ATH1* gene. This enzyme is believed to be localized in the vacuoles and it has a pH optimum for activity at 4.5. The *ATH1* gene has no sequence similarity with the *NTH1* and *NTH2* genes, and its regulation appears to be different [16,17]. Recent evidence shows that the Ath1p is responsible for utilization and uptake of extracellular trehalose [18,19]. The molecular biology of the trehalases in *S. cerevisiae* has been reviewed recently [20].

In a further study on the role of trehalose in heat tolerance using the neutral trehalase mutants Δ*nth1* and Δ*nth2*, it was shown that high trehalose concentration and thermotolerance are not always correlated [13,14,21]. Using mutants deficient in various steps in trehalose metabolism and certain heat-shock proteins, some groups have also shown lack of correlation between high trehalose accumulation and acquisition of thermotolerance under some conditions [20,22–24]. Furthermore, it was demonstrated that expression of the *NTH1* and *NTH2* genes shows increased inducibility at 40°C compared to 30°C and in so far behaves like the 'classical' heat stress-induced expression of heat-shock proteins [2,13,14]. As expected from the heat stress inducibility, we have shown, based on a sensitive and simple heat-shock recovery growth assay on solid media, that the *NTH1* and *NTH2* genes protect yeast cells from heat-shock damage [13,14]. As a result of these findings, we became interested in studying the expression of the *NTH1* gene under the influence of other stressors, with a view to

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determining whether the Nth1p is a multiple stress responsive protein.

In the present study we show that not only heat but other stressors such as H_2O_2 which causes oxidative injury, cycloheximide (CHX) which is a metabolic stressor and other toxic chemicals ($CuSO_4$, and $NaAsO_2$) increase the expression of the *NTH1* gene both at the mRNA and at the protein level. In contrast, NaCl which causes osmotic stress did not increase the production of neutral trehalase. It is also shown that under the conditions that led to increased mRNA expression of *NTH1* and its protein Nth1p, the toxic chemicals did not lead to an increased trehalose level in the cell.

2. Materials and methods

2.1. Yeast strains, growth conditions and stress treatment

The *NTH1* deletion strain YSN1 (*Mata his3-11,15 leu2-3,112 ura3Δ5 CanR gal⁻ nth1Δ LEU2*) [12,13,21] and its isogenic wild-type strain YS18 (*Mata his3-11,15 leu2-3,112 ura3Δ5 CanR gal⁻*) [25] were grown on YEPD medium (1% yeast extract, 2% peptone and 2% glucose) at 30°C to OD_{600} 0.5–1.0. For stress treatment, the cells were separated into aliquots and exposed to heat stress as described before [14], or to other stressors at the following end concentrations: $CuSO_4$ (5.5 mM), $NaAsO_2$ (1 mM), H_2O_2 (0.4 mM), NaCl (1.5 M) and CHX (1 μg/ml, 10 μg/ml, 50 μg/ml) for 40 min or for various time periods (5, 15 and 40 min). The control cells were not exposed to these chemicals. The cultures were incubated at 30°C with the respective chemicals and after the indicated time cells were immediately harvested and washed with ice-cold water, prior to crude extract preparation for enzyme assay, immuno-blot and total RNA extraction.

2.2. Preparation of the Nth1p antiserum and immunoblotting

To produce antisera to neutral trehalase Nth1p, a synthetic peptide corresponding to amino acid residues 499–515 of the Nth1p deduced from the sequence of the *NTH1* gene [12,26] was synthesized. The peptide was then coupled to keyhole limpet hemocyanine (KLH) as described [27]. Standard procedures were used to generate antiserum in rabbits and mice [28]. The serum was tested for antibody response by Western blot using the pre-immune serum as control.

For the immunoblotting experiments, crude extracts were prepared with glass beads from exponentially growing cells at 30°C, heat stressed cells at 40°C and cells treated with chemical stressors. Protein concentrations were measured as described [29]. Equal amounts of protein (≈50 μg/lane) were loaded onto 10% polyacrylamide–SDS gel. Following electrophoresis, the proteins were transfered to a nitrocellulose membrane (Amersham Buchler, Germany) by electroblotting, and neutral trehalase Nth1p was detected using Nth1p antiserum by standard procedures.

