Volume 13, Issue 2, Page 106-113, 2024 https://doi.org/10.46810/tdfd.1394491 Research Article

BİNGÖL UNIVERSITY

Türk Doğa ve Fen Dergisi
Turkish Journal of Nature and Science
www.dergipark.gov.tr/tdfd

# Effect of cAMP-dependent Phosphodiesterase Activity on *NTH1* Gene Expression and Reserve Carbohydrate Metabolism

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(Received: 22.11.2023, Accepted: 10.06.2024, Online Publication: 28.06.2024)

**Keywords**
Heat stress, Nitrogen starvation, *PDE1*, *PDE2*, *Saccharomyces cerevisiae*, Stress recovery

**Abstract:** The regulation of Protein kinase A signaling pathway is controlled by the cellular cAMP level. The level of cAMP in the cell is regulated by the enzymes adenylate cyclase, which synthesizes the cAMP from ATP, and cAMP phosphodiesterase, which degrades the cAMP to AMP. The *PDE1* and *PDE2* genes encode two phosphodiesterases with low and high affinity for cAMP, respectively. The *NTH1* gene encodes the neutral trehalase enzyme, which is responsible for stress-accumulated trehalose degradation, and its expression is regulated by PKA. The aim of this study was to examine the effect of *PDE1* and *PDE2* gene products on the expression of the *NTH1* gene and reserve carbohydrate metabolism in both stressful and restoring conditions. Thus, the expression of the *NTH1* was assessed under nitrogen starvation, heat stress, and recovery period by inserting the plasmid containing the Nth1-LacZ gene fusion into *pde1*Δ, *pde1*Δ, and wild-type yeast strains. The expression of the *NTH1* gene was shown to be lower than that of the wild-type under normal conditions, heat stress, nitrogen starvation, and also during the replenishment period in *pde1*Δ and *pde1*Δ yeast cells. The accumulation of trehalose and glycogen was shown to be dramatically enhanced in *pde1*Δ yeast cells. However, deletion of the *PDE2* gene did not lead to a significant change in trehalose and glycogen accumulation comparable to that found in the wild-type. These results indicate that the *PDE1* gene product, rather than *PDE2*, is required for the downregulation of reserve carbohydrate metabolism. Consequently, the Pde1 protein is considered to exert yet-unidentified regulatory control over the Pde2 protein.



# cAMP-bağımlı Fosfodiesteraz Aktivitesinin *NTH1* Gen Ekspresyonu ve Depo Karbonhidrat Metabolizması Üzerine Etkisi

**Anahtar Kelimeler**
Isı stresi, Azot açlığı, *PDE1*, *PDE2*, *Saccharomyces cerevisiae*, Stresten kurtulma

**Öz:** Protein kinaz A sinyal yolağının düzenlenmesi hücresel cAMP seviyesi tarafından kontrol edilir. Hücredeki cAMP seviyesi ise, cAMP'yi ATP'den sentezleyen adenilat siklaz ve cAMP'yi AMP'ye indirgeyen cAMP fosfodiesteraz enzimleri tarafından düzenlenir. *PDE1* ve *PDE2* genleri, cAMP için düşük ve yüksek afiniteye sahip iki fosfodiesterazı kodlar. *NTH1* geni stres şartlarında biriktirilen trehalozun parçalanmasından sorumlu olan nötral trehalaz enzimini kodlar ve ekspresyonu PKA tarafından kontrol edilir. Bu çalışmada, stres koşullarında ve stres sonrasında, *PDE1* ve *PDE2* genlerinin *NTH1* gen ekspresyonu ve depo karbonhidrat metabolizması üzerine etkisinin belirlenmesi amaçlanmıştır. Bu nedenle Nth1-LacZ gen füzyonu taşıyan plazmid *pde1*Δ, *pde1*Δ ve yaban tip maya suşlarına transforme edilerek azot açlığında, ısı stresinde ve stres sonrasında *NTH1* gen ekspresyonu belirlendi. Normal koşullarda, ısı stresinde, azot açlığında ve stresten kurtulma sonrasında, *pde1*Δ ve *pde1*Δ maya hücrelerinde belirlenen *NTH1* gen ekspresyonunun yaban tipten daha düşük olduğu gözlendi. Trehaloz ve glikojen birikiminin *pde1*Δ maya hücrelerinde oldukça yüksek oranda arttığı gözlendi. Bununla birlikte, *PDE2* yokluğunun trehaloz ve glikojen birikiminde önemli bir değişikliğe yol açmadığı gözlendi. Bu sonuçlar, depo karbonhidrat metabolizmasının aşağı regülasyonu için *PDE2*'nin değil de *PDE1* gen ürününün gerekli olduğunu göstermektedir. Sonuç olarak, Pde1 proteininin, Pde2 proteini üzerinde henüz tanımlanamayan düzenleyici kontrol uyguladığı düşünülmektedir.

