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Sensitivity of a Hyperactivated Ras Mutant in Response to Hydrogen Peroxide, Menadione and Paraquat

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We have explored the impact of altering the Ras-cAMP pathway on cell survival upon oxidative exposures. A hyperactivated Ras mutant of *Saccharomyces cerevisiae*, intrinsically more sensitive to heat shock than the wild type, was investigated with regard to oxidative stress. In this paper we report that the response of *ira1, ira2*-deleted mutant (IR2.53) to an oxidant, such as hydrogen peroxide (H_2O_2) or menadione is more sensitive than that of the wild type. IR2.53 showed a dramatic decrease in survival rate when challenged with 0.1 mM H_2O_2 for 30 min. The greater sensitivity of IR2.53 was also noticed with treatment of 0.01 mM menadione. Prior to oxidative stresses by these oxidants, both the wild type and the mutant were preconditioned with a mild heat shock (37 °C, 30 min), resulting in improved survivals against oxidative stresses. Rescue of IR2.53 from menadione stress by heat pretreatment was more clearly demonstrated than that from H_2O_2 treatment. On the other hand, no significant difference was observed between the wild type and the IR2.53 mutant in their survival rates upon paraquat treatments. These findings imply that the mechanism by which H_2O_2 and menadione put forth their oxidative effects may be closely associated with the cAMP-Ras pathway whereas that of paraquat is independent of the Ras pathway. Finally, the level of glutathione (GSH) was measured enzymatically as an indicator of antioxidation and compared with the survival rate. Taken all these together, this study provides an insight into a mechanism of the Ras pathway regulated by several oxidants and suggests that the Ras pathway plays a crucial role in protection of cell damage following oxidative stress.

Introduction

Most living cells are very susceptible to oxygen toxicity, which is mostly caused by a highly reactive superoxide anion radical ($O_2^{\cdot-}$), a hydroxy radical (OH·), hydrogen peroxide (H_2O_2).[1] These reactive oxygen species (ROS) are very reactive and sources of damages to DNA, lipids, and proteins.[2] Aerobic cells have therefore developed multiple defence mechanisms, by which cellular antioxidants and enzymes are capable of removing ROS to avoid oxidative damages. For example, superoxide anions produced in yeast are enzymatically reduced to H_2O_2 by superoxide dismutase (SOD),[3] and subsequently removed by catalase.[4] As an antioxidant, GSH reacts with H_2O_2 and superoxide radicals to protect cells from oxidative stress and xenobiotic toxicity.[5] Glutathione reductase provides sufficient GSH from oxidized glutathione (GSSG) in the presence of NADPH. The yeast strain defective in γ-glutamylcysteine synthetase activity was found to be hypersensitive to H_2O_2 and superoxide anions.[6] Either changes in GSH level or in the ratio of GSH/GSSG

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may serve as a signal to play a vital role in buffering the cell against ROS.

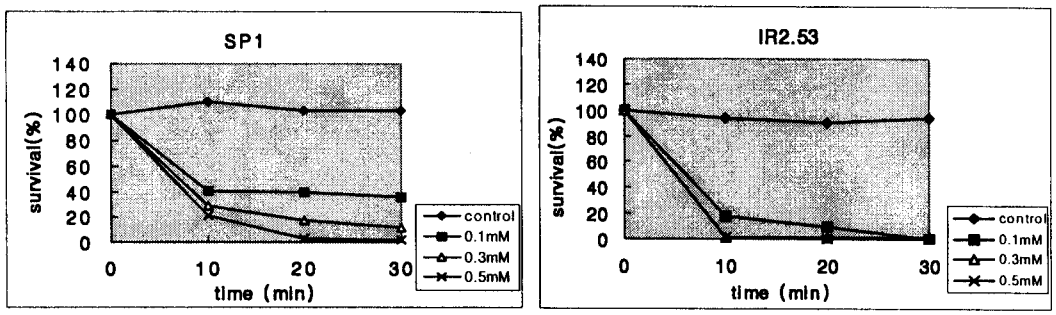
ROS have been previously implicated in mediating many signal transduction pathways.[7] The exact targets by which ROS transmits signals in human T cell line were identified as G proteins, and particularly $p21^{ras}$. The proto-oncogene Ras proteins are participated in diverse cellular processes, including the control of proliferation and differentiation in mammalian cells as well as yeast cells.[8] The activity of Ras protein is controlled by binding and hydrolyzing guanine nucleotides. Upon bound to GTP, Ras becomes activated whereas GTP hydrolysis inactivates Ras. Once activated, Ras proteins control the activity of adenylate cyclase,[9] which generates the second messenger, cAMP. cAMP in turn activates cAMP-dependent protein kinase A (PKA), whose activity plays a crucial role on cell cycle progression. Like those in mammalian system, Ras proteins in yeasts are positively regulated by CDC25[10] and negatively modulated by IRA-1 and IRA-2.[11,12] IRA-1 and IRA-2 proteins promote the hydrolysis of Ras-GTP form and results in inactivation of Ras. With *ira1* or *ira2* genes deleted, mutant (IR2.53) accumulates the activated Ras-GTP form and causes an increased level of intracellular cAMP.[11] Consequently, IR2.53 shows increased sensitivity to heat shock, nitrogen starvation and sporulation defects.

Here, we report the response of aerobic *S. cerevisiae* cells with an altered Ras activity to several oxidative stresses, which are profoundly induced by H_2O_2, menadione or paraquat. A mild heat pretreatment was also applied prior to oxidative challenge in order to explore a cross protection role of heat treatment for oxidative stress. Finally, an intracellular level of GSH was enzymatically measured and compared with the survival rate upon oxidative stress.

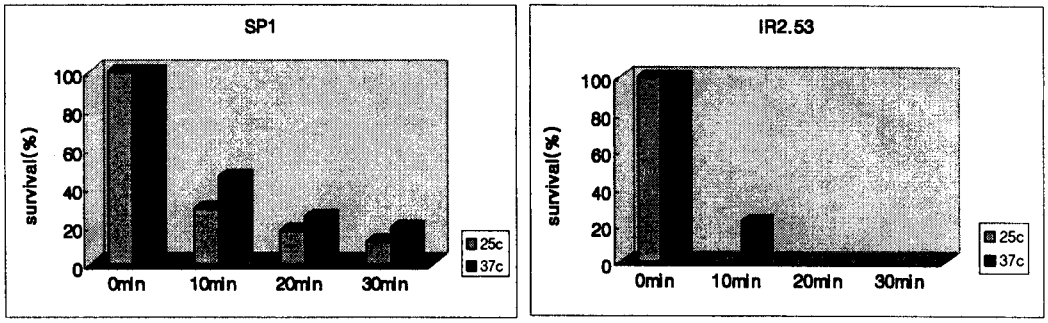
Results and Discussion

Sensitivity of Yeast Cells to H_2O_2 Treatment. Cell viabilities of the wild type SP1 and the mutant IR2.53 were determined after treatment with various concentrations of H_2O_2. Both SP1 and IR2.53 displayed a time-dependent dose response curve as shown in Figure 1A. IR2.53 appeared to be more susceptible to H_2O_2 toxicity than SP1, according to a dramatic decrease in the cell survival curve even when treated with 0.1 mM H_2O_2. These results indicate that H_2O_2 may exert its oxidative stress to yeast cells *via* direct regulation of the Ras-cAMP pathway.

Davidson *et al.* has reported that oxidative stress is involved in heat-induced cell death in yeast.[13] Mutants deleted for SOD, catalase and cytochrome peroxidase were more sensitive to heat shock than the wild type. Overexpression of catalase and SOD genes resulted in elevated thermotolerance as expected. Therefore it is worth while investigating a cross protection between heat shock response and oxidative stress in our study. Treatment condition with 0.3 mM H_2O_2 was chosen to examine an effect of heat pretreatment for providing protection against H_2O_2 oxidative challenge. Pretreatment of SP1 at 37 °C for 30 min resulted in a better survival as shown in Figure 1B. However, IR2.53 was rescued by heat pretreatment only at the beginning when the cells were incubated wtih 0.3 mM H_2O_2. As the exposure time to H_2O_2 extended, the protection effect by mild heat pretreatment was completely abolished. Therefore the heat pretreatment seems to provide protection against H_2O_2, absolutely depending on the presence of *ira1* and *ira2*



(A)



(B)

Figure 1. Sensitivity to H_2O_2. (A) Survival rates for SP1 and IR2.53 were measured as a function of the incubation time at various concentrations of H_2O_2. The survival of untreated cells was monitored as a control. (B) Cells were preconditioned either at 25 °C for 30 min (left columns) or at 37 °C for 30 min (right olumns) prior to oxidative stress with 0.3 mM H_2O_2.

gene products or the lethal response of the hyperactivated Ras mutant against H_2O_2 is too severe to be overcome by heat treatment.

Sensitivity of Yeast Cells to Menadione. We have examined the effect of menadione (2-methyl-1,4-naphthoquinone) to compare with that of H_2O_2 in SP1 and IR2.53.[14]

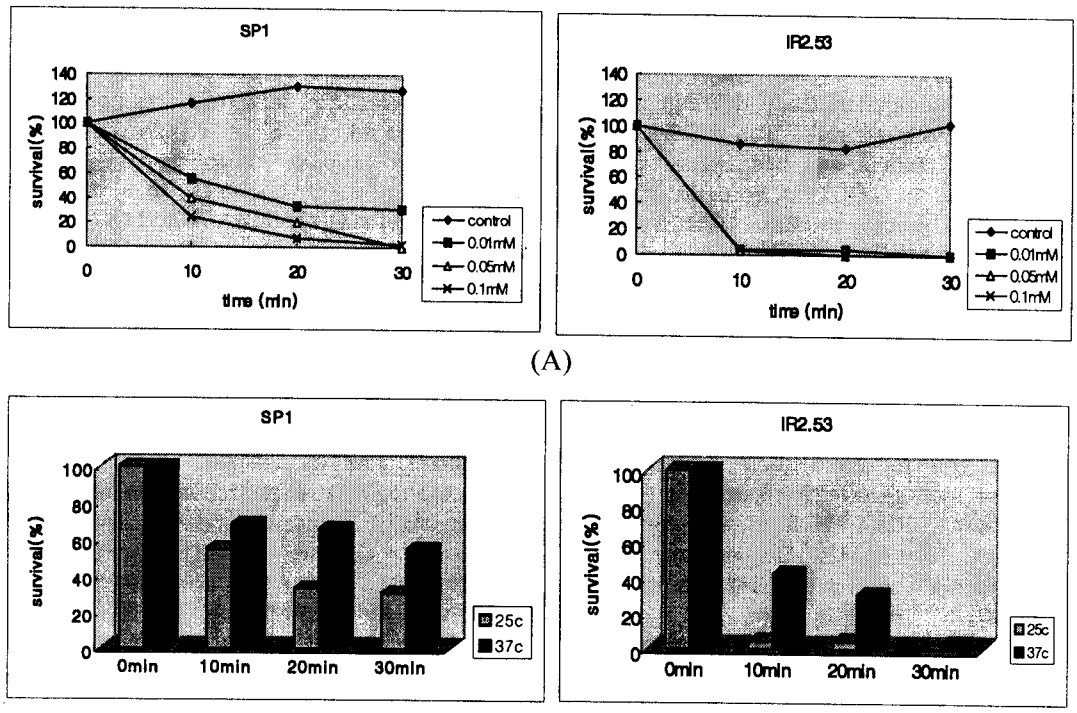


Figure 2. Sensitivity to menadione. (A) Dose responses of SP1 and IR2.53 were measured after incubations with various concentrations of menadione. (B) Cells were preincubated either at 25 °C for 30 min (left columns) or at 37 °C for 30 min (right columns) before incubated with 0.01 mM menadione.

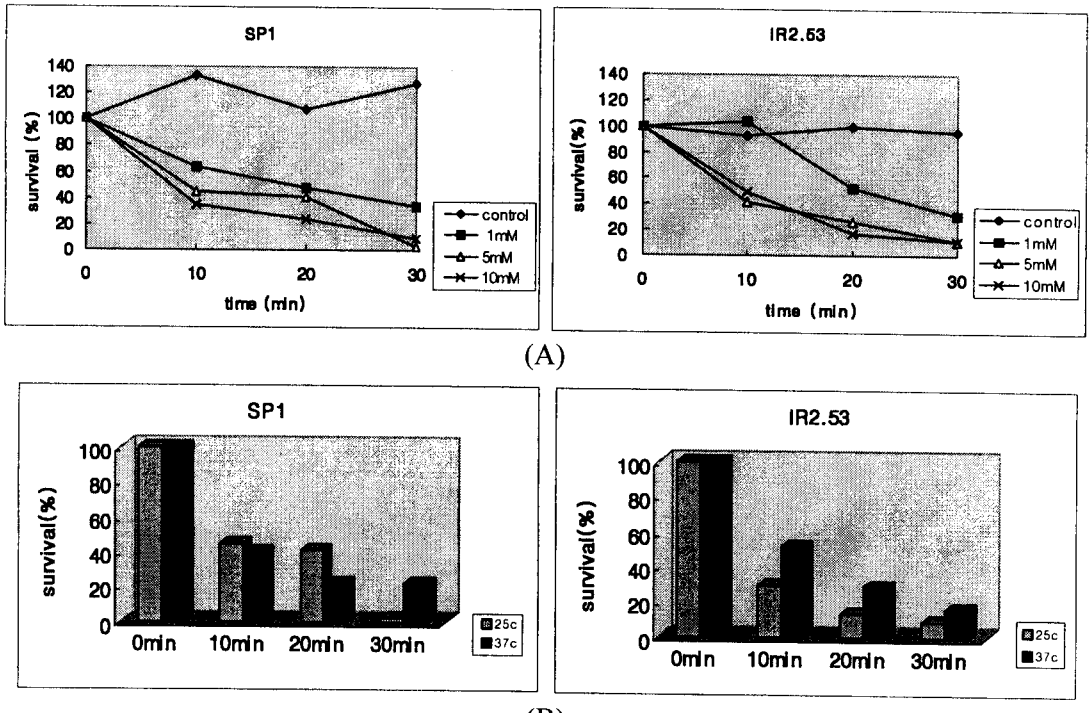


Figure 3. Sensitivity to paraquat. (A) SP1 and IR2.53 were compared for their survival rates after incubation with various concentrations of paraquat. (B) Preincubation of cells either at 25 °C for 30 min (left columns) or at 37 °C for 30 min (right columns) was followed by treatment with 5 mM paraquat.

Menadione generates superoxide radicals *via* a redox cycling, which can be converted to H_2O_2 and subsequently to the hydroxyl radical through enzyme reactions.[15] In our study the lethality of menadione was largely augmented when *ira1* and *ira2* genes were deleted as in IR2.53 (Figure 2A). The dose response curve indicated that 0.01 mM menadione was completely toxic to IR2.53 within 10 min whereas more than 30% of SP1 cells survived over 30 min incubation. When preincubated at 37 °C, both SP1 and IR2.53 also showed significant protections against menadione stress (Figure 2B). Especially rescue of IR2.53 from menadione stress by heat pretreatment in Figure 2B was much more clearly demonstrated than that from H_2O_2 stress (Figure 1B).

Sensitivity of Yeast Cells to Paraquat. Paraquat (1, 1'-dimethyl-4,4'-bipyridium dichloride) has been known to increase the cyanide-resistant respiration and caused a profound increase in the biosynthesis of the manganese-containing SOD.[15] The lethality of paraquat is entirely dependent upon the presence of dioxygen and the availability of an abundant source of electrons. In our study unlike other oxidants, such as H_2O_2 and menadione, no significant difference in cell survival was detected between SP1 and IR 2.53 when incubated with as high as 10 mM paraquat (Figure 3A). This implies that the mechanism of paraquat action is totally independent of the Ras pathway whereas the mechanisms by which H_2O_2 and menadione cause their oxidative effects may be closely related to the cAMP-Ras pathway. It was rather surprising to find that SP1 was not protected from paraquat stress by a mild heat pretreatment (Figure 3B). In contrast, IR2.53 showed a slight recovery in cell viability upon heat pretreatment followed by 5 mM paraquat exposure. In this sense the oxidative stress by paraquat again appears to be mediated by a mechanism distinct from that which regulates H_2O_2 and menadione oxidative responses.

GSH Levels for Antioxidation. Cells produce GSH in the process of oxidative responses and protected themselves against oxidative stresses.[16] Therefore the level of intracellular GSH as an antioxidant (Figure 4A) was measured to be correlated with the degree of cell survival upon treatment with oxidants (Figure 4B). For this study both SP1 and IR2.53 were treated with heat pretreatment (37 °C for 30 min), H_2O_2 (0.3 mM for 30 min), menadione (0.01 mM for 30 min) and paraquat (5 mM for 30 min). Since GSH can be interconverted to GSSG inside the cells, the ratio between GSH and GSSG can be meaningful to determine how efficiently intracellular ROS can be removed. With heat pretreatment, SP1 has a remarkably increased ratio of GSH/GSSG whereas IR2.53 has only 70% of control. The ratio decreased when SP1 was incubated with H_2O_2 or menadione, consistent with poor survival rates (Figure 4B). These findings indicate that GSH is an important antioxidant molecule in normal yeast cells, providing a good protection against H_2O_2 or menadione stress. However IR2.53 showed rather higher ratios of GSH/GSSG than those of SP1, which are contradictory to the survival patterns. Finally, paraquat treatment of SP1 is accompanied by a significant increase in the GSH/GSSG ratio. However, in the case of IR2.53 the ratio was not high enough to explain the similar pattern of cell survival to that of SP1. So far no direct correlation between GSH levels and development of oxidative stresses

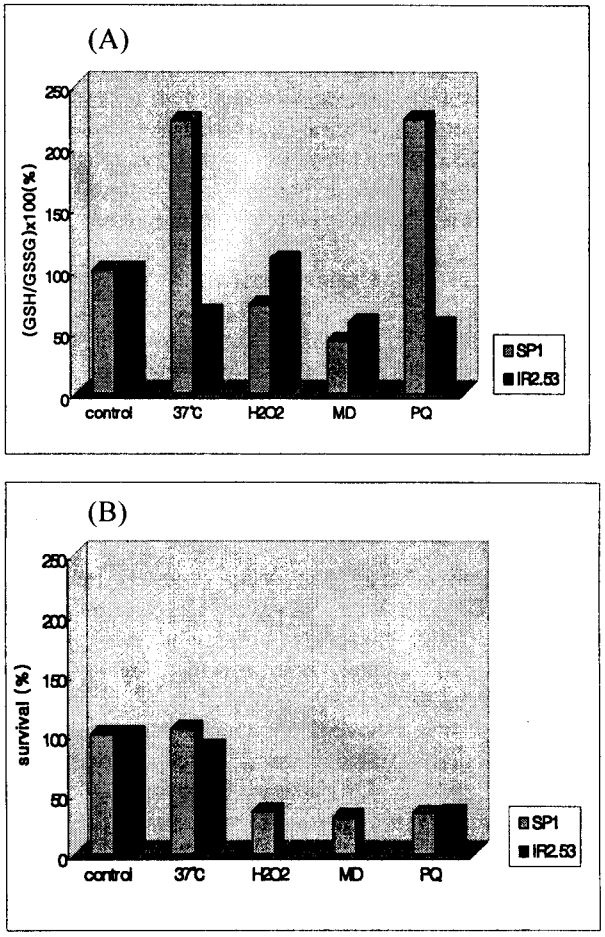


Figure 4. GSH levels for antioxidation. (A) The ratio between GSH and GSSG was compared as an indicator of protection against oxidative stress in SP1 (left columns) and IR2.53 (right columns). Cells were treated at 37 °C, 0.3 mM H_2O_2, 0.01 mM menadione or 5 mM paraquat for 30 min before preparation of cell crude extracts. (B) Cell survivals were compared as an indicator of oxidative stress for the same treatment as in (A).

in IR2.53 was found in our study. Therefore it is suggested that IR2.53 may experience oxidative stresses by H_2O_2, menadione or paraquat with unknown mechanisms beyond the presence of GSH protection machinery.

Our study shows that IRA1 or IRA2 protein expressed in SP1 protects yeast cells from oxidative stress induced by H_2O_2 or menadione, but not by paraquat. Both SP1 and IR2.53 are significantly rescued from oxidative stress once pretreated with mild heat shock. Taken together, this study provides an insight into diverse mechanisms by which the Ras pathway is involved in controlling oxidative damages as well as heat shock responses in yeasts. It was reported that the HSP104 protein responsible for thermotolerance[17] is also crucial in withstanding detrimental exposure to a high concentration of ethanol in yeasts.[18] As demonstrated in our previous study using ^{13}C NMR study, HSP104 protein can regulate the respiration pathway in yeast.[19] Therefore it can be envisioned that HSP104 protein may provide a protection against heat shock as well as oxidative stress by dealing with ROS produced as byproducts resulting from inefficient reduction of dioxygen in the respiration pathway. It would

be interesting to examine if HSP104 protein plays a pivotal role in coping with oxidative stress in IR2.53, which exhibits higher sensitivity to H_2O_2 and menadione than the wild type. In order to demonstrate that HSP104 protein helps yeast cells survive against oxidative stress in Ras mutants, IR2.53 was transformed with various plasmids containing *hsp104* gene derivatives and characterization of the resulting Ras mutants is currently in progress.

Experimental

Yeast Strains. The yeast strains used in this study were as follows: SP1 (*MATa his3 leu2 ura3 trp1 ade8*) and IR2.53 (*MATa his3 leu2 ura3 trp1 ade8 ira1::HIS3 ira2::ADE8*).[20] YPD medium containing 1% (w/v) yeast extract, 2% (w/v) bacto-peptone and 2% (w/v) dextrose was used for cell growth. All the chemicals used for oxidative stress and enzymatic analysis were purchased from either Sigma or Aldrich.

Plating Procedure. Following chemical treatment of cells in YPD, cells were harvested by centrifugation and washed twice with phosphate buffer, and then appropriate dilutions of cell cultures were spreaded onto YPD agar plates. The petri dishes were incubated at 28 °C for 2 days. Cell Survival was estimated by comparing the number of survived colonies with that of the untreated control cells.

Survival After Oxidative Stresses. Cells were grown to an early log phase (~5×10^6 cells/mL) at 25 °C with shaking in YPD media and harvested by centrifugation. To observe the sensitivity of yeast cells to H_2O_2, menadione and paraquat, various concentrations of oxidants were added to 10 mL samples and incubated further. For the mild heat pretreatment, cells were incubated at 37 °C for 30 min prior to oxidative stress. Cell survival was monitored by taking samples out at every 10 min interval. Aliquots of cells were plated on YPD plates to monitor cell viability after 2 day incubation at 28 °C. The survival rate was reported as a percent of control averaged from at least three independent experiments.

Preparation of Cell-free Extracts. Yeast cells treated with some oxidants were resuspended in 0.1 M potassium phosphate (pH 7.0) containing 2 mM EDTA. Cells were extracted by adding equal volumes of 2 M $HClO_4$ and disrupted by vigorous vortexing in the presence of glass beads for 30 sec ten times at 4 °C. After 1 hr precipitation in ice, cell debris was removed by centrifugation and the supernatant was neutralized to pH 7.0 by 2 M KOH/0.3 M MOPS. After another centrifugation, the supernatant was collected in a 15 ml tube and frozen immediately with liquid nitrogen.

Determination of Cellular GSH and GSSG. Total amount of glutathione (GSH+GSSG) in cell extracts was measured by adding 5 μL of glutathione reductase into a reaction mixture containing 700 μL of 0.3 mM NADPH and 100 μL of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and 200 μL of resuspended extract.[21] An increase of A_{412nm} was monitored over 20 min. A blocking procedure should be performed to measure GSSG alone. For the purpose of preventing reduction of GSSG into GSH, 100 μL sample was vigorously mixed with 2 μL 2-vinylpyridine for 1 min and the reaction was allowed to proceed for 60 min at room temperature. Following the blocking procedure, the amount of free GSH in the cell extract was measured as above. An actual concentration of either GSH or GSSG was converted from the calibration curve obtained from known concentrations of GSH or GSSG.

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