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Effect of Mitosis on the Resistance to Oxidative and Osmotic Stresses in Yeast

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Abstract

Cancer cells are defined by abnormal and unrestricted mitotic divisions, therefore targeting mitosis is a useful strategy for cancer treatment. Two groups of drugs that are most successfully used in the treatment of several types of cancer, taxanes and vinca alkaloids, exhibit their anti-tumor effects by causing a mitotic arrest. However, not much is known about whether being arrested in mitosis affects the sensitivity of cells to tumor-related stresses, such as oxidative and osmotic stresses. In this study, we investigated whether mitosis affects the sensitivity of S*accharomyces cerevisiae* cells to H_2O_2-induced oxidative stress and sorbitol-induced osmotic stress. Mitotic and G1-arrests were induced by nocodazole and alpha factor, respectively. The effects of nocodazole or alpha factor treatments on the sensitivity of wild type (WT) and *MAD3* deletion (*mad3Δ*) strains to oxidative stress and osmotic stress were evaluated by the spotting and coloni forming unit (cfu) assays as well as detection of reactive oxygen species (ROS) production. Data were analyzed using Student's t-test and expressed as standard deviation (std), $p<0.05$ was considered significant. Our data indicate that mitosis significantly increases resistance to oxidative stress, however it does not have any significant effect on the osmotic stress resistance in yeast.

Keywords: Oxidative stress, Osmotic stress, Mitosis, *Saccharomyces cerevisiae*.

1. INTRODUCTION

Mitosis is a cell cycle phase, which involves equal segregation of the genetic material (karyokinesis) and appropriate distribution of the cytoplasm (cytokinesis) into two identical daughter cells. Equal chromosome segregation requires each sister kinetochore to bind microtubules emanating from opposing poles of the cell (bipolar attachment). Since failure in proper chromosome segregation in mitosis may lead to aneuploidy, which is a hallmark of cancer cells, it is critical to establish proper attachments between kinetochores and microtubules in mitosis to avoid carcinogenesis [1].

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The status of kinetochore-microtubule attachments is monitored by an evolutionarily conserved major cell cycle checkpoint in mitosis, called the spindle assembly checkpoint (SAC). Presence of improperly attached/unattached kinetochores activates the SAC. SAC activation leads to a mitotic arrest in metaphase, until all kinetochores establish proper attachments prior to proceeding to anaphase, thus prevents aneuploidy [2]. Since cancer cells display abnormal, unrestricted divisions, targeting mitosis is a useful strategy for cancer treatment [3]. Two groups of drugs that are successfully used in the treatment of several types of cancer, taxanes (paclitaxel/taxol, docetaxel) and vinca alkaloids (vinblastine, vincristine) impede mitosis by targeting microtubules. These drugs interfere with microtubule structure/dynamics, thus prevent formation of proper kinetochore-microtubule attachments, leading to chronic SAC activation [4]. Exposure to anti-microtubule cancer drugs led to a prolonged mitotic arrest in all cell lines tested [5]. Consistently, mitotic index in tumors increases significantly in response to the treatment with anti-microtubule cancer drugs [6].

A major outcome of the prolonged mitotic arrest induced by microtubule targeting drugs is apoptosis [7]. Besides, despite being the shortest cell cycle phase, mitosis is the most vulnerable phase to various external signals, including chemical exposure [8] and radiation [9]. For these reasons, increasing the number of mitotic cells by the induction of prolonged mitotic arrest has been useful for cancer treatment [6]. The number of mitotic cells increases significantly in response to the treatment with anti-microtubule cancer drugs [6], however not much is known about how mitosis affects sensitivity of cancer cells to tumor related-stresses such as oxidative and osmotic stresses.

Oxidative stress, the imbalance between the production of reactive oxygen species (ROS) and antioxidant defense mechanisms in the favor of the former [10], is tightly associated with carcinogenesis [11]. Abnormally high levels of ROS have been detected in almost all types of cancer cells due to increased metabolic activity, oncogene activity, etc. Elevated ROS levels in cancer cells are associated with their oncogenic properties including proliferation, survival and migration [12]. Progression of solid tumors largely depends on the compressive stress that is generated as the tumor grows and presses against the surrounding tissue [13]. Tumor cells must overcome the compressive stress to be able to survive [14], which requires modulation of their tonicity by osmotic regulation [15]. Consistently, osmotic pressure, the underlying cause of osmotic stress, is increased in tumors [16]. However, not much is known about the effect of mitosis induced by anti-microtubule cancer drugs on the sensitivity of cancer cells to oxidative and osmotic stresses, which may alter the efficiency of cancer therapy.