## 1. INTRODUCTION

The budding yeast *Saccharomyces cerevisiae* has the ability to respond to environmental alterations by using different sensing and signaling mechanisms. Depending on nutrient availability, yeast cells alter their gene expression patterns and related metabolic pathways. The stress response system is a good example of how to analyze these alterations in nutrient-deficient conditions. The stress tolerance of yeast cells begins to increase whenever they enter the stationary phase of growth, where essential nutrients are depleted [1, 2]. One of these signaling pathways is the RAS-cAMP-PKA pathway, which is involved in different stress responses such as oxidative stress, osmotic stress, and STRE-related stress. Protein kinase A (PKA) is a tetrameric protein that is composed of two regulatory subunits (encoded by *BCY1*) and two catalytic subunits (encoded by *TPK1*, *TPK2*, and *TPK3*) [2, 3]. The activity of PKA is regulated by the binding of cyclic AMP (cAMP) to Bcy1p, which results in the release of catalytic subunits. The level of cAMP in the cell is regulated by the activity of RAS-associated adenylate cyclase (encoded by *CYR1*) and cAMP phosphodiesterase (encoded by *PDE1* and *PDE2*) enzymes. The adenylate cyclase enzyme is responsible for the synthesis of cAMP from ATP, and the phosphodiesterase enzyme degrades the cAMP to AMP [4, 5].

The primary amino acid sequences of Pde1 and Pde2 cAMP phosphodiesterases are not similar. *PDE1* and *PDE2* genes were coded for 526 and 369 amino acids, respectively. Both phosphodiesterase enzymes have different affinities for cAMP. The monomeric Pde1p has low affinity for cAMP, with its Michaelis-Menten constant ($K_m$) changing between 20 and 250 μM. The dimeric Pde2p has a high affinity for cAMP, with a $K_m$ for cAMP of 170 nM. Pde1 protein has dual activity on the hydrolysis of both cAMP and cGMP with similar enzymatic efficiencies. Pde1p is required for lowering the concentration of intracellular cAMP levels upon the addition of glucose. In addition, Pde1p has a specific role in agonist-induced cAMP signaling in response to intracellular acidification. Pde2p is responsible for breaking down exogenous cAMP. Pde2p controls the basal cAMP level in the cell. Deletion of the *PDE2* gene causes the accumulation of cAMP and continuously active PKA, which results in irregularities in cell wall integrity and stress response pathways [5-7].

The level of the stress metabolite, trehalose, is controlled by cAMP in *S. cerevisiae* yeast cells. Trehalose accumulation starts in the diauxic phase and continues during the stationary phase in normal growth conditions. But in a stressful environment, trehalose is accumulated at the logarithmic phase. The stress-accumulated trehalose must be rapidly degraded whenever the stress is terminated. The level of trehalose in yeast cells is maintained by means of the TPS enzyme complex and the trehalase enzyme. The hydrolysis of trehalose is fulfilled by the neutral trehalase enzyme encoded by the *NTH1* gene. The promoter region of *NTH1* contains three STRE motifs that are the targets of transcriptional activator proteins, Msn2 and Msn4. *NTH1* expression is induced by various stresses as well as by cAMP-dependent protein kinase [8]. Therefore, the inhibition of Msn2p and Msn4p activation via the highly activated RAS-cAMP-PKA pathway or some other interconnected pathways directly effects the stress response of the yeast *S. cerevisiae* [9-11].

We investigated the role of cAMP phosphodiesterases in *NTH1* gene expression and reserve carbohydrate deposits under nitrogen deficiency and heat stress. Our results indicated that the deletion of *PDE1* or *PDE2* genes downregulates *NTH1* gene expression both in normal growth conditions and stressful environments. In addition, the *PDE1* gene product may be involved in the downregulation of reserve carbohydrate metabolism.

## 2. MATERIAL AND METHOD

### 2.1. Yeast Strains and Plasmids

Yeast strains BY4741 (*Mata, his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*) and mutant derivatives *pde1Δ* (*Mata, his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*; YGL248w::kanMX4) and *pde2Δ* (*Mata, his3Δ1; leu2Δ0; met15Δ0; ura3Δ0;* YOR360c::kanMX4) were used in this study. The yeast strains were purchased from EUROSCARF (Frankfurt, Germany). *PDE1* and *PDE2* genes were completely replaced with the KanMX4 module in both *pde1Δ* and *pde2Δ* strains, respectively. The pNL1 plasmid, including the Nth1-lacZ gene fusion, was used to quantify the promoter activity of the *NTH1* gene. The other plasmid, including the Suc2-lacZ gene fusion, was used as a control.