2.3. Assay of Nth1p activity

Enzymatic activity assay for neutral trehalase Nth1p was performed in crude extracts of exponentially growing cells on YEPD at 30°C, heat stressed cells at 40°C, and cells treated with stress agents using standard method as described before [11–13].

2.4. Extraction of total RNA and mRNA analysis

Total RNA from control or stressed yeast cells was extracted after the indicated times according to the method of Elder et al. [30], except that three organic extractions were done with water-equilibrated phenol/chloroform/isoamylalcohol (25:24:1) as described before [14]. RNA was electrophoresed on formaldehyde gel containing 1% agarose, followed by transfer to a nylon membrane by capillary force. Random priming kit, [α-^{32}P]dCTP for DNA labelling and Hybond N^+ membrane for blot analysis were purchased from Amersham Buchler, Germany, and hybridization was performed in the presence of 50% formamide according to manufacturers' instructions.

2.5. Determination of trehalose levels

Yeast cells were harvested by centrifugation at 5000×*g* for 5 min. After washing with ice-cold water the cells were resuspended in water (for example, 1 mg yeast wet weight plus 1 μl H_2O) and heated for 20 min at 95°C. The cell suspension was centrifuged at 10 000×*g* for 5 min and 5 μl of the supernatant were loaded on Silica Gel 60 plates (Merck). Plates were exposed to butanol/ethanol/water (5:3:2, v/v/v) as solvent. Trehalose spots were visualized with 20% H_2SO_4 and heating at 95°C for 15 min [31].

3. Results

3.1. Nth1p enzymatic activity and antibody response are increased by H_2O_2, $CuSO_4$, and $NaAsO_2$

Our previous demonstration that the neutral trehalase Nth1p activity is increased by heat stress [14] prompted us to study Nth1p activity in the presence of some toxic chemicals (H_2O_2, $CuSO_4$, and $NaAsO_2$), which are known to induce expression of stress proteins at the given concentrations [32]. As shown in Table 1 (first line), the Nth1p activity increases 2–3-fold when control cells are exposed to heat stress (40°C), H_2O_2 (0.4 mM), $NaAsO_2$ (1 mM) and $CuSO_4$ (5.5 mM) for 40 min. In contrast, NaCl (1.5 M or lower) did not increase Nth1p activity. The about 3 times increase in Nth1p activity resulting from heat stress (40°C treatment) is consistent with previously published data [13,22,33]. The non-treated control cells (Table 1) exhibited specific Nth1p activity of 12 mU/mg protein which is within the normal level reported before for exponentially growing cells on glucose [12,13,22].

Using antibodies raised against the Nth1p (see Section 2), it is shown in Table 1 (second line) and the picture below, that the increase in Nth1p activity (first line of Table 1) is the result of increase in concentration of Nth1p. Nth1p levels increase when cells are exposed to heat, H_2O_2, $CuSO_4$ and, to some extent, $NaAsO_2$. In contrast, the Nth1p level in cells exposed to NaCl did not increase similar to Nth1p activity.

3.2. mRNA expression of the NTH1 gene in the presence of H_2O_2, $CuSO_4$, and $NaAsO_2$

mRNA expression of the *NTH1* gene was shown to be induced by heat stress [14]. The additional results (Table 1) that Nth1p activity and immunoreactivity is increased by H_2O_2, $CuSO_4$, and $NaAsO_2$ led us to study the mRNA expression of the *NTH1* gene under the same conditions. As shown in Fig. 1, H_2O_2 strongly increased mRNA of *NTH1* gene compared to control after 40 min. $NaAsO_2$ led to an increase in mRNA of *NTH1* after 5 and 15 min in contrast to H_2O_2 which did not increase the expression of *NTH1* within 15 min. Furthermore, $CuSo_4$ increased the mRNA in a short time but the mRNA gradually decayed with time (data not shown). These data (together with that presented in Table 1), support the participation of de novo protein synthesis in the increase in Nth1p activity after exposure to heat, H_2O_2, $CuSO_4$ and $NaAsO_2$.

