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Adaptive Stress Response to Menadione-Induced Oxidative Stress in *Saccharomyces cerevisiae* KNU5377

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The molecular mechanisms involved in the ability of yeast cells to adapt and respond to oxidative stress are of great interest to the pharmaceutical, medical, food, and fermentation industries. In this study, we investigated the time-dependent, cellular redox homeostasis ability to adapt to menadione-induced oxidative stress, using biochemical and proteomic approaches in *Saccharomyces cerevisiae* KNU5377. Time-dependent cell viability was inversely proportional to endogenous amounts of ROS measured by a fluorescence assay with 2′,7′-dichlorofluorescin diacetate (DCFHDA), and was hypersensitive when cells were exposed to the compound for 60 min. Morphological changes, protein oxidation and lipid peroxidation were also observed. To overcome the unfavorable conditions due to the presence of menadione, yeast cells activated a variety of cell rescue proteins including antioxidant enzymes, molecular chaperones, energy-generating metabolic enzymes, and antioxidant molecules such as trehalose. Thus, these results show that menadione causes ROS generation and high accumulation of cellular ROS levels, which affects cell viability and cell morphology and there is a correlation between resistance to menadione and the high induction of cell rescue proteins after cells enter into this physiological state, which provides a clue about the complex and dynamic stress response in yeast cells.

***Keywords*:** *Saccharomyces cerevisiae* KNU5377, menadione stress, adaptive response, cell rescue proteins, trehalose

Yeast cells have striking similarities to mammalian cells with respect to macromolecular and cellular organelles, and a large number of yeast proteins have been shown to have functional homology with human counterparts (Sirisattha *et al.*, 2004; Lopez-Mirabal and Winther, 2008). On this basis, the yeast *Saccharomyces cerevisiae* has long been studied as a useful model for research on stress response. Although yeasts can survive in aerobic environments by producing energy through an aerobic respiratory process, they face major oxidative stress that is caused by reactive oxygen species (ROS) such as the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH^-) (Costa and Moradas-Ferreira, 2001; Gibson *et al.*, 2007). ROS may also be generated by environmental changes such as heat, ethanol, hydrogen peroxide, superoxide-generating agents [e.g., menadione (MD) and paraquat], and irradiation. ROS damages a variety of cellular components such as DNA fragmentation, protein or enzyme inactivation, modification of carbohydrate compounds, and change of membrane fluidity by lipid peroxidation (Jamieson, 1998; Le Moan *et al.*, 2006; Castro *et al.*, 2007). In order to overcome transient or continuous ROS challenges, cells have evolved a variety of enzymatic (catalase, superoxide dismutase, thioredoxin-dependent thiol peroxidases) and non-enzymatic (trehalose) antioxidant defense systems, which are capable of protecting them from free radicals and their by-products, leading to the repair of stress-induced cellular damage, and therefore can protect the cellular constituents (Moradas-Ferreira and Costa, 2000; Querol *et al.*, 2003; Fernandes *et al.*, 2007; Herrero *et al.*, 2008; Lopez-Mirabal and Winther, 2008).

Although considerable progress has been achieved, we still have limited information on yeast's response to oxidative stress. When cells confront a change in their environment, a fast alteration in gene expression is expected in order to adapt and survive in the new environment (Alic *et al.*, 2004). Under oxidative and other stresses such as heat or ethanol, *S. cerevisiae* induces a large number of cell rescue proteins, including antioxidant proteins and heat shock proteins (Hsps) (Jamieson, 1998). Functional and physiological roles of stress-induced proteins are very different even whithin the same *S. cerevisiae* species. Expression monitoring in yeast provides three different types of information. First, the set of genes expressed at a given time reflects the cellular processes that a yeast cell is undergoing. Second, expression studies can help elucidate the function of characterized genes and uncharacterized genes. Finally, information can be deduced about gene regulation (Jelinsky and Samson, 1999; Horak and Snyder, 2002). Therefore, comprehensive analysis of differential expression is becoming an important area of functional genomics. Up-regulated genes/proteins are principal candidates for being important in stress adaptation and mitigation and are potentially important targets for developing yeast fermentation capacity as well (Zuzuarregui and del Olmo, 2004; Gibson *et al.*, 2007).

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S. cerevisiae KNU5377 was isolated from sludge and found to have higher resistance to various stresses, and an ability to ferment at high temperature (Kim *et al.*, 2007a). Despite its biotechnological interest, a systematic study of intrinsic stress tolerance in this yeast strain has not been done. In this study, we examined its stress response to menadione, a compound extensively used in studies of cellular oxidative stress. Our results show that the cell protection strategy of this yeast strain correlates with cell survival during the different stages of menadione exposure and with the accumulation of cell rescue proteins produced under menadione stress.

Materials and Methods

Growth conditions

S. cerevisiae KNU5377 pre-cultures were grown in YPD medium (1% yeast extract, 2% peptone, and 2% dextrose) at 30°C overnight, transferred to fresh YPD medium, and further cultured with shaking at 160 rpm. Once mid-log phase to 1.0 of OD_{600} was reached, cells were challenged directly with 0.6 mM menadione at 30°C and samples were taken at 30 min intervals up to 120 min. Cells from each treatment were harvested by centrifugation (2,500 rpm, 3 min, 4°C) and the pellets were washed once with cold washing buffer (10 mM sodium azide, 10 mM potassium fluoride, and 50 mM Tris-HCl, pH 7.5) followed by chilled distilled water. These cells were used for subsequent experiments.