*S. cerevisiae* strains were cultured in YPD medium (1% yeast extract, 2% peptone, and 2% glucose) for plasmid transformation. The plasmids were transformed into wild-type, *pde1Δ*, and *pde2Δ* yeast strains using a procedure as described previously [12]. Yeast cells were plated on Yeast Synthetic Minimal (YSM) medium without uracil (0.17% yeast nitrogen base (w/o amino acids and ammonium sulphate) + 0.5% ammonium sulphate + 20 mg $L^{-1}$ histidine + 60 mg $L^{-1}$leucine + 20 mg $L^{-1}$ methionine) supplemented with 2% glucose and grown at 30 °C till well-grown transformant colonies were obtained.

### 2.2. Growth Conditions

Transformant yeast cells were grown overnight in YSM medium at 120 rpm and 30 °C. The overnight cultures were resuspended in fresh YSM culture and grown to an exponential phase under the same conditions. The yeast cultures were then separated into two groups, and one part of the culture was transferred directly to a 42 °C incubator without changing the other growth conditions. The second part was harvested, washed, refreshed in YSM culture supplemented with proline (0.1%), and then incubated at 30 °C. All yeast cultures were further incubated for four hours. At the end of incubation periods, half of the cultures were harvested and used for measurements of β-galactosidase activities, trehalose, and glycogen contents

of the yeast cells. The second half of the cultures grown at 42 °C were transferred to 30 °C for heat stress recovery, and 0.5% ammonium sulphate was added to rescue yeast cells from nitrogen starvation. Then all yeast cultures were grown at these conditions for further 4 hours.

### 2.3. Enzyme Assays

The harvested yeast cells were used for determining β-galactosidase activities as described previously [13]. Beta galactosidase units (U) were given in nanomoles of ONPG (o-Nitrophenyl β-D-Galactopyranoside) cleaved per minute per milligram of protein in permeabilized yeast cells. Trehalose and glycogen assays of yeast cells were determined as described previously [14]. The yeast transformants were removed, washed with ice-cold water, and then resuspended in 125 µL of 0.25 M $Na_2CO_3$ and incubated at 95 °C for about 3 hours. Then 75 µL of 1 M acetic acid and 300 µL of 0.2 M sodium acetate, pH 5.2 were added and divided into two parts. One half of the cell mixture was incubated at 37 °C overnight in the presence of 3 mU trehalase enzyme (Sigma, T8778) for the trehalose assay. The second half of the suspension was incubated at 57 °C for overnight with continuous shaking in the presence of 70 U $mg^{-1}$ amyloglucosidase enzyme from *Aspergillus niger* (Sigma, 10115) for the glycogen assay. The quantity of liberated glucose was measured enzymatically using the glucose oxidase-peroxidase system (GOD-POD assay) (Fluitest®-GLU, Biocon, Germany). The amounts of trehalose and glycogen in the yeast cells were calculated and expressed as micrograms of glucose equivalent per milligram of wet mass (µg $mg^{-1}$).

### 2.4. Statistical Analysis

The results were reported as the means ± standard deviation (SD) of at least three independent transformants and three independent experiments. Data analysis was performed using GraphPad Prism software 5 (GraphPad Software Inc., La Jolla, USA). Differences were considered statistically significant when a P value was less than 0.05.

## 3. RESULTS AND DISCUSSION

### 3.1. Effects of Pde1 and Pde2 on *NTH1* Transcription

In order to determine the effects of low- and high-affinity cyclic AMP phosphodiesterases on regulation of *NTH1* gene transcription, Nth1-lacZ gene fusion was transformed into wild-type, *pde1Δ*, and *pde2Δ* strains. The expression of Nth1-lacZ gene fusion in wild-type, *pde1Δ*, and *pde2Δ* yeast cells was found to be 857.7±98.6 U, 437.2±40.5 U, and 226.3±27.3 U, respectively (Figure 1). The *NTH1* gene expression decreased 2-fold in the absence of Pde1 protein and 2-fold in the absence of Pde2 protein at normal growth conditions. This result indicated that the absence of PDE genes caused a significant reduction in *NTH1* transcription under normal growth conditions. Also, the promotor activation in *pde2Δ* yeast cells was 2-fold lower than that in *pde1Δ* yeast cells.

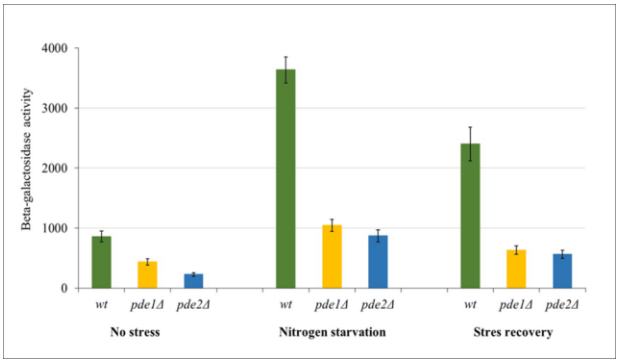


**Figure 1.** The effect of Pde1 and Pde2 proteins on the expression of *NTH1* gene during nitrogen starvation and recovery. For nitrogen starvation yeast cells transferred to 0.1% proline and for recovery 0.5% ammonium sulfate was added. Incubation period for nitrogen starvation and recovery period was 4 hours.