3.3. mRNA expression of the NTH1 gene and Nth1p activity in the presence of CHX

The increased mRNA expression of *NTH1* and Nth1p resulting from heat stress, oxidative stress and other chemicals, lead us to study the effect of metabolic stress on *NTH1* expression by using CHX. In yeast, CHX leads to metabolic shock [34], probably due to its inhibition of protein synthesis [35,36]. As shown in Fig. 2, the following concentrations of CHX (1, 10 and 50 μg/ml) led to high level expression of the *NTH1* gene after 40 min compared to control cells. The extent

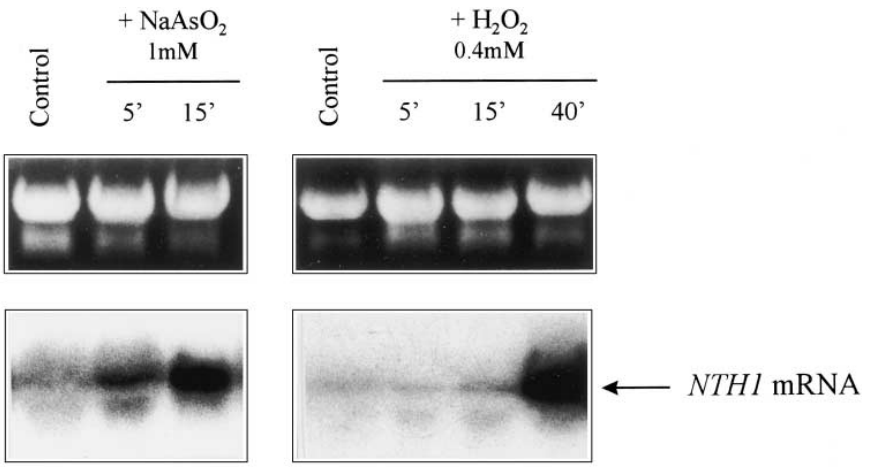


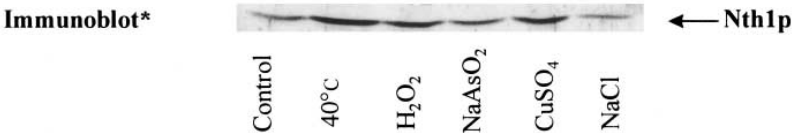
Fig. 1. mRNA expression of the *NTH1* gene in the presence of $NaAsO_2$ and H_2O_2. Cells were grown on YEPD medium at 30°C to OD_{600} 0.5–1.0. The cells were separated into aliquots and exposed to the indicated concentration of the indicated stressors for the indicated time. Cells used as controls did not undergo stress treatment. Upper panel: Ethidium bromide-stained formaldehyde gel to show that equal amounts of RNA were loaded. Lower panel: *NTH1*-specific mRNA as determined by Northern blot using a 1.7 kb (*Bam*HI–*Hin*dIII) fragment of the *NTH1* gene as a radiolabelled probe.

of increase is shown to depend on the concentration of CHX and the time of exposure (Fig. 2).

In accordance to the increased mRNA expression of *NTH1* by CHX, it is shown in Fig. 3 (open bar on the right), that CHX increased the Nth1p activity about 2 times compared to control. A control experiment showed that the concentrations of CHX used in this study inhibited cell growth, consistent with inhibition of protein synthesis (data not shown). In a previous study [37,38], it was shown that CHX increases the activity of neutral trehalase in derepressed cells. It is also shown in Fig. 3 (closed bars on the right) that CHX has no significant effect on the heat- and chemical stress-induced increase in Nth1p activity, similar to what was observed for heat in *S. Pombe* [39].

Table 1
Nth1p activity and antibody response after exposure to heat, H2O2, $NaAsO_2$, and $CuSO_4$

	40 minutes exposure					
	30°C Contr.	40°C	0.4mM H_2O_2	1mM $NaAsO_2$	5.5mM $CuSO_4$	1.5M NaCl
Relative increase in Nth1p activity	1	3	2.0	2.5	2.8	1.1
Relative band intensity* (immunoblot)	1	2.7	2.3	1.3	2.1	0.9



Exponentially growing wild-type cells on YEPD at 30°C were exposed to the respective stressors for 40 min as indicated in the table. The relative increase in Nth1p activity was worked out using activity of control cells (12 mU/mg protein) as factor 1. The immunoblot* data (below table) was quantified using the ImageMaster (Pharmacia) to generate the relative band intensity* numbers in the table, using control as factor 1. Preparation of crude extracts and assay method is described in Section 2.