Stress sensitivity assay

For cell viability, mid-log phase cells were challenged with 0.6 mM menadione. Samples were taken from the culture at 30 min intervals for 120 min at 30°C, diluted, and spread onto YPD agar plates. Viable cell numbers were determined by counting the colonies that formed on YPD agar plates after stressful conditions at 30°C. Sensitivities were determined as survival percentage values, which were calculated by dividing the viable-cell numbers after stress application by the viable-cell numbers determined under the same growth conditions but without the stress exposure.

Preparation of yeast extracts and native polyacrylamide gel electrophoresis

Yeast cells were washed in 0.85% NaCl, centrifuged, and resuspended in cell storage buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM phenylmethylsulphonyl fluoride (PMSF), and 1 mM DTT. Cells were disrupted by a micromixer for 5 min with 1 min intervals. The cell-free extract (CFE) was centrifuged at 10,000×g for 10 min at 4°C. Protein concentrations were measured by the Bradford method using the Bio-Rad Protein Assay reagent. In-gel activity was measured according to the method reported previously (Beriault *et al.*, 2005).

SDS-PAGE and Western blot analysis

SDS-PAGE was performed by a method reported previously (Kim *et al.*, 2006). Forty microgram of denatured proteins were separated in a 10 or 12% polyacrylamide gel, and transferred to PVDF membranes (Bio-Rad, USA) in the transfer buffer (25 mM Tris-base, 192 mM glycine, and 20% methanol). The PVDF membranes were blocked at room temperature for 60 min in TTBS buffer (0.05% Tween 20, 10 mM Tris-HCl; pH 7.6, 150 mM NaCl) containing 5% non-fat skim milk and 0.02% sodium azide. The blotted membranes were incubated at 4°C overnight with primary antibodies. The types and sources of primary antibodies were as follows: rabbit anti-DNP and rabbit anti-glucose-6-phosphate dehydrogenase (Sigma, USA), rabbit anti-Hsp104 and mouse anti-Hsp60 (Stressgen, Canada), rabbit anti-isocitrate dehydrogenase (Cell Signaling, USA), rabbit anti-alcohol dehydrogenase, rabbit anti-aldehyde dehydrogenase, and rabbit anti-hexokinase (Rockland, USA), rabbit anti-tubulin (SantaCruz, USA), rabbit anti-Hsp90, rabbit anti-Ssa1 and rabbit anti-Ssb1 antibody (provided by Dr. E.A. Craig), rabbit anti-Tsa1p, -Sod1, and -Sod2 antibody (provided by Dr. Jeen-Woo Park), rabbit anti-Trr1p, -Trx2p, and -Trx3p antibody (provided from Dr. Kang-Hwa Kim), and rabbit anti-Hsp42 and -Hsp26 antibody (kindly provided by Dr. Johannes Buchner). Rabbit anti-Hsp30 antibody was prepared using a synthetic peptide as antigen. After incubation with primary antibodies, membranes were washed four times with TTBS, horse radish peroxidase-conjugated anti-rabbit IgG (H+L) (Promega, USA) or anti-mouse IgG (GE Healthcare, USA) secondary antibodies were added and further incubated for 90 min at room temperature. Membranes were washed 4 times with TTBS. Color was developed by enhanced chemiluminescence (ECL kit, GE Healthcare).

Trehalose assay and trehalase activity

Cell pellets (4-10 mg) were put on ice, washed twice with 10-20 volumes of cold-distilled water, resuspended in 0.25 ml of 0.25 M sodium carbonate, and incubated at 95°C for 4 h. The pH of the mixture was brought to 5.2 by adding 0.15 ml of 1.0 M acetic acid and 0.6 ml of 0.2 M Na-acetate (pH 5.2). The suspension was incubated overnight with trehalase (0.05 U/ml) at 37°C under constant agitation. The suspension was centrifuged for 3 min at 5,000×g (Parrou and Francois, 1997). The resulting glucose was measured by the Somogy-Nelson method. Enzyme activity of neutral trehalase was measured by incubating 20 μl of crude extract (100 μg of protein) with 200 μl of 200 mM trehalose (Sigma) prepared in 25 mM MES (pH 7.1) and 125 μM $CaCl_2$ (final pH 6.0). Acid trehalase activity was determined by incubating 50 μl of protein extract (200 μg of protein) with 200 μl of 200 mM trehalose prepared in 200 mM sodium citrate (pH 4.5) plus 1 mM EDTA (final pH 4.6). Both reaction tubes were incubated for 30 min at 30°C and stopped by putting in boiling water for 3 min (San Miguel and Arguelles, 1994). The glucose concentration was determined by using the glucose oxidase-peroxidase procedure. One unit of trehalase is defined as the amount of enzyme that yields 1 μM of glucose per min by hydrolysis of trehalose under the assay conditions. Specific activity was expressed as units per mg protein.