In glucose-grown yeast cells, PKA is activated through the RAS-cAMP pathway, depending on the cAMP concentration. High cAMP accumulation results in a continuously active PKA that phosphorylates Rim15, Yak1, and Msn2/4 and localizes them in the cytoplasm [2, 10, 15, 16]. Also, nuclear Msn2/4 proteins are phosphorylated by active Yak1 and Rim15 kinases from sites apart from the PKA phosphorylation site, and then they bind to the relevant stress elements (STRE sequences) and activate transcription. Therefore, in the absence of *PDE1* and *PDE2* gene products, continuously active PKA blocks Msn2/4 activation, resulting in reduced *NTH1* transcription in a stress-free environment. As a result, even under normal growth circumstances, cAMP phosphodiesterase enzyme activity is required for *NTH1* gene regulation. Furthermore, it was shown that the Pde2 protein exhibited greater efficacy in facilitating *NTH1* transcription compared to the Pde1 protein.



The exponentially growing yeast cells were transferred to the nitrogen starvation condition, and the promoter activations were determined (Figure 1). The expression of Nth1-lacZ gene fusion in the wild-type, *pde1Δ* and *pde2Δ* yeast cells was determined to be 3636.2±218.5 U, 1044.5±98.6 U, and 869.8±95.2 U, respectively. The promoter activity of wild-type yeast cells increased nearly 4-fold in nitrogen starvation. Similarly, when *pde1Δ* and *pde2Δ* mutant yeast cells were transferred to poor nitrogen sources, the expression level increased nearly 2-fold in *pde1Δ* and 4-fold in *pde2Δ* mutants. The *NTH1* transcription level in *pde1Δ* and *pde2Δ* yeast cells was 2-fold lower than in wild-type yeast cells under nitrogen starvation conditions. When the yeast cells shifted to a nitrogen-rich environment, Nth1-lacZ gene expression became 2400.4±278.6 U, 631.8±72.6 U, and 561.6.6±65.3 U in the wild-type, *pde1Δ*, and *pde2Δ* yeast cells, respectively. Although the *NTH1* promoter activity in wild-type and mutant yeast cells decreased nearly 2-fold after the replenishment period, it was still higher than in the normal growth condition. Yeast strains did not show a dramatic fall in *NTH1* gene expression after the 4-hour post-stress period, but it was observed that there was a gradual recovery of gene expression back to pre-stress patterns after 8 hours (data not given).

When heat stress was applied to yeast cells, Nth1-lacZ expression of wild-type, *pde1Δ*, and *pde2Δ* yeast cells was measured as 1789.6±214.6 U, 679.7±71.8 U, and 288.6±50.3 U, respectively (Figure 2). The shifting of wild-type and *pde2Δ* yeast cells to 42 °C increased the expression of Nth1-LacZ gene fusion nearly twofold. However, the shifting of *pde1Δ* yeast cells to heat stress did not cause a change in *NTH1* promoter activity. After the 4-hour recovery period, the *NTH1* promoter activities of wild-type (766.5±72.7 U) and *pde1Δ* yeast cells (458.3±62.8 U) were returned to the pre-stress level. Interestingly, in the absence of the *PDE2* gene, *NTH1* transcription was not affected during heat stress and stress recovery. Generally, *NTH1* transcription levels in *pde1Δ* and *pde2Δ* yeast cells became lower than wild-type yeast cells under both normal and stressful conditions. In addition, regulation of *NTH1* gene expression in stress conditions is faster than in stress recovery.

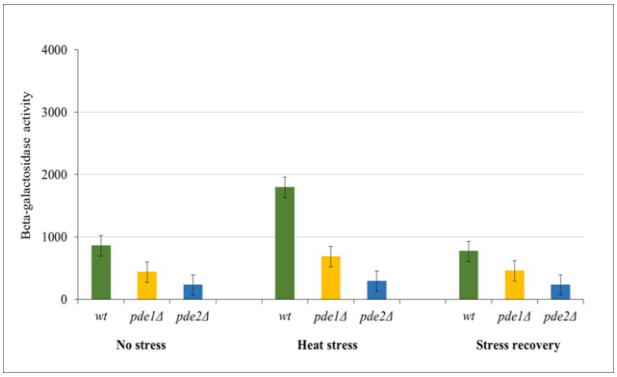


**Figure 2.** The effect of Pde1 and Pde2 proteins on the expression of *NTH1* gene during heat stress and recovery. For heat stress yeast cells transferred to 42 °C and for recovery yeast cells retreated to 30 °C. Incubation period for heat stress and recovery period was 4 hours.