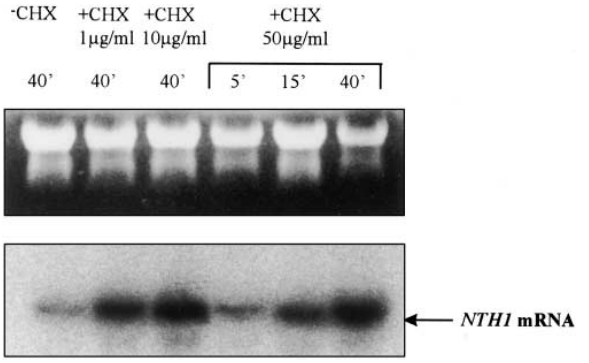


Fig. 2. Increased mRNA expression of the *NTH1* gene in the presence of different concentrations of CHX at different times. Cells were grown under the same conditions as described in Fig. 1, and the indicated concentrations of CHX were added to the cells for the given time. Upper panel: Ethidium bromide-stained formaldehyde gel of the total RNA showing that equal amounts of RNA (≈15 μg) were loaded on each lane. Lower panel: *NTH1*-specific mRNA as determined by Northern blot using a 1.7 kb (*Bam*HI–*Hin*dIII) fragment of the *NTH1* gene as a radiolabelled probe. Total RNA was extracted and probed as described in Section 2. CHX, cycloheximide.

3.4. *Trehalose levels after exposure of wild-type and Δnth1 cells to heat stress, H_2O_2, $CuSO_4$, and $NaAsO_2$*

The increase in expression of the *NTH1* gene and Nth1p protein level in the presence of the stressors under consideration raised the question if the level of trehalose changes reciprocally to the activity of neutral trehalase. As shown in Fig. 4 (left side), exponentially growing wild-type cells on glucose show low or no detectable trehalose (control 30°C). As the cells are exposed to heat stress (40°C), trehalose level increases. This observation has also been reported by several authors for heat-stressed cells [13,21,22,33,40,41]. In contrast to heat stress, H_2O_2 (0.4 mM), $CuSO_4$ (5.5 mM), $NaAsO_2$ (1 mM) and CHX (50 μg/ml) did not lead to a detectable trehalose accumulation within 40 min (Fig. 4). To examine if the non-detectable trehalose level observed in the presence of H_2O_2, $CuSO_4$, $NaAsO_2$, and CHX is due to rapid hydrolysis of trehalose by the neutral trehalase Nth1p, we performed the same experiment with Δ*nth1* mutant cells which cannot hydrolyze internal trehalose [12,13,21]. As shown in Fig. 4 (right side), exponentially growing Δ*nth1* cells at 30°C (control) and heat-stressed cells have higher steady-state levels of trehalose compared to wild-type (see also [12,13]). The Δ*nth1* cells exposed to the various stress chemicals show low levels of trehalose similar to the control. Therefore, these chemicals in contrast to heat stress did not cause a significant increase in trehalose level. Some workers have shown increased trehalose accumulation in the presence of $CuSO_4$ and CHX under special conditions, for example, after a longer incubation time or with different concentrations of the chemicals or different growth conditions [34,40]. Similar to our data, Coote et al. [42] showed that CHX did not lead to increase in trehalose level in growing cells. In another study [43], it was shown that a non-lethal concentration of the chemical stressor called tetrachloroisophthalonitrile (TPN) led to induction of expression of stress proteins; however, trehalose level did not increase.