NADPH and malondialdehyde (MDA) assay

The cleared supernatants were collected and protein concentration was determined by the Bradford method (Bio-Rad). NADPH and MDA were measured using a NADPH assay kit (Abcam, UK) and a MDA assay kit (Merck, USA), respectively.

Protein carbonylation analysis using two-dimensional gel electrophoresis

Yeast cells grown to the mid-log phase were exposed to menadione at 30°C with shaking. Cells were harvested by centrifugation, washed twice with cold PBS buffer, resuspended in lysis buffer containing 50 mM Tris-HCl, pH 7.5, 5% glycerol, 1 mM EDTA, 2 mM DTT plus 1 mM PMSF, and vortexed with MicroMixer. Protein crude extracts were obtained by high speed centrifugation, incubated with DNase/RNase/Mg mix on ice for 15 min, boiled for 5 min, and cooled on ice for 5 min. Sample preparation by TCA/acetone precipitation, isoelectric focusing, equilibration, SDS-PAGE, and immunoblotting

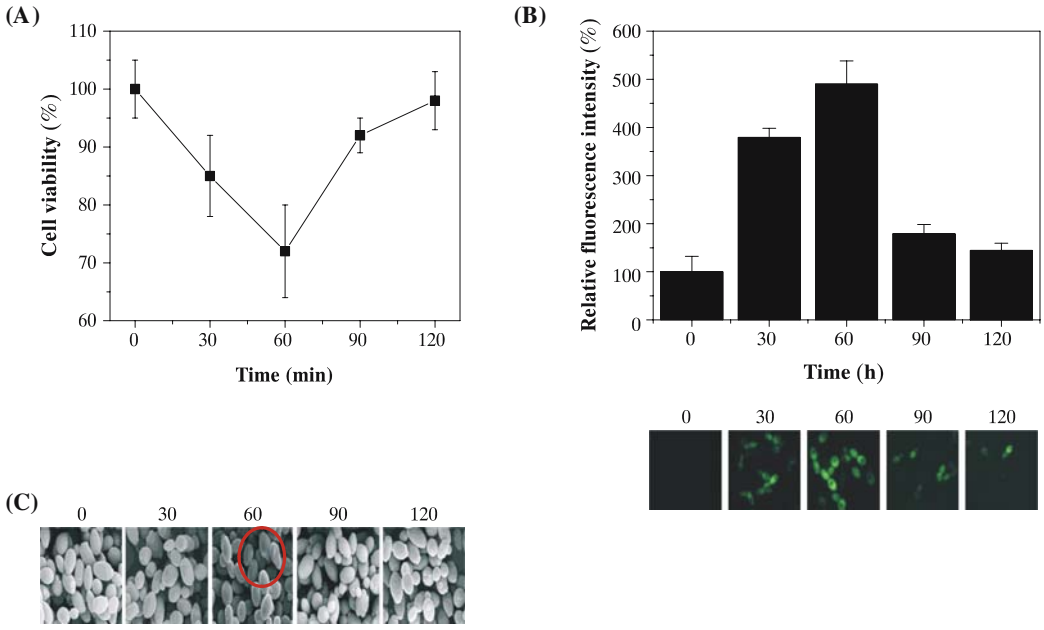

Fig. 1. Cell viability, intracellular peroxide level and cell morphology analysis. (A) Logarithmically growing KNU5377 cells (approximately OD_{600}=1.0) were inoculated into YPD liquid media and then challenged with 0.6 mM menadione and sampled at 30 min intervals for 120 min. The cell viability was determined by measuring colony forming units (CFU) and expressing survival as % survival relative to the untreated control cells. (B) Fluorescence of cell extracts from KNU5377 following 120 min exposure to 0.6 mM menadione in the presence of 100 μM dichlorofluorescin diacetate (DCFHDA). Results obtainted are a representative of triplicate determinations. (C) Cell morphology of yeast cells in the absence and in the presence of 0.6 mM menadione for 120 min was examined by phase contrast microscopy without staining.