The variable stress conditions activate Msn2/4 and Hsf1 transcription factors via different signaling pathways. Msn2/4 and Hsf1 transcription factors bind to the STRE sequences and heat shock elements (HSE), respectively. Msn2/4 transcription factors are localized both in the cytoplasm and nucleus, depending on environmental conditions [15]. The nuclear localization and activity of Msn2/4 proteins increase after nutrient starvation or other stress treatments [15-17]. Hsf1 transcription factor is localized in the nucleus and bound to HSE under normal growth conditions. The occupancy and activity of Hsf1 increase after heat shock treatment. The activity of Msn2/4 and Hsf1 transcription factors is regulated by different signaling pathways. Yak1 and Rim15 kinases are regulated by cAMP-dependent PKA and TOR (Target of Rapamycine) signaling pathways [18]. Rapamycin treatment or nitrogen starvation causes the inactivation of the TOR pathway, which results in the nuclear accumulation of Msn2/4 proteins and the localization of Yak1 and Rim15 kinases in the nucleus [2, 10, 16, 18, 19]. Thus, Yak1 kinase activates the nuclear Msn2/4 and Hsf1 proteins for binding to STRE and HSE sequences, respectively. Therefore, in our research, *NTH1* gene expression increased in wild-type yeast cells in response to nitrogen starvation and heat stress. The constitutive activation of the RAS/cAMP pathway blocks several rapamycin-induced responses, such as the activation of stress transcription [18, 20]. The high cAMP level in the absence of cAMP phosphodiesterase enzyme activity caused constitutive activation of PKA and may have blocked STRE-mediated *NTH1* gene expression. Therefore, *NTH1* transcription in *pde1Δ* and *pde2Δ* yeast strains may have been lower than in wild-type yeast cells.

*SUC2* gene expression is independent of stress regulation, so it was used as a control. The expression of Suc2-lacZ gene fusion at normal growth conditions was found to be 1.48±0.02 U, 1.12±0.02 U, and 1.53±0.03 U in wild-type, *pde1Δ*, and *pde2Δ* yeast strains, respectively. The Suc2-lacZ expressions in wild-type, *pde1Δ*, and *pde2Δ* yeast strains were found to vary between 1.88±0.01 U and 4.72±0.05 U during nitrogen starvation, heat stress, and replenishment periods.

### 3.2. Effects of Pde1 and Pde2 on Trehalose and Glycogen Accumulation

*S. cerevisiae* yeast cells accumulate trehalose and glycogen as an energy source when environmental conditions become unfavorable. The accumulation of both carbohydrates is regulated by different signaling pathways, such as the RAS/cAMP pathway, depending on the growth rate of yeast cells. Therefore, the effect of cAMP phosphodiesterase activity on reserve carbohydrate accumulation was determined in wild-type, *pde1Δ*, and *pde2Δ* yeast strains during the exponential phase. As expected, the trehalose level was 2-fold higher than the glycogen level in wild-type cells under normal growth conditions (Figure 3). In *pde1Δ* yeast cells, the level of trehalose was almost four times higher than the level of glycogen. However, the levels of trehalose and glycogen in *pde1Δ* yeast cells were 17-fold and 9-fold higher, respectively, than in wild-type cells. The trehalose level in *pde2Δ* yeast cells was nearly 2-fold higher than the glycogen level, and the level of these carbohydrates in *pde2Δ* yeast cells was similar to the wild-type level. Both trehalose and glycogen levels in the yeast cells were quite high in the presence of solo *PDE2* gene. But the presence of a solo *PDE1* gene (*pde2Δ*) or the presence of both *PDE1* and *PDE2* genes (wt) resulted in basal-level accumulation of these reserve carbohydrates. These results suggest that *PDE1* and *PDE2* gene products may be involved in down- and up-regulation of reserve carbohydrate metabolism, respectively.

It was previously reported that *PDE2* deletion did not result in a significant reduction in expected PKA-dependent phosphorylation events [21]. Furthermore, deletion of the *PDE1* gene results in significantly larger cAMP accumulation in response to glucose addition, whereas deletion of the *PDE2* gene results in low cAMP accumulation [22]. The phosphorylation of the Pde1 protein by PKA does not change its affinity for cAMP but may change its interaction with other proteins and subcellular localization [22]. The cAMP-phosphodiesterase Pde2 exhibits physical interaction with several stress-regulated transcription factors that are targeted by PKA [23]. The role of Pde2 is crucial in the proper initiation of Msn2/4 target genes [21]. Consequently, the Pde2 protein interacts with the Msn2/4 transcription factors, creating a stress signal that induces

the accumulation of reserve carbohydrates. High levels of trehalose and glycogen were therefore observed in *pde1Δ* mutant cells.