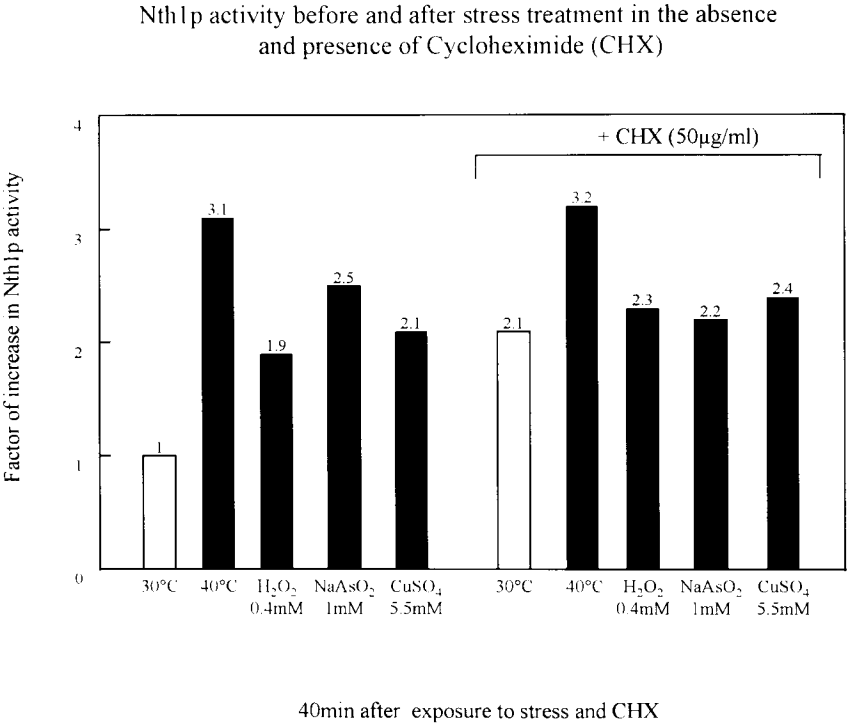


Fig. 3. CHX increases Nth1p activity but has no effect on the increase resulting from heat, H_2O_2, $NaAsO_2$ and $CuSo_4$. The condition for the experiment is same as described in Figs. 1 and 2. The bars on the left represent the Nth1p activity in the presence of the indicated stressors without CHX. The bars on the right represent the same Nth1p activity in the presence of the stressors plus CHX (cells were pre-incubated with CHX for 15 min before the stress treatments). The control cells (open column, left side) without CHX show a normal activity of Nth1p (factor 1), while in the presence of CHX (open column, right side) the activity increased by a factor of about 2.

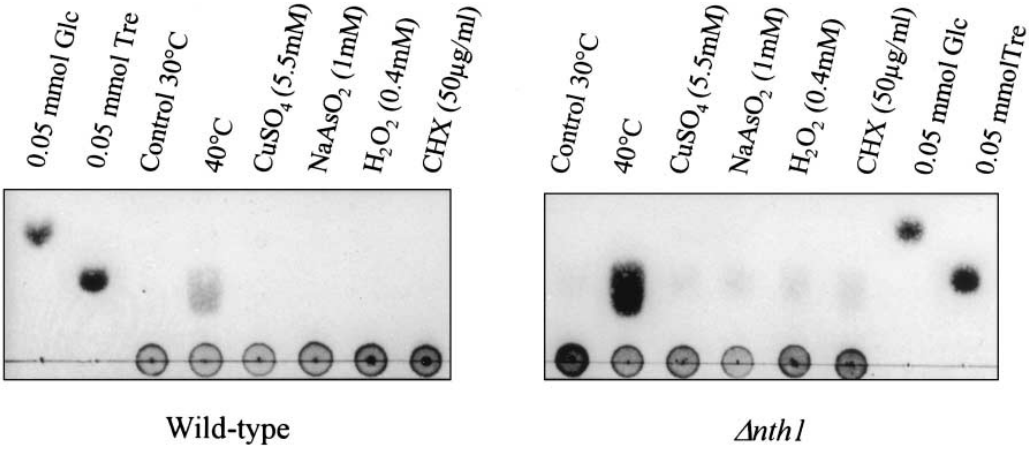


Fig. 4. Trehalose level after exposure to heat, $CuSO_4$, $NaAsO_2$, and H_2O_2. Cells were grown as described in the other figures. The concentration of stressors are indicated in the figure. The cells were exposed to the various stressors for 40 min. Trehalose was extracted by boiling a cell suspension and equal samples were loaded on thin layer chromatography plates as described in Section 2. 5 µl of 10 mM glucose or trehalose samples served as control to detect the corresponding sugar.