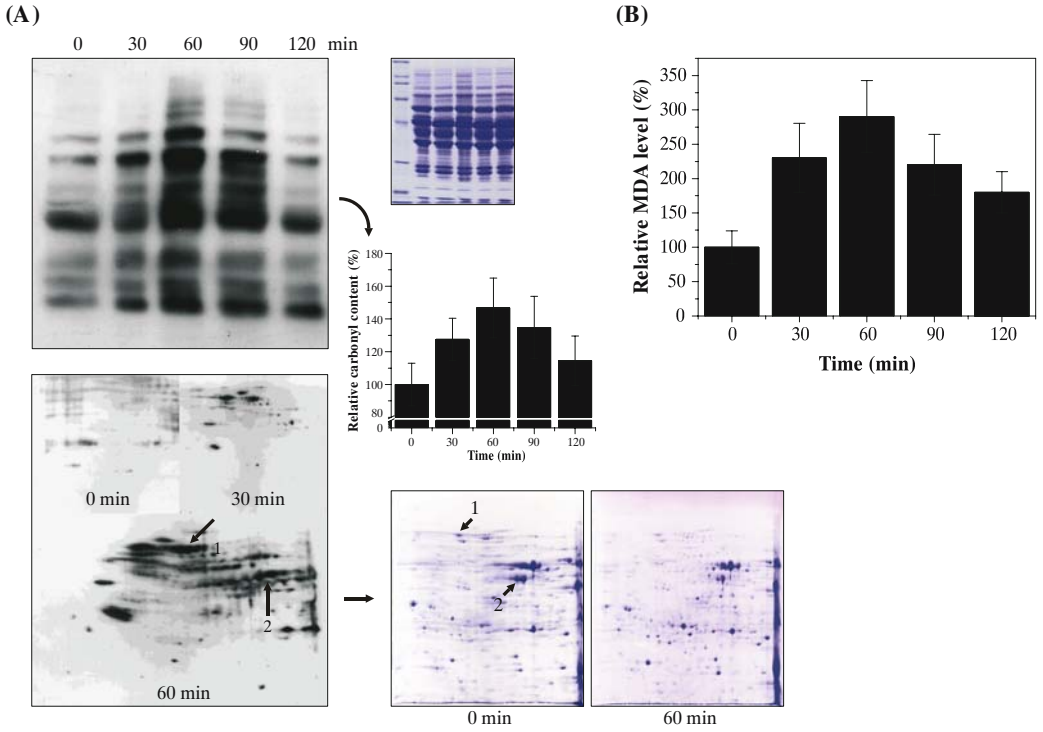

Fig. 2. The degree of protein oxidation and lipid peroxidation from the action of menadione in yeast cells. (A) Yeast cells were exposed to menadione for 120 min at 30 min intervals. Protein carbonylation was examined by using SDS-PAGE (upper panel) and 2-DE (lower panel) for separation of the proteins. The main oxidized proteins were identified via peptide mass fingerprinting by MALDI-TOF. Spot 1, Ssb1 protein; Spot 2, enolase protein. (B) Malondialdehyde (MDA) was set to be 100% that of cells without menadione treatment.

analysis was performed as previously reported (Kim *et al.*, 2007a).

Redox state analysis

Cellular ROS production was examined by a method based on intracellular deacetylation and oxidation of 2′,7′-dichlorodihydrofluorescein diacetate (DCFHDA) to the fluorescent compound 2′,7′-dichlorofluorescein (DCF). Overnight cultures of KNU5377 in YPD were subcultured into fresh medium and incubated at 30°C for 5 h with shaking (160 rpm) until they reached a concentration of approximately 5×10^7 cells/ml (OD_{600}=1.0). Cells were exposed to a final 100 μM DCFHDA from a fresh 10 mM stock in ethanol for 15 min to allow uptake and deacetylation of the dye and then treated with 0.6 mM menadione for 120 min with samples taken at 30 min intervals. Cells were collected by centrifugation and suspended in lysis buffer containing 50 mM HEPES buffer (pH 7.5), 2 mM PMSF, 2 μM pepstatin A and protease inhibitor cocktails (Roche, Switzerland). After cell lysis using glass beads, cleared supernatants were transferred into new tubes and protein concentration was measured by Protein Dye Reagent (Bio-Rad). DCF fluorescence intensity was measured at an excitation wavelength of 488 nm and an emission wavelength of 525 nm using a PerkinElmer SPEX fluorometer (Machida *et al.*, 1999) and then normalized to protein level. The fluorescence intensity in arbitrary units was thus directly read. Also, cells loaded with the fluorescent probes were washed twice with PBS buffer and imaged by fluorescence microscopy (excitation, 488 nm; emission, 525 nm).

Scanning electron microscope analysis

Yeast cells exposed to menadione were rinsed with distilled deionized water, air-dried, sectioned with a razor blade, and mounted on SEM (JOEL, JSM 5400) stubs using carbon paint. At least two randomly selected sections of each microorganism were examined.

Statistical analysis

All experiments were carried out with at least three independent repetitions. Results were expressed as Mean±SD.

Results

Cell survival, cell morphology, and redox state in the presence of menadione

Cell viability was observed at intervals of 30 min for 2 h after initiation of the menadione challenge. After exposure to 0.6 mM of menadione for 60 min, cell viability decreased to 25% when compared to control cells without exogenous oxidant, and then gradually increased for 120 min (Fig. 1A). The viability of cells after 2 h with menadione was almost similar to control cells. The difficulty of maintaining redox homeostasis in the yeast cells could render it unprotected against