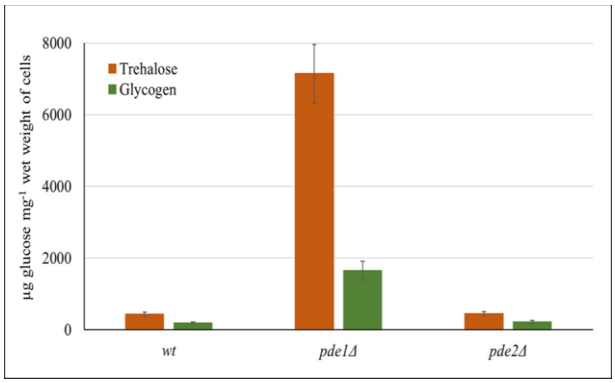


**Figure 3.** The effect of cAMP phosphodiesterase activity on trehalose and glycogen accumulations at normal growth condition.

In a recent study, the hyperactivation of the Ras/cAMP/PKA pathway in *pde2Δ* mutant cells resulted in decreased glycogen accumulation and thermotolerance in the pathogenic fungus *Candida auris* [24]. As a result, they suggested that *C. auris* primarily uses high-affinity phosphodiesterase (Pde2) as the primary regulator of the cAMP/PKA signaling pathway, with the low-affinity Pde1 enzyme playing a minor role. However, the low levels of trehalose and glycogen in the wild-type and *pde2Δ* yeast cells in this study show that the Pde1 protein can regulate the activity of the Pde2 protein under normal growth conditions via an unidentified mechanism. It has been reported that the creation of deterministic mathematical models of the PKA module and complete cAMP pathway reveals that Pde1p is more important than Pde2p for controlling the cAMP levels following glucose pulses, and the proportion of active PKA is not directly proportional to the cAMP level, allowing PKA to exert negative feedback by activating Pde1p [25]. Our findings suggest that, in addition to the enzymatic activity of Pde1, its regulatory role confers upon it a heightened significance comparable to that of the Pde2 protein.

### 3.3. Trehalose and Glycogen Accumulation Under Nitrogen Starvation and Recovery

In order to determine the effect of Pde1 and Pde2 on trehalose and glycogen accumulation, yeast cells were grown at a strong nitrogen source until the exponential stage and then transferred to a poor nitrogen source. Trehalose and glycogen levels increased 3-fold and 26-fold in wild-type yeast cells during nitrogen starvation, respectively (Figure 4). Glycogen accumulation during starvation was fourfold higher than the trehalose level in wild-type yeast cells. During starvation, half of the stored trehalose was broken down in *pde1Δ* yeast cells, resulting in a decline in the trehalose level, whereas the glycogen level increased two-fold. Trehalose level in *pde1Δ* yeast cells was still 3-fold higher than that of wild-type. The nitrogen deficiency induced trehalose and glycogen accumulation about 2-fold and 3-fold, respectively, in *pde2Δ* yeast cells. These results indicate that nitrogen starvation induces more glycogen accumulation than trehalose, both in wild-type and mutant yeast cells. Additionally, *pde2Δ* yeast cells were more susceptible to nitrogen deficiency than *pde1Δ* yeast cells, which indicates that Pde2 protein has a more significant function than Pde1 protein in the regulation of reserve carbohydrate metabolism. It was previously shown that the deletion of the *PDE2* gene made cells sensitive to freeze-thawing and oxidative stress, whereas the deletion of the *PDE1* gene has no effect on cellular stress resistance [6]. Furthermore, it was shown that glycogen accumulation and stress tolerance were lower in *C. auris pde2Δ* cells compared to *Δpde1* mutant cells [24]. Since *pde2Δ* yeast cells accumulate fewer stress metabolites than *pde1Δ* yeast cells and are thus more sensitive to environmental stresses, our results are consistent with these findings.

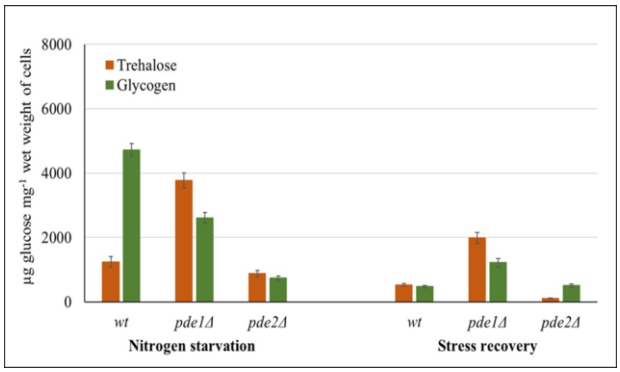


**Figure 4.** The effect of Pde1 and Pde2 proteins on trehalose and glycogen accumulation during nitrogen starvation and recovery. For nitrogen starvation yeast cells transferred to 0.1% proline and for recovery 0.5% ammonium sulfate was added. Incubation period for nitrogen starvation and recovery period was 4 hours.