4. Discussion

Stress protein inducers are bewildering in their variety, but many have in common the capacity to produce protein damage [32,44,45]. It has been proposed that a common signal for heat-shock protein induction is protein denaturation and one function of the induced proteins is to prevent or repair denaturation damage [46]. This calls into question the kind of damage caused by certain toxic chemicals. H_2O_2 is a known reactive oxygen species which can cause oxidation of proteins, DNA lession and lipid peroxidation [47,48]. The effect of such oxidants has been implicated in cancer, cardiovascular diseases, ageing and, more recently, HIV-1 expression and replication [49]. The known anti-oxidative defense systems employed by the cell include enzymes such as superoxide dismutase and catalase, and nonenzymatic molecules such as glutathion and vitamin C [50]. The strong increase of the *NTH1* gene expression induced by H_2O_2 similar to the *CTT1* gene encoding the cytosolic catalase T [4,51] suggests that Nth1p-like catalase T may participate in protection against oxidative stress in yeast. Interestingly, *CTT1* and *NTH1* genes share some feature such as: regulation by a cAMP-dependent phosphorylation and presence of STRE in their promotor different from HSE (reviewed in [20,38,52]). In contrast, while NaCl increases the activity of catalase T, no increase in the activity or protein level of the Nth1p in the presence of NaCl could be detected under our conditions. This suggests that the Nth1p does not participate in osmotic stress tolerance in *S. cerevisiae*.

The type of damage caused to cells on exposure to $NaAsO_2$ and $CuSO_4$ is not very clear. Arsenite may modify protein activity or structure by reacting with thiol groups and transiently affect cell growth [53], while $CuSO_4$ may resemble H_2O_2 in formation of free radicals or reactive oxygen intermediates [54]. These chemicals are known to induce the expression of Hsp104 and certain genes involved in metal homeostasis [32,53,54]. Therefore increased expression of the *NTH1* gene in the presence of $NaAsO_2$ or $CuSO_4$ indicates either a direct or indirect participation of Nth1p in the defence mechanism against the toxic effect of these chemicals.

Our data show that CHX causes increased mRNA expression of the *NTH1* gene, as well as Nth1p activity. In *S. cerevisiae*, cycloheximide increases mRNA expression of the *TPS2* gene encoding the trehalose-6-phosphate phosphatase [34], the DNA damage-inducible gene *RAD2* [55], and the multidrug resistance genes *YDR1* and *SNQ2* [56]. In mammalian cells, the transcriptional activator NF-κB which has some stress-related function is also induced by CHX [57,58]. This extends the spectra of response of the *NTH1* gene to drugs.

Recently it was demonstrated that the zinc finger proteins Msn2p and Msn4p participate in the transcriptional activation of the *CTT1*, *TPS2*, *DDR2* and *HSP12* genes during stress by interacting with the STRE (CCCCT) present in their promoter [5,6]. STRE is found 3 times in the promoters of the *NTH1* and *NTH2* genes [14,20]. We are working on the mutagenesis of STRE in the *NTH1* and *NTH2* promoters.

During heat stress, the activities of the trehalose hydrolyzing (neutral trehalase) and synthesizing (trehalose synthase) enzymes increase, as well as mRNA expression of their genes. In addition, trehalose level increases simultaneously [14,41,59–61]. This seemingly futile cycling of trehalose turnover during heat stress was suggested to be necessary for maintenance of a constant glucose concentration in the cytosol [33]. Subsequently it was proposed that glucose from trehalose might be necessary for the cell to survive heat shock because a mutant of the *Δnth1* was defective in survival after heat shock [13,14]. The much higher concentration of trehalose at heat stress, as compared to chemical stress, indicates an important role of the steady-state concentrations of trehalose mediated by synthesis and hydrolysis during heat stress.

Our results with heat compared to chemical stress show that high trehalase activity and/or expression of the *NTH1* gene does not always correlate with high trehalose level during stress. The mechanism of stress protection afforded by the trehalases during heat stress may therefore be different from those resulting from oxidative stress (H_2O_2) or other chemical stress. The time-dependent differences in the induction of *NTH1* by the various chemicals indicate different effects of these chemicals. Furthermore, in contrast to heat, no significant differences in survival have so far been observed between wild-type and *Δnth1* mutant in the presence of higher concentrations of these chemicals (data not shown).

In conclusion, our results support a role of the Nth1p in multiple stress response. Like some stress proteins, we propose that Nth1p may participate in stress response by preventing protein damage resulting from stress and/or by its role in trehalose hydrolysis.

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