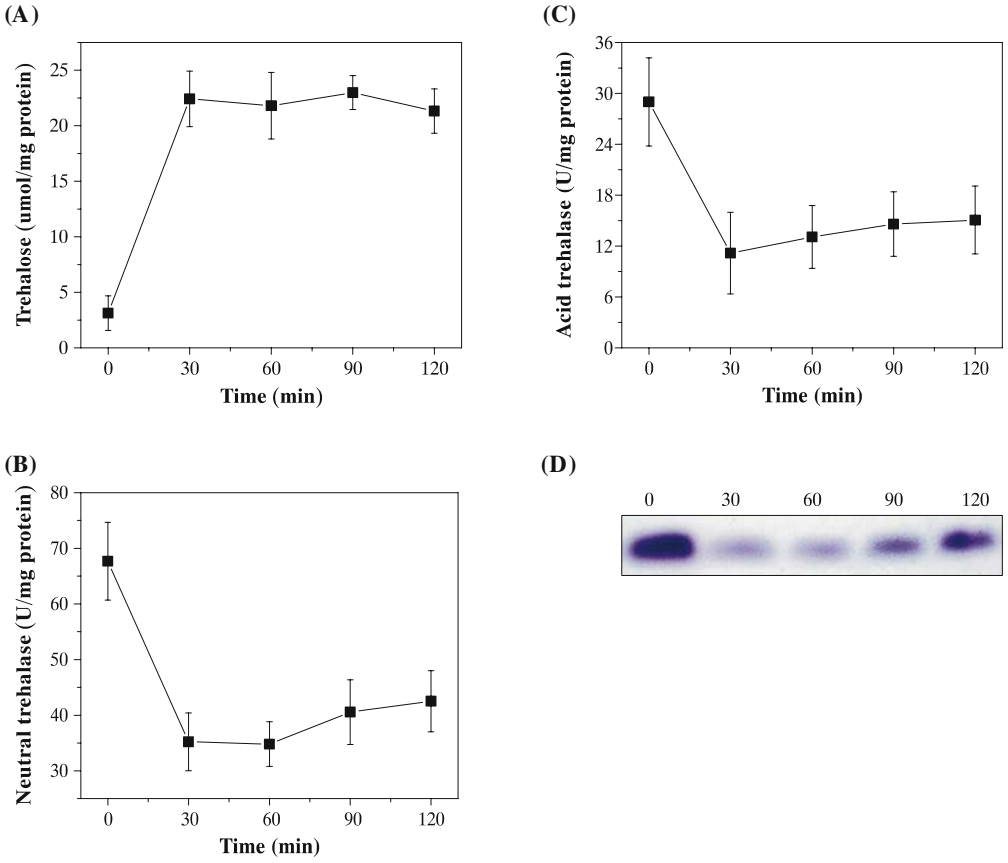

Fig. 3. Trehalose level and trehalase activity in relation to menadione stress. Yeast cells were aerobically grown in nutrient rich YPD medium at 30°C with shaking at 160 rpm. Once mid-log phase to OD_{600} of 1.0 was reached, cells were challenged directly with 0.6 mM menadione at 30°C for 120 min with sampling at 30 min intervals. At the indicated times, cell cultures were collected to analyze trehalose accumulation (A), neutral trehalase activity (B), acidic trehalase activity (C), and in-gel activity of neutral trehalase (D).