Reserve carbohydrate accumulation was also analyzed during the 4 hours' recovery period. In wild-type and mutant yeast cells, starvation-induced trehalose and glycogen synthesis stopped during recovery. Within 4 hours, 57% of the accumulated trehalose and 90% of the accumulated glycogen were broken down into glucose and restored to normal growth conditions in wild-type yeast cells (Figure 4). In wild-type cells, the rate of glycogen breakdown was seen to be higher compared to that of trehalose. In the presence of a solo *PDE1* gene (*pde2Δ*), the levels of both trehalose and glycogen were shown to decrease by approximately 50% during the replenishing phase. In contrast to *pde1Δ* cells, *pde2Δ* cells showed 90% and 30% degradation in stress-induced accumulation of trehalose and glycogen, respectively. The rate of trehalose breakdown was shown to be faster in *pde2Δ* cells in comparison to both wild-type and *pde1Δ* cells.

Trehalose levels in *pde1Δ* cells were high under normal growth conditions, but when stress was applied, yeast cells immediately began to break it down and continued to do so even after the stress was removed. However, the accumulation of glycogen continued through the starvation period and was subsequently metabolized during the recovery phase. These findings indicate that the Pde2 protein plays a crucial role in the regulation of intracellular trehalose accumulation, both in normal and stressful environments.

### 3.4. Trehalose and Glycogen Accumulation During Heat Stress and Recovery

Intracellular trehalose levels are very low during normal growth conditions and increase during different stress conditions and heat shock. The heat shock treatment of yeast cells causes a decrease in the growth rate and the concomitant accumulation of reserve carbohydrates [26, 27]. Therefore, the effects of Pde1 and Pde2 proteins on reserve carbohydrate accumulation during heat shock and recovery were analyzed. When wild-type yeast cells grown under favorable growth conditions were shifted to 42 °C, the trehalose level increased 7-fold but the glycogen content remained unchanged (Figure 5). The reserve carbohydrate accumulations in *pde1Δ* cells decreased when exposed to heat shock, but they were still 2-fold and 12-fold higher than the trehalose and glycogen levels of wild-type cells, respectively. As opposed to *pde1Δ* yeast cells, trehalose and glycogen levels increased 3- and 2-fold in *pde2Δ* cells, respectively. During heat shock, the amount of trehalose in *pde2Δ* cells was 2-fold lower than in wild-type cells, whereas the amount of glycogen was 5-fold greater.

The glycogen accumulation exhibited an upward trend in *pde1Δ* and *pde2Δ* cells after heat stress, while remaining unchanged in wild-type cells. As in nitrogen starvation, trehalose continued to be synthesized in wild-type and *pde2Δ* cells during heat stress, but it was degraded in *pde1Δ* cells. These results indicate that both Pde1 and Pde2 activity are essential for the proper accumulation of trehalose and glycogen during nitrogen starvation and heat stress. Furthermore, in the presence of Pde2 protein (in *pde1Δ* cells), trehalose levels were very high in normal and stressful conditions. This finding suggests that the Pde2 protein is capable of initiating stress signaling pathways even in the absence of external stress. Consequently, it can be inferred that the *PDE1* gene product plays a crucial function in the regulation of PKA activity and, indirectly, in the regulation of Pde2 protein activity, thereby enabling the detection of stress signals.

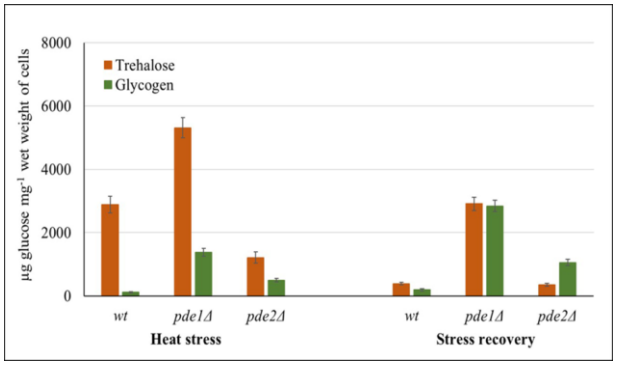


**Figure 5.** The effect of Pde1 and Pde2 proteins on trehalose and glycogen accumulation during heat stress and recovery. For heat stress yeast cells transferred to 42 °C and for recovery yeast cells retreated to 30 °C. Incubation period for heat stress and recovery period was 4 hours.