The aim of this study was to investigate the effect of mitotic arrest on the sensitivity to H_2O_2-induced oxidative stress and sorbitol-induced osmotic stress in *Saccharomyces cerevisiae.* Our data revealed that mitotic arrest induced by nocodazole treatment significantly increases resistance to oxidative stress. However, nocodazole-induced mitotic arrest does not have any significant effect on the sensitivity to osmotic stress in yeast.

2. MATERIALS AND METHODS

2.1. Strains and Growth Conditions

Wild type (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 PDS1-3HA-URA3*) and *mad3Δ* (*MATα can1Δ his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 mfa1Δ::*P_{MFA1} *his5*$^+$ *mad3::NAT) S. cerevisiae* strains, kindly gifted from Dr. Daniel Burke, were used in this study. Yeast cells were maintained on YPD agar plates containing 2% (wt/vol) glucose, 1% (wt/vol) yeast extract, 2% (wt/vol) peptone, and 2% (wt/vol) agar and cultured in liquid YPD medium (2% wt/vol) glucose, 1% (wt/vol) yeast extract and 2% (wt/vol) peptone). Both strains were grown and all experiments were performed at 30^0C.

2.2. Cell Cycle Arrest and Stress Inductions

Wild type and *mad3Δ* yeast cells grown to early mid-logarithmic phase ($O.D_{600}$=~0.3) were incubated with 15 μg/ml nocodazole (Sigma) in liquid YPD on a mechanical shaker (175 rpm) at 30^0C for 3 hours to induce mitotic arrest. For G1 arrest induction, wild type cells were treated with α-factor (25 μg/ml.) in acidic liquid YPD (pH=3.4) for 2-3 hours. Oxidative stress was induced by a 3-hour incubation with 8 mM H_2O_2. 2 hour and 3 hour incubations with 2 M sorbitol was used to induce osmotic stress.

2.3. Detection of Reactive Oxygen Species (ROS)

Intracellular ROS levels were analyzed as reported previously [17]. In summary, 200 μl of cells were resuspended in fresh YPD medium (200 μl) and incubated with 10 μg/ml 2′,7′-di chlorodihydrofluorescein diacetate (H2DCFDA; Molecular Probes) at 30^0C for 40 minutes. Immediately after the incubation, 5 μl of cells were applied onto microscope slides and examined under a fluorescence microscope (Leica DM1000 LED, Leica Microsystems, Germany). At least 200 cells were evaluated and categorized as "DCF positive" or "DCF negative". Average % DCF positive cells from at least three independent experiments was graphed with standard deviations (std).

2.4. Spotting and Colony Forming Unit (CFU) Assays

For the spotting assay, 10-fold serial dilutions were prepared for each sample and 5 μl of each aliquot was spotted on YPD plates. Pictures were taken following a 2-day incubation at 30^0C. Spotting assays were repeated at least twice and pictures of a representative experiment was reported. For CFU determination, appropriate dilutions were plated onto YPD plates and colonies on each plate was counted following a 2-day incubation at 30^0C. Survival (% cfu/ml) was determined by dividing the number of colonie forming units (CFU's) by the dilution factor and the volume of the plated dilution. Average % cfu/ml or % cfu/ml fold change from at least 3 independent experiments was graphed with standard deviations (std).

2.5. Statistical Analysis

Statistical analyses were performed using two-tailed Student's t-test. Data are expressed as standard deviation (std). $p<0.05$ was considered significant.

3. RESULTS AND DISCUSSION

3.1. Nocodazole Increases Resistance to H_2O_2-induced Oxidative Stress

Almost all types of cancer cells display high levels of reactive oxygen species, which is involved in cancer cell proliferation, survival and migration [12]. Although, high ROS levels are counteracted by antioxidant defense mechanisms in cancer cells, ROS levels still remain higher compared to normal cells, leading to oxidative stress [18]. We investigated whether treatment with nocodazole, a microtubule-targeting drug, prior to the induction of oxidative stress by H_2O_2 effects resistance of budding yeast cells to oxidative stress. For this purpose a wild type *Saccharomyces cerevisiae* strain grown to early mid-log phase (OD_{600}=~0.3) was divided into two and treated with or without nocodazole (15 μg/ml) for 3 hours. Immediately after the treatment with or without nocodazole (0 hr), both cultures (OD_{600}=~0.8) were incubated with 0 mM or 8 mM H_2O_2 for 3 hours (3 hrs). Samples taken from each culture at 0 and 3 hours were examined for ROS levels using the H2DCFDA assay. In the presence of ROS, carboxy-H2DCFDA, which is normally a non-fluorescent reagent, is oxidized and becomes green fluorescent [19]. Nocodazole treatment alone increased ROS production as %DCF positive cells in nocodazole-treated culture incubated with 0 mM H_2O_2 was significantly higher (19.4 %) compared to nocodazole untreated cells incubated with 0 mM H_2O_2 (1.8 %) (*$p<0.05$) (Fig. 1A). Interestingly, although nocodazole treatment alone significantly increased ROS production, it decreased ROS production in response to oxidative stress: % DCF

positive cells in nocodazole-treated cells incubated with 8 mM H_2O_2 were significantly lower compared to that of nocodazole untreated cells incubated with 8 mM H_2O_2 (*p<0.05) (Fig. 1A).
Next we examined the viability of nocodazole-treated and untreated cells to 8 mM H_2O_2 by the spotting and CFU assays. An early mid-log culture was divided into two and treated with or without nocodazole for 3 hours. Following the treatments with/without nocodazole (0 hr), both cultures were incubated with 0 mM/8 mM H_2O_2 for 3 hours (3 hrs). After the H_2O_2 incubations, 10-fold serial dilutions of each culture were spotted onto YPD plates. After a 2-day incubation at 30^0C, we observed that nocodazole-treated cells were more resistant to 8mM H_2O_2 treatment compared to nocodazole-untreated cells (Fig. 1B). We also plated appropriate dilutions onto YPD plates and calculated the survival (% cfu/ml) for each culture. Consistent with the spotting assay, survival of cells treated with 8 mM H_2O_2 was significantly higher (*p<0.05) in nocodazole-treated culture compared to the nocodazole-untreated culture (Fig. 1C). These data together suggest that nocodazole treatment significantly increases resistance to H_2O_2-induced oxidative stress by decreasing ROS production significantly.

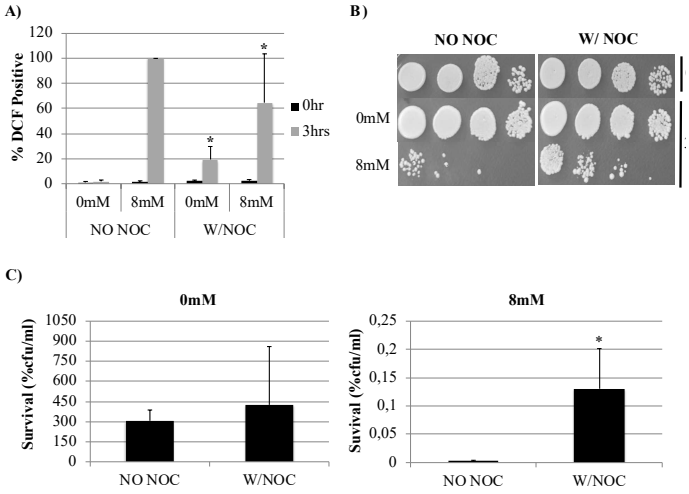

Figure 1. Sensitivity of nocodazole treated and untreated cells to H_2O_2 incubation.

3.2. Mitosis is Required for the Increased Resistance to H_2O_2-induced Oxidative Stress

Since nocodazole induces a mitotic arrest, we next tested whether mitosis is required for the resistance to H_2O_2-induced oxidative stress. Mad3 is a SAC protein required for SAC-activation dependent mitotic arrest [20]. Therefore, *mad3Δ* cells are unable to arrest in mitosis in response to anti-microtubule drugs. We grew *mad3Δ* culture to early mid-log (OD_{600}=~0.3) and divided the culture into two and treated with or without nocodazole for 3 hours. Following the nocodazole treatment (0 hr), both cultures (OD_{600}=~0.8) were incubated with 0 mM, 6 mM or 8 mM H_2O_2 for 3 hours (3 hrs). After the H_2O_2 treatments, 10 fold serial dilutions of the cultures were spotted onto YPD plates. At the end of a 2-day incubation, we observed that nocodazole treatment slightly reduced the resistance to 6 mM and 8 mM H_2O_2 (Fig. 2A). Consistently, although not statistically significant, % DCF positive cells in nocodazole-treated cells incubated with 8 mM H_2O_2 (80 %) was higher compared to nocodazole-untreated cells incubated with 8 mM H_2O_2 (60 %) (p>0.05) (Fig. 2B). These results suggest that nocodazole increases resistance to H_2O_2-induced oxidative stress through mitotic arrest induction. Thus, mitosis is required for the increased resistance to H_2O_2. In support of this, we observed by spotting assay that wild type cells arrested in G1 with alpha factor were more sensitive to 8 mM H_2O_2 incubation compared to cycling cells that were not treated with alpha factor (Fig. 2C). Consistently, 8 mM H_2O_2 incubation led to a significantly higher ROS production in G1-arrested cells (35.9 %) compared to alpha factor-untreated cells (9.2 %) (*p<0.05) (Fig. 2D). These data together suggest that unlike mitosis, G1 decreases resistance to oxidative stress.

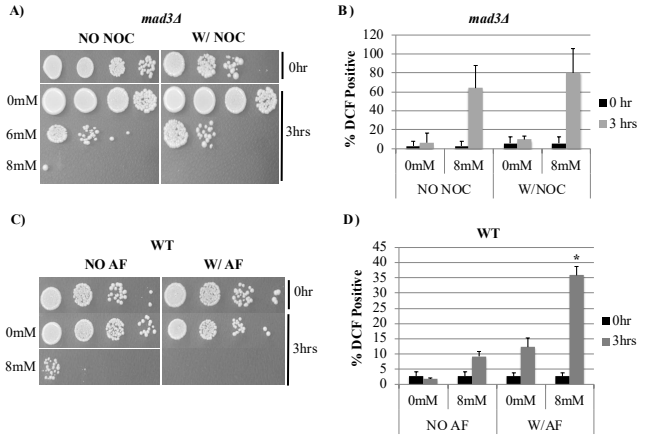

Figure 2. Requirement of mitosis for H_2O_2 resistance.

Next, we investigated whether nocodazole treatment alter the H_2O_2 sensitivity of G1-arrested cells. For this purpose, we induced a G1 arrest in the early mid-log phase wild type strain with alpha factor. Alpha factor was re-added before the arrested culture was divided into two and treated with or without nocodazole (0 hr). Both nocodazole treated and untreated G1-arrested cultures were then incubated with either 0 mM or 8 mM H_2O_2 for 3 hours (3 hrs). Spotting assay revealed that nocodazole treatment did not have a remarkable effect on the sensitivity of G1-arrested cells to 8 mM H_2O_2 (Fig. 3A). In support of these data, ROS production (%DCF positive cell), due to 8 mM H_2O_2 incubation, was not significantly different in G1-arrested cells treated with nocodazole (AF+NOC) (35.3 %) compared to that of G1 arrested cells not treated with nocodazole (NOC) (44.3 %) ($p>0.05$). These data further support the hypothesis that it is nocodazole treatment increases resistance to oxidative stress through induction of mitosis.

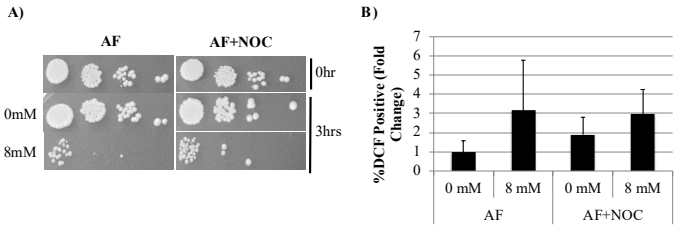

Figure 3. Effect of nocodazole treatment in G1 cells on H_2O_2 resistance.

3.3. Effect of Mitosis on the Sensitivity to Sorbitol-induced Osmotic Stress

Cancer cells have increased osmotic stress to modulate their tonicity to be able to endure the compressive stress that is generated by the surrounding tissues [14, 15]. To investigate whether mitosis effects sensitivity to sorbitol-induced osmotic stress, an early mid-log phase wild type culture was incubated with 0 M or 2 M sorbitol (0 hrs) for 2 (2 hrs) and 3 hours (3 hrs). Following the incubations, viability was assayed by the spotting assay and intracellular ROS production was detected using H2DCFDA. Spotting assay revealed that the viability of the nocodazole-treated culture incubated with sorbitol was only a little higher compared to that of nocodazole-untreated culture incubated with sorbitol (Fig. 4A). In support of these data, although not statistically significant, intracellular ROS production due to 2 M sorbitol incubation in nocodazole treated cells (40.7 %) was lower compared to not that of nocodazole untreated cells (47.3 %) ($p>0.05$) (Fig. 4B). These results suggest that mitosis has a little effect on the sensitivity to sorbitol-induced osmotic stress.

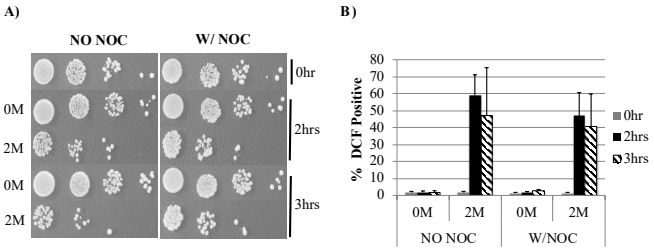

Figure 4. Effect of mitosis on sorbitol resistance.

H_2O_2 has been shown to induce a G2 checkpoint dependent arrest in different types of eukaryotic cells as well as *S.cerevisiae* [21, 22]. This suggests that the time required for the H_2O_2-induced DNA damage is mainly provided by the G2/M phase of the cell cycle and may explain why increasing the number of mitotic cells leads to an increased resistance to H_2O_2. Osmotic stress, on the other hand, leads to a delay in both G1 and G2 phases of the cell cycle [23]. Therefore, mitosis may not be sufficient to confer