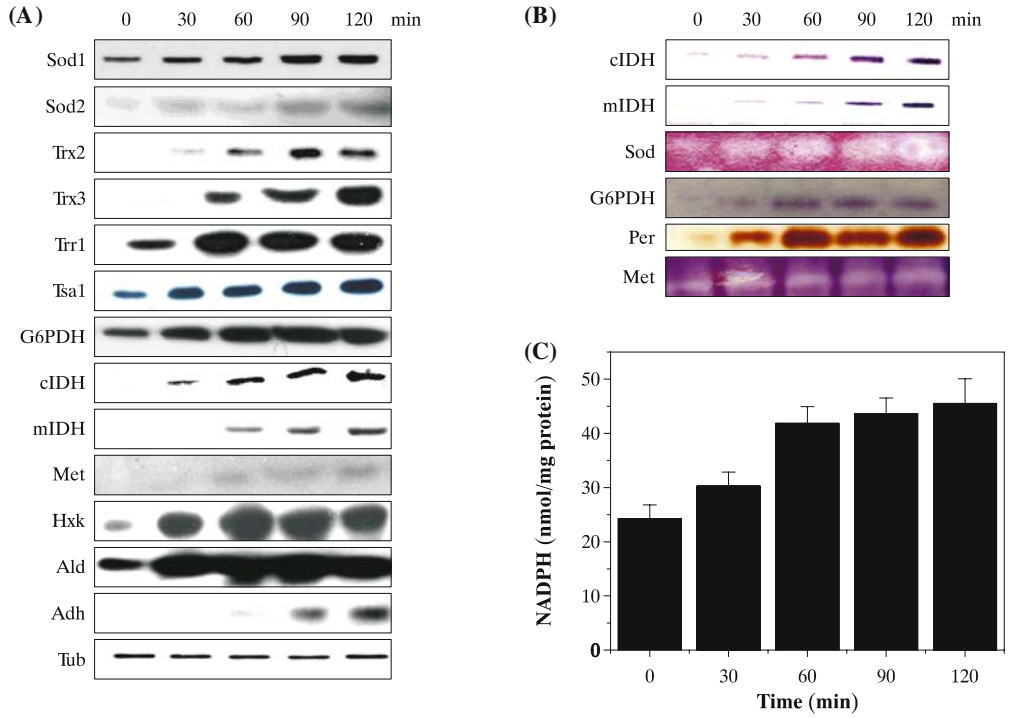

Fig. 4. Expression changes of antioxidant proteins, metabolic enzymes, and NADPH under menadione stress. At the indicated times, cell cultures collected for analysis by Western blot (A). (B) In-gel activity was done as described in 'Material and Methods'. Anti-tubulin (Tub) antibody was used for a housekeeping control. Sod1, Cu/Zn superoxide dismutase; Sod2, Mn superoxide dismutase; Trx2, thioredoxin isoform 2; Trx3, thioredoxin isoform 3; Trr1, thioredoxin reductase 1; Tsa1, cytosolic thioredoxin peroxidase 1; G6PDH, glucose-6-phosphate dehydrogenase (Zwf1); cIDH, cytosolic isocitrate dehydrogenase; mIDH, mitochondrial isocitrate dehydrogenase; Met, metallothionein; Hxk, hexokinase; Ald, aldehyde dehydrogenase; Adh, alcohol dehydrogenase; Per, peroxidase. (C) NADPH level was represented as nmol/mg protein.

oxidative stress caused by menadione. The breakdown of cellular redox balance involves the modulation of cellular oxidative biomarkers such as hydrogen peroxide. As seen in Fig. 1B, hydrogen peroxide levels in the yeast cells, measured using the oxidant-sensitive probe DCFHDA increased up to 5-fold for 60 min after initiation of menadione treatment, and thereafter the levels declined gradually. The hydrogen peroxide levels continued to decline until it reached the level prior to the menadione challenge (upper panel). Figure 1B (lower panel) shows the fluorescence of extracts from growing cells treated with 100 μM DCFHDA and incubated for 120 min in the presence of menadione. The DCF fluorescence was maximal (4.1-fold increase) for 60 min after initiation of menadione treatment and thereafter decreased gradually. At the end of the treatment, the intensity of the DCF fluorescence in the menadione-treated cells was very close to that of the control cells without oxidant treatment. For cell morphology, scanning electron microscopy (SEM) analysis was done to observe cell surface variations of KNU5377 during menadione treatment (Fig. 1C). Prior to the MD challenge, KNU5377 cells had an unimpaired shape, but the cell shape was slightly deformed after 60 min of MD treatment, and subsequently most of the cells recovered the shape of control cells without menadione. The result shows that menadione stress influences cell morphology in the KNU5377 strain. These results also show that menadione causes ROS generation and that cellular ROS levels are inversely proportional to cell viability and cell morphology changes.

Analysis of oxidatively damaged proteins after menadione stress

The ROS produced by menadione can damage a wide range of cellular biological molecules including carbohydrates, nucleic acids, lipids, and proteins. As shown in Fig. 2A, carbonyl contents were gradually augmented for 1 h after exposure to 0.6 mM menadione. The main oxidized proteins were visualized by Western blot following two-dimensional gel electrophoresis, and identified via peptide mass fingerprinting by MALDI-TOF. The major proteins were Hsp70 family (Ssb1) (Spot No. 1) and enolase (Spot No. 2). Malondialdehyde (MDA) levels as a result of lipid peroxidation were also measured. The change of the MDA concentration was almost similar to other oxidative biomarkers such as ROS level and carbonyl content (Fig. 2B). Thus, these results suggest that the increase of cellular oxidative biomarkers such as protein oxidation and lipid peroxidation induces the breakdown of cellular redox homeostasis.

Trehalose assay and trehalase enzyme activity

Trehalose has been implicated in the response to various environmental parameters such as heat and osmotic pressure (Alvarez-Peral *et al.*, 2002; Voit, 2003). Based on these results, trehalose level and trehalase activity were measured during menadione treatment. Trehalose accumulated rapidly for the

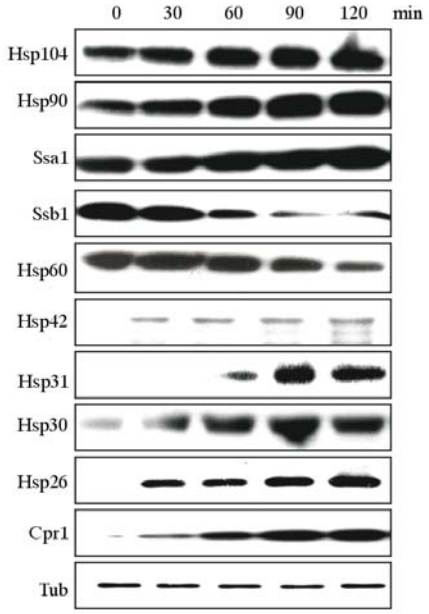

Fig. 5. Expression changes of molecular chaperones to menadione stress. Cells grown aerobically (OD_{600} of 1.0) were treated directly with 0.6 mM MD at 30°C for 120 min and sampled at 30 min intervals. Cell cultures were collected for analysis by Western blot. Anti-tubulin (Tub) antibody was used as a standard control. Hsp, heat shock protein; Ssa1, Hsp70 family; Ssb1, Hsp70 family; Cpr1, cyclophilin A isoform 1.

initial 30 min after exposure to menadione and remained at a high steady state level thereafter in the treated cells (Fig. 3A). Activity of neutral- and acid-trehalase, enzymes involved in trehalose degradation, dropped rapidly during the first 30 min after initiation of MD treatment, remained unchanged at 60 min, and thereafter gradually increased (Figs. 3B and C). These results were strongly supported by in-gel activity of trehalase. As seen in Fig. 3D, up to 60 min after menadione exposure, trehalase activity rapidly declined and then showed a mild recovery of enzyme activity, although the enzyme activity did not rebound to its normal basal level. The trehalose concentration had an inverse relationship to trehalase activity. These results imply that yeast cells respond to stress by trehalose accumulation via fine regulation of trehalase.