For recovery, yeast cells were transferred to 42 °C and incubated for 4 hours, then trehalose and glycogen levels were detected. Approximately 87% and 71% of stress-accumulated trehalose were hydrolyzed in wild-type and *Δpde2* yeast cells, respectively, during the recovery period. (Figure 5). Interestingly, in *pde1Δ* cells, 50% of the stress-accumulated trehalose was degraded, whereas glycogen synthesis continued twofold throughout the replenishment period. It was shown that *pde2Δ* cells recovered to a similar extent as the wild-type, whereas *pde1Δ* failed to recover properly in terms of trehalose. Additionally, unlike wild-type yeast cells, *pde1Δ* and *pde2Δ* yeast cells continued to synthesize glycogen during the recovery phase. These results indicate that both *PDE1* and *PDE2* gene products are crucial for the regulation of trehalose and glycogen metabolism under normal circumstances, stress conditions, and replenishment.

It is known that low levels of cAMP and repression of cAMP-dependent protein phosphorylation show enhanced heat resistance in yeast strains [19]. The overexpression of *PDE2* genes showed enhanced heat resistance, whereas the overexpression of the Pde1 enzyme did not show a significant change in heat resistance compared with the control yeast strain [22]. The ability of *pde2Δ* yeast strains to survive in stress conditions such as heat stress and nitrogen starvation was decreased [28, 29]. In addition, deletion of the *PDE2* gene made cells sensitive to different stresses, whereas deletion of the *PDE1* gene did not affect cellular stress resistance [6]. Our results showed that the trehalose level in *pde1Δ* yeast cells was too high and did not change during stress or the recovery period compared to the wild-type. Consistent with these results, the *PDE2* gene product is essential for the accumulation of the stress metabolite trehalose, thus making the yeast cells more resistant to stress conditions. Interestingly, the level of glycogen in *pde1Δ* yeast cells decreased during heat stress and quietly resynthesized in the recovery period. Conversely, in *pde2Δ* yeast cells, glycogen synthesis was triggered during stress and the recovery period. Therefore, the *PDE2* gene product has a distinct regulatory role in glycogen metabolism, even if the exact mechanism is unknown yet.

Different external and internal factors affect the stress response production rate and the duration of the response in yeast cells. In the absence of cAMP phosphodiesterases, cAMP clearance is altered, affecting the overall timing of cellular decisions. Therefore, Pde1 and Pde2 proteins affecting stress response duration [22, 30]. The findings of our study indicate that Pde1 and Pde2 proteins also play a crucial role in the sensing and production of stress responses.

## 4. CONCLUSION

In yeast cells, PKA activity has been linked to a variety of cellular functions, including stress response. The activity of PKA is regulated by the cAMP level, which is controlled by adenylate cyclase and cAMP phosphodiesterase enzymes. *PDE1* and *PDE2* genes encode the low- and high-affinity cAMP phosphodiesterases, respectively. Inactivation of PKA activates the stress response pathways and causes the accumulation of storage carbohydrates, which leads to increased tolerance to diverse environmental stresses such as nutrient deficiency, oxidative stress, and heat stress.

The activation of PKA represses the expression of genes, including STRE sequences on their promoters. The genes involved in trehalose metabolism and glycogen synthesis include STRE elements on their promoters. The neutral trehalase enzyme, encoded by the *NTH1* gene, is responsible for the breakdown of stress-accumulated trehalose. In this study, the effect of Pde1 and Pde2 proteins on *NTH1* gene expression and storage carbohydrate accumulation was investigated at nitrogen starvation, heat stress, and during stress recovery. *NTH1* transcription levels in *pde1Δ* and *pde2Δ* yeast cells were lower than in wild-type yeast cells under normal conditions, nitrogen starvation, heat stress, and also during the recovery phase. In the absence of *PDE1* and *PDE2* gene products, the high cAMP level caused constitutive activation of PKA and may have blocked STRE-mediated *NTH1* gene expression. Both trehalose and glycogen levels in the yeast cells were quite high in *pde1Δ* cells at normal growth conditions. However, in *pde2Δ* yeast cells, the accumulation of trehalose and glycogen was at the level of wild-type cells. Under conditions of nitrogen deficiency, heat stress, and subsequent replenishment, the concentration of trehalose in *pde1Δ* cells remained higher than in *pde2Δ* and wild type cells. In addition, *pde2Δ* cells recovered to a similar extent as the wild-type, whereas *pde1Δ* cells failed to recover properly. The Pde2 protein may interact with Msn2/4, creating a long-lasting stress signal to induce the accumulation of reserve carbohydrates. So, *PDE1* is necessary for the downregulation of reserve carbohydrate metabolism, and/or Pde1 may regulate the activity of Pde2 via an unknown mechanism. These findings suggest that Pde1 and Pde2 proteins are essential for the regulation of trehalose and glycogen metabolism in both normal and stressful circumstances, as well as throughout the replenishment process.

**Acknowledgement**

The work is an outcome of research project number FHD-2017-1325 financed by the Scientific Research Coordination Unit of Çanakkale Onsekiz Mart University, Türkiye. We thank to Professor Jean Marie François for gift of LacZ fusion systems.

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