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Original Research

Thiamine leads to oxidative stress resistance via regulation of the glucose metabolism

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Abstract: Thiamine diphosphate (ThDP) is an essential cofactor for important enzymes in carbohydrate, amino acid and lipid metabolisms. It is also known that thiamine plays an important role in stress response of some organisms. In this study, we focused on the effect of thiamine on stress responses triggered by various stress agents. For this purpose, firstly, viability of *Schizosaccharomyces pombe* cell cultures was examined under oxidative, osmotic and heat stresses. The highest tolerance observed in cell viability due to the presence of extracellular thiamine (1.5 µM) was found only against oxidative stress. Then, enzyme activity of catalase and superoxide dismutase (SOD) involved in antioxidant defense mechanism and the expression analysis of genes encoding enzymes related to glucose metabolism and stress response pathways were investigated under oxidative stress. In this condition, it was not observed any difference in SOD and catalase activities, and their gene expressions due to the presence of thiamine, whereas the upregulation of *pyruvate dehydrogenase (pdb1), transketolase (SPBC2G5.05), fructose-1,6-bis-phosphatase (fbp1)* and the downregulation of *pyruvate decarboxylase (pdc201)* were observed. In conclusion, these findings suggest that extracellular thiamine leading to oxidative stress resistance have an impact on the regulation of glucose metabolism by shifting the energy generation from fermentation to respiration.

Key words: *Schizosaccharomyces pombe*; Thiamine; Stress response; Glucose metabolism; Oxidative stress.

Introduction

Thiamine (vitamin B_1) is a member of vitamin B family that is water soluble (1). There are many forms of thiamine in cells: I) Free thiamine, II) Thiamine monophosphate (ThMP), III) Thiamine diphosphate (ThDP), IV) Thiamine triphosphate (ThTP), V) Adenosine thiamine triphosphate (AThTP). ThDP, biologically active form of thiamine, is a cofactor for enzymes of carbohydrate, lipid and aminoacid metabolism with Mg (2). The most important enzymes of these are pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, α-ketoacid dehydrogenase, transketolase and pyruvate decarboxylase (3).

Schizosaccharomyces pombe usually undergoes fermentation even in aerobic conditions. Glucose is phosphorylated by the hexokinase enzyme after being taken into the cell and glycolysis is induced. Subsequently, depending on glucose concentration, pyruvate is directed either to the fermentation by pyruvate decarboxylase or to the mitochondrial respiration by pyruvate dehydrogenase. Further, pentose phosphate pathway which is an important metabolic process for the synthesis of NADPH, ribose-5-phosphate and erythrocyte 4-phosphate is also proceeded by transketolase (4, 5). There are 4 predicted pyruvate decarboxylase genes (SPAC1F8.07c; SPAC186.09; SPAC3G9.11c; SPAC13A11.06); 6 pyruvate dehydrogenase genes (SPAC1002.09c; SPCC794.07; SPAC26F1.03; SPBC30D10.13c; SPCC1259.09c; SPAC644.11c) and one predicted transketolase gene (SPBC2G5.05) in *S. pombe* database (6).

Core environmental stress response (CESR) genes which are either repressed or induced by various environmental stresses have been defined via using DNA microarrays. These genes, such as catalase (*ctt1*), superoxide dismutase (*sod*), glutathione peroxidase (*gpx*), are regulated by mitogen-activated protein kinase Sty1p and transcription factor Atf1 in fission yeast (7).

It was emphasized that stress response mechanisms are still poorly understood and already known processes are not the only possible mechanisms to protect the cells (8). Unlike the cofactor property, thiamine is involved in stress responses in various organisms. Particularly, it provides resistance against oxidative agents in bacteria and plants (9-11). Wolak et al. (12) have revealed that thiamine has a therapeutic effect against cellular stresses by reducing cellular levels of free radicals and preventing protein oxidases in *S. cerevisiae*. In addition, due to its cofactor property, it is known that thiamine protects cellular redox balance by producing NADPH and glutathione (13). Palabiyik et al. (14) have shown that expression of genes encoding some enzymes involved in thiamine metabolism increased under oxidative stress in *S. pombe ird11* mutant which is resistant to glucose suppression and oxidative stress.

Hence, we aimed to investigate the effect of extracellular thiamine on stress responses triggered by various stress agents. Besides, the role of thiamine dependent enzymes in this process was investigated via gene expression analysis.

Materials and Methods

Yeast strain and growth conditions

S. pombe Lindner liquifaciens wild type *972h*$^-$ strain was used in this study. The strain was grown as recommended by Gutz et al. (15) in Edinburg Minimal Medium (EMM) without thiamine on shaker (30°C, 180 rpm) until the mid-logarithmic phase.

Cell viability

Primarily, 0.05, 1.5 and 15 µM thiamine concenrations were examined at cells exposing to oxidative stress (2 mM, H_2O_2, (16)) to determine effective thiamine concentration. After one hour of stress treatment, cells were diluted to final OD_{600} (optical density at λ=600 nm) value of 0.2 in fresh media with or without thiamine. Cell growth rates were monitored by measuring OD_{600} value in every two hours for a total of 12 hours. Then, determined thiamine concentration was also examined under osmotic stress induced with 1 M Sorbitol and heat stresses induced with 39°C for an hour. We compared thiamine-rich and thiamine-free media for each stress conditions and control group to determine the effect of thiamine on cell viability.

Crude extract preparation

The cells were harvested after 10 min. centrifugation at 7000 rpm and pellets were washed with 800 µL isolation buffer. Isolation buffer, containing tris HCl (100 mM), dithiothreitol (1 mM), glycerol (20% v/v), was used for protein extraction. Cell disruption was performed with glass beads (0.45-0.50 mm) for 1 minute at 3000 rpm (Sartorius Mikro-Dismembrator S). Then, the homogenate was transferred to a new tube and separated by centrifugation at 10000 rpm for 10 minutes. The supernatant was used for enzyme assays. All the centrifugation processes were done at +4°C. Protein concentration of crude extract was determined by Lowry method (17).

Catalase and SOD activity

Enzyme activity assays in the crude extracts that obtained from *S. pombe* cell cultures were based on spectrophotometry. Catalase activity assay was conducted by monitoring the decrease in absorbance of H_2O_2 at 240 nm (18). 96 µL of 40 mM H_2O_2 was added into the mixture which contains 136 µL of 50 mM phosphate buffer and 15 µL of the crude extract. The decrease in absorbance of H_2O_2 was measured with 10 second intervals for two minutes at 240 nm. The specific catalase activity was represented as ΔA_{240}/min/mg protein.

Spectrophotometric SOD activity assay was performed by using the method based on the inhibition of nitroblue tetrazolium chloride reduction by O_2- under light intensity (19) in a final volume of 1 mL at 24-well plate. 50 mM phosphate buffer, 50 mM sodium carbonate (Na_2CO_3), 100 µM ethylenediaminetetraacetic acid (EDTA), 13 mM methionine, 75 µM nitro blue tetrazolium chloride (NBT) and 50 µg the crude extract was added respectively. Enzymatic reaction was initiated by adding 2 µM riboflavin. It was incubated under fluorescent light for 10 minutes. After incubation, the absorbance of the samples at 560 nm was measured. SOD activity was expressed as U/mg, where one unit of SOD is defined as the amount of enzyme causing 50% inhibition of the rate of NBT reduction.

RNA isolation and Real-Time polymerase chain reaction (RT-PCR)

Total RNA was isolated from control and test group using PureLink® RNA Mini Kit (Ambion® by Life Technologies). The quality of total RNA was assessed by agarose gel electrophoresis and by measuring A_{260}/A_{280} ratio. First-strand cDNA was synthesized from total RNA by using 2 µg of total RNA according to the manufacturer's instructions (High-Capacity cDNA Reverse Transcription Kits, Applied Biosystems). RT-PCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems) by using diluted cDNA (100 ng) as a template in 20 µL final volume. The PCR conditions were as follows; 95°C for 10 min (pre-incubation), followed by 40 cycles of 95°C for 15 s, 53°C for 60 s, and 72°C for 30 s. The gene-specific primers, which are used throughout the study, are listed in Table1. *gpd3*, a

Table 1. Primers that used throughout the study.

Group	SEQ_ID	Name	Primer sequence (5'-3')
Reference gene	SPBC354.12	Glyceraldehyde 3 phosphate dehydrogenase / *gpd3*	GGTGACAACCACTCCTCCAT[1] TCAACAACACGGTGGGAGTA[2]
Thiamine-dependent enzyme	SPAC644.11c	Mitochondrial pyruvate dehydrogenase (lipoamide) kinase / *pkp1* (predicted)	CTTCATAATCCCTCGCTTGC[1] ATTCGGGCCAATTACAATCA[2]
	SPBC30D10.13c	Pyruvate dehydrogenase e1 component beta subunit/ *pdb1*	CGTGATCCTAATCCCGTTGT[1] CAAAGGGAAGCACAAAGTCC[2]
	SPAC3G9.11c	Pyruvate decarboxylase (predicted) / *pdc201*	TTAAGGGCATTTCTGCCATC[1] AACACCGACAATGTGAACGA[2]
	SPBC2G5.05	Transketolase (predicted)	GAAGCCATGTCTTGCACTGA[1] AGAGGACTGCCGTGAACACT[2]
Glucose metabolism	SPBC1198.14c	Fructose-1,6-bisphosphatase / *fbp1*	GTATGGTGCTTCGGCTCATT[1] TTCATGTTTCGATGGGTCAA[2]
	SPAC4F8.07c	Hexokinase 2 / *hxk2*	CAACAAGGACTTTGCCCAAT[1] AAGGTGTCGCTCTCCTTTGA[2]
Stress response	SPAC24B11.06c	MAP kinase / *sty1*	TGTTCATTCTGCCGGTGTTA[1] GAATACGAGCCAAACCGAAA[2]
	SPAC821.10c	Superoxide dismutase / *sod1*	ATTGGCCGTACCATTGTCAT[1] GACACCACAAGCGTTACGTG[2]
	SPCC757.07c	Catalase / *ctt1*	ATCCTCAATCCGACCACTTG[1] AACGTCGGTAATTTCGTCCA[2]

[1]: Forward sequence; [2]: Reverse sequence.

housekeeping gene, was used as the reference gene. The variation in gene expression levels was calculated according to Pfaffl equation (20).

Statistical analysis

GraphPad Prism 6 software was used for statistical analysis. Analysis of variance (ANOVA) was employed to comparison among gene expression of control and test groups. All gene expression analyses were expressed as the means ± standard deviation (SD). Biochemical analyses were expressed as the means ± standard error of mean (SEM).

Results

The effect of thiamine on cell viability against different stresses

To investigate the effect of thiamine, wild type cells, which were exposed to oxidative stress, were monitored for 12 hours in thiamine-rich (0.05, 1.5 and 15 µM) and thiamine-free media. The highest tolerance against oxidative stress was observed in minimal media supplemented with 1.5 µM thiamine (thiamine-rich media) and the difference between thiamine-rich media and thiamine-free media was also found to be statistically significant. Therefore, we selected 1.5 µM thiamine as an effective dose for further steps (Figure 1). This dose was also used to determine whether thiamine had a similar effect on both osmotic and heat stresses. However, no differences were observed in these conditions depending on the presence of thiamine (Figure 2). We selected the cells, that exposed to oxidative stress, for biochemical and molecular analyses at their 8th hour in which the cells grown up to 0.4-0.5 $OD_{600.}$

The relationship between the effect of thiamine and stress response pathways

In this study, as probable mechanism underlying the increased resistance of cells to oxidative stress, we examined SOD and catalase activities along with the expression levels of *sty1*, *sod1* and *ctt1* genes. For this purpose, enzyme activities of crude extracts, which were obtained from cells exposed to oxidative stress in the thiamine-rich and thiamine-free medium, were measured. No significant difference has been observed in catalase activity (data not shown). Although SOD activity increased in thiamine-rich medium, comparing to thiamine-free medium, it was calculated that these differences were not statistically significant (Table 2). Under non-stressed condition, expression of *sty1* and *ctt1* in wild type grown in thiamine-rich media decreased approximately 1.4-fold compared with that in thiamine-free media, while *sod1* gene expression increased approximately 1.74-fold (Figure 3a). On the other hand, the expression levels of these genes (*sty1*, *sod1*, *ctt1*) were found as insignificant under oxidative stressed condition (Figure 3b).

Expression profiles of the genes that related to glucose metabolism.

We examined expression profiles of *fbp1* and *hxk2* in both non-stressed and oxidative-stressed conditions to check whether the glucose repression and glycolysis maintenance are processed, respectively. We observed

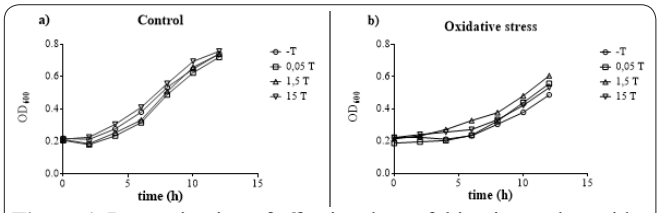

Figure 1. Determination of effective dose of thiamine under oxidative stress. The absorbance of the cells at 600 nm was measured in thiamine-rich (0.05, 1.5 and 15 µM) and thiamine-free media throughout 12 hours. **a)** Control: Untreated, **b)** Oxidative stress: The cells treated with 2 mM H_2O_2.

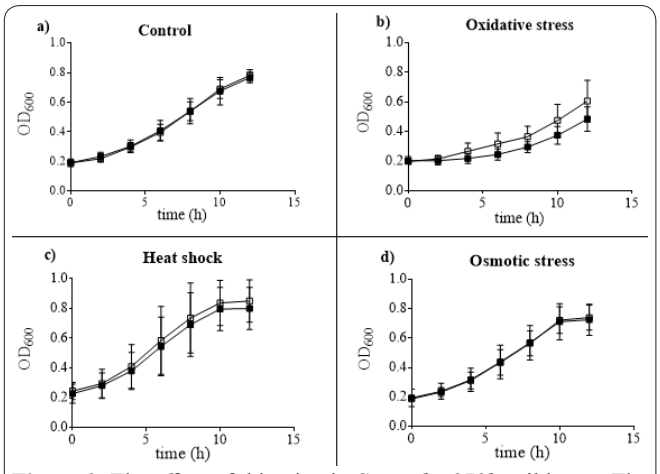

Figure 2. The effect of thiamine in *S. pombe 972h*$^-$ wild type. The absorbance of the cells at 600 nm was measured at presence and absence thiamine throughout 12 hours. **a)** Control: Untreated, **b)** Oxidative stress: The cells treated with 2 mM H_2O_2, **c)** Heat shock: The cells treated in 39 °C, **d)** Osmotic stress: The cells treated with 1 M sorbitol. ■ - Thiamine, □+ Thiamine.

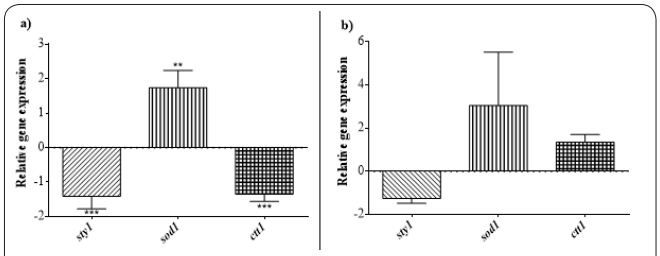

Figure 3. Expression levels of genes of oxidative stress response proteins in **a)** control and **b)** oxidative stress groups. Relative expression for each gene in both control and stress groups comparing thiamine-rich condition to thiamine-free condition was normalized by using Pfaffl method relative to *gpd3* gene. *sty1:* MAP kinase (SPAC24B11.06c); *sod1:* superoxide dismutase (SPAC821.10c); *ctt1:* catalase (SPCC757.07c). Error bars indicate SD (n = 3–5). (Dunnett's test, P<0.05*, P<0,01**, P<0,001***).

Table 2. Spectrophotometrically detected SOD activity of the cells.

	SOD activity (units/mg)	
Group	- Thiamine	+ Thiamine
Control	11,3 ± 3,92	10 ± 2,26
Oxidative stress	5,6 ± 1,2	9,75 ± 0,45

that the expression level of *fbp1* upregulated under oxidative stress (Figure 4a), whereas *hxk2* upregulated in non-stressed conditions (Figure 4b). Expression levels of genes which encode thiamine-dependent enzymes were examined both in non-stressed and oxidative-stressed conditions. We showed that the expression of *pdb1* significantly increased whereas *pdc201* decreased, but there was not any significant change in *pkp1* and

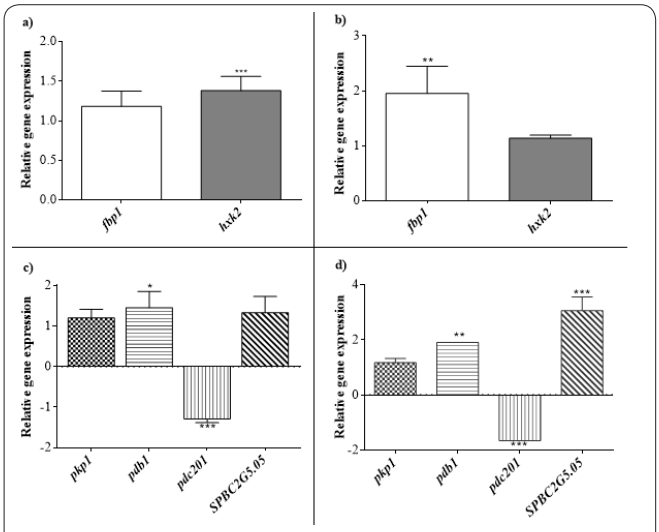

Figure 4. Expression levels of genes of glucose marker enzymes, and thiamine-dependent enzymes in both control and oxidative stress groups. Relative expression for each gene in both control and stress groups comparing thiamine-rich condition to thiamine-free condition was normalized by using Pfaffl method relative to *gpd3* gene. **a)** Glucose marker enzymes in control group, **b)** Glucose marker enzymes in oxidative stress group, **c)** Thiamine-dependent enzymes in control group, **d)** Thiamine-dependent enzymes in oxidative stress group. *fbp1*: fructose-1,6-biphosphatase (SPBC1198.14c); *hxk2*: hexokinase2 (SPAC4F8.07c). *pkp1*: mitochondrial pyruvate dehydrogenase (lipoamide) kinase (SPAC644.11c); *pdb1*: pyruvate dehydrogenase e1 component β subunit (SPBC30D10.13c); *pdc201*: pyruvate decarboxylase (SPAC3G9.11c); SPBC2G5.05: transketolase. Error bars indicate SD (n = 3–5). (Dunnett's test, P<0.05*, P<0,01**, P<0,001***)

SPBC2G5.05 genes in the control group (Figure 4c). Similar expression profiles of these genes were observed under oxidative stress except SPBC2G5.05 (Figure 4d). The expression of SPBC2G5.05 gene was significantly upregulated (3-fold).

Discussion

In all organisms, ThDP has an important role due to cofactor property for significant enzymes of glucose, aminoacid and lipid metabolisms. In addition to cellular metabolism, there are many recent studies about protective effect of thiamine in various organisms. It has been shown that ThDP protects tissues against oxidative damage together with reduced $NADP^+$ (21). Nagae et al. (22) have suggested that *de novo* synthesis of thiamine can lead to normal nodule formation in infected plants. In the study conducted by Li et al. (23) has shown that upregulation of the genes involved in thiamine and thiamine pirophosphate (TPP) biosynthetic processes are important against ethanol stress in *S. cerevisiae*. In the present study, after the effective thiamine concentration was determined by using H_2O_2-induced oxidative stress (Figure 1b), we examined the effect of 1.5 µM thiamine on cell viability under osmotic and heat stresses, which were triggered with sorbitol and high-temperature, respectively. As it was revealed in previous study which was conducted with *S. cerevisiae* (12), the highest tolerance observed in cell viability due to the presence of extracellular thiamine was found to be against only oxidative stress among different stress conditions (Figure 2b). It might arise from either not being adequate dose of thiamine for osmotic and heat stresses or not associated with the pathways of these stresses.

Concerning the increased cell viability due to the presence of extracellular thiamine against oxidative stress, we have raised the question of whether this elevated tolerance might be arising from the stress response pathway. No changes in the activity of SOD (Table 2) and in the expression profiles of *sod1*, *ctt1* and *sty1* genes depended on thiamine against oxidative stress were observed (Figure 3b). On the other hand, since we found that oxidative stress increased expression of some genes belonging to the thiamine biosynthetic and transport pathway in our previous study (24). In this study, it was confirmed the extracellular thiamine do not make any difference in the activity of the enzymes and the transcription levels of the genes associated with the stress pathway.

S. pombe fbp1 gene (25) is used as a marker gene in glucose suppression studies because of suppressed at the level of transcription by protein kinase A activation in the presence of glucose (26). Besides, the *hxk2* gene encoding the hexokinase (27), the first enzyme of the glycolysis process, is also used as a marker for the glucose metabolic process. In this context, increased expression of *hxk2* gene indicates that glucose might be efficiently metabolized under each condition (Figure 4a-4b). Furthermore, upregulation of the *fbp1* gene expression under oxidative stress suggest that the glucose repression might shift to glucose derepression condition (Figure 4b). *S. pombe* (Crabtree-positive yeast) undergoes aerobic fermentation by following glycolysis in optimum conditions (4, 5). In the current study, when cells were grown in optimal conditions with extracellular thiamine, pyruvate decarboxylase gene (*pdc201*), coding the fermentative enzyme, was downregulated, while pyruvate dehydrogenase gene was upregulated (Figure 4c). Since similar profiles were shown in oxidative stress condition (Figure 4d) suggest that these two genes encoding thiamine-dependent enzymes, responsible for glucose metabolism, are affected by environmental thiamine. It has been reported that *S. cerevisiae* PDC5 gene which encodes minor isoform of pyruvate decarboxylase (ortholog of *S. pombe pdc201*) was repressed by thiamine (28). Moreover, the expression of the transketolase gene, which is known to increase expression under derepressed conditions (29), has also elevated significantly in these conditions (Figure 4d). It has been suggested that thiamin may be effective in stress response by inducing structural changes in cellular redox balance of enzymes such as transketolase, α-ketoglutarate dehydrogenase as a cofactor (30, 31).

This study suggested that extracellular thiamine leading to oxidative stress resistance have an impact on the regulation of the glucose metabolism. It was seen that this effect seems to be occurred through directing the energy generation from fermentation to respiration.

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Compliance with Ethical Standards

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Conflict of interest
The authors declare that they have no conflict of interest.

Author's contribution
Burcu Kartal: Carried out all experimental steps, literature search and write the manuscript; Bedia Palabıyık: Designed the research plan, contributed to literature search and results analysis.

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