Expression changes of cell rescue proteins and metabolic enzymes during menadione stress

Yeast cells must operate a variety of cell rescue programs to protect itself from oxidative damage by ROS produced during menadione stress. First, antioxidant enzymes and metabolic enzymes were analyzed by Western blot, and in-gel activity. Most of the antioxidant enzymes were up-regulated by more than two-fold under menadione exposure, as compared to cells without menadione, and their expression was time-dependent. The up-regulated enzymes were as follows: superoxide dismutase (Sod1 and Sod2), thioredoxin (Trx2 and Trx3), thioredoxin peroxidase (Tsa1), thioredoxin reductase (Trr1), and metallothionein (Met). In addition, proteins involved in energy-generating system were also induced. An accumulation shift at the protein level was confirmed by Western blotting for hexokinase (Hxk), aldehyde dehydrogenase (Ald), alcohol dehydrogenase (Adh), glucose-6-phosphate dehydrogenase (G6PDH or Zwf1), and cytosolic/mitochondrial isocitrate dehydrogenase (cIDH and mIDH) (Fig. 4A). These results were supported by in-gel activity for isocitrate dehydrogenase (IDH), superoxide dismutase (Sod), G6PDH and peroxidase (Per) (Fig. 4B). Induction of the energy-generating system led to an increase in the NADPH concentration. The concentration was time-dependent after menadione exposure (Fig. 4C). Also, transition analysis of heat shock proteins (Hsps) and molecular chaperones was conducted by immunoblotting. Hsp104, Hsp90, Ssa1, Hsp42, Hsp31, Hsp30, Hsp26, and cyclophilin A Cpr1 were significantly elevated in the drug-treated cells for the indicated time, while Hsp60 and Ssb1 gradually decreased for 2 h when cells were exposed to menadione (Fig. 5). The proteins analyzed were time-dependently expressed. These results show that yeast cells require various rescue/repair proteins to protect themselves from toxic ROS.

Discussion

Development of *S. cerevisiae* yeast strains that have resistance to various stresses is important in the fermentation and brewing industries. *S. cerevisiae* KNU5377 has been characterized to be highly resistant to various stresses including heat shock (Kim *et al.*, 2007a, 2007b). In this respect, *S. cerevisiae* strain KNU5377 is a good model. However, information on the yeast's defenses against adverse environments would shed light on its biology and assist the engineering of stress-resistant strains. This study obtained evidence for differential expression of cell rescue proteins in *S. cerevisiae* KNU5377 after treatment with the oxidant menadione, a quinone extensively used in studies of cellular oxidative stress (Zadzinski *et al.*, 1998; Castro *et al.*, 2007).

Menadione stress causes oxidative stress by ROS (Fig. 1B), which affects cellular viability (Fig. 1A) and shape (Fig. 1C), and increases oxidative-protein damage (Fig. 2A) and lipid peroxidation (Fig. 2B), which can serve as oxidative biomarkers (Fig. 2). Under unfavorable conditions, yeast cells must activate cell protection mechanisms to adapt to the environmental changes or repair the damaged cellular components (Herdeiro *et al.*, 2006). First, the physiological hallmark of menadione stress response in yeast strain KNU5377 strain is a rapid, enormous increase in the concentration of trehalose through down-regulation of trehalase (Fig. 3). Normally found in growing yeast cells and other organisms only as traces, trehalose becomes a crucial protector of proteins and membranes against a variety of stresses, including heat, cold, starvation, desiccation, osmotic or oxidative stress, and exposure to toxicants (Benaroudj *et al.*, 2001; Francois and Parrou, 2001; Querol *et al.*, 2003; Herdeiro *et al.*, 2006). Recently, it has been reported that the disaccharide trehalose is capable of protecting cells and proteins against oxidative damage caused by challenge to H_2O_2 (Benaroudj *et al.*, 2001), as well as guarding cell membranes against oxidative injuries by lipid peroxidation (Herdeiro *et al.*, 2006). Taken together, rapid accumulation and maintenance of trehalose at a high concentration, as a component of antioxidant substrates appear to be highly beneficial to the cells by protecting them from oxidative damage under menadione-induced stress.

Second, antioxidant enzymes are activated during oxidative stress in *S. cerevisiae* (Estruch, 2000; Castro *et al.*, 2007; Fernandes *et al.*, 2007; Herrero *et al.*, 2008; Lopez-Mirabal and Winther, 2008). *S. cerevisiae* KNU5377 cells induced a large number of antioxidant proteins involved in internal redox homeostasis (Fig. 4), including superoxide dismutase (Sod) which converts the superoxide anion produced by menadione to H_2O_2, thioredoxin system (Trx2, Trx3, Trr1, and Tsa1) capable of scavenging ROS, NADPH-generating enzymes (G6PDH and IDH), metallothionein (Met) and peroxidase (Per), and metabolic enzymes containing hexokinase (Hxk), aldehyde dehydrogenase (Ald), and alcohol dehydrogenase (Adh). There are multiple evidences for the importance of these systems in defense of oxidative stress. For example, yeast strains deficient in antioxidant systems, such as thioredoxin peroxidase (*tsa1*), and thioredoxin II (*trx2*), become very sensitive to hydrogen peroxide and/or menadione (Tucker and Fields, 2004). Overexpression of catalase and superoxide dismutase increases thermotolerance and protects cells from oxidative stress (Thorpe *et al.*, 2004; Lushchak and Gospodaryov, 2005). Aldehyde dehydrogenase (Ald) is induced by a variety of stresses, including osmotic stress and glucose exhaustion (Navarro-Avino *et al.*, 1999) and is indispensable for providing NADPH in yeast cells lacking glucose-6-phosphate dehydrogenase activity (Grabowska and Chelstowska, 2003). In addition, alcohol dehydrogenase has recently been shown to have a novel antioxidant role the *E. coli*. Cells with a deletion of the *adhE* gene were extremely sensitive to oxidative stress and displayed increased levels of internal peroxides and increased protein carbonyl content (Echave *et al.*, 2003). As seen in *S. cerevisiae* KNU5377, rapid and effective activation of metabolic enzymes within the pentose phosphate and glycolysis pathways promptly supplies the cellular energy, such as reducing power through NADPH (Fig. 4C) and ATP, required to maintain a reduced redox state as an adaptive stress response.

Finally, yeast cells express heat shock proteins (Hsps) to maintain proteostasis via folding and/or refolding proteins during stress conditions. In general, Hsps are molecular chaperones, which are critical for survival under various types of stresses (Brosnan *et al.*, 2000). In the menadione challenge, a spectrum of Hsps were induced in *S. cerevisiae* KNU5377 including Hsp104, Hsp90, Hsp70 family (Ssa1), Hsp42, Hsp30, Hsp26, and Cpr1 (Fig. 5). Hsp104, Hsp82 (Hsp90), Hsp42, and Hsp26 adaptively respond to comprehensive stress and participate in oxidative stress (H_2O_2) tolerance via an emergent rescue function as a molecular chaperone in laboratory yeast (Fujita *et al.*, 1998; Haslbeck *et al.*, 2004) or industrial wine yeast (Carrasco *et al.*, 2001; Aranda *et al.*, 2002). By contrast, transcriptional activation of Hsp104, Hsp42, and Hsp78 was induced by menadione stress, whereas transcripts of Hsp26, Ssa4, Ssa3, and Sse3 were unchanged or repressed under the same conditions (Gasch *et al.*, 2000). As mentioned above, our results showed the induction of Ssa and Hsp26 in the presence of menadione. Hsp104 facilitates disaggregation and reactivates aggregated proteins with assistance from Hsp70 (Ssa1) and Hsp40 (Ydj1). The small heat shock proteins, Hsp26 and Hsp42, also function in the recovery of misfolded proteins and prevent aggregation *in vitro*, but their *in vivo* roles in protein homeostasis remain elusive (Cashikar *et al.*, 2005). Interestingly, the level of the Hsp70 family (Ssb1) and Hsp60, an essential mitochondrial chaperone (Cabiscol *et al.*, 2002), were decreased. The reduction of these proteins is likely due to protein carbonylation, an early marker during protein oxidation. The Hsp70 family (Fig. 2) and Hsp60 are major targets of oxidative damage (Reverter-Branchat *et al.*, 2004). Carbonylated proteins cannot be repaired. Hence, they must be removed by proteolytic degradation systems. Time-dependent accumulation of protein damage was inversely proportional to cell viability under menadione stress. Also, KNU5377 cells upregulated cyclophilin A Cpr1, a housekeeping protein with many roles, including PPIase and protein folding, interaction and trafficking. The Cpr1-deleted mutant was hypersensitive under menadione-induced oxidative stress, which increased carbonyl content following an increased ROS level (Kim *et al.*, 2006). Although a comprehensive knowledge of chaperone interaction with chaperones and their cofactors (or substrates) has not yet been publicized, our results show a new model of the chaperone network of physiological interactions under menadione stress. Clearly, induction of a combination of Hsps can contribute to yeast's stress tolerance by acting as "molecular chaperones".

In conclusion, most of the information on how cell rescue proteins containing antioxidant enzymes and Hsps function in yeast has been obtained from studies of laboratory strains. There is little information regarding the induction of the stress response under alternative physiological conditions. It would be interesting to carry out analyses of this kind using yeast *S. cerevisiae* strain KNU5377 strain isolated from sludge. Moreover, an accurate analysis of the global expression patterns in yeast during transient stress conditions or alcoholic fermentation could contribute to a more complete understanding of the mechanisms that affect the evolution of yeast populations. These results also would be useful for the selection of a valuable gene source and genetic manipulation of industrial yeasts to improve bioenergy production using biomass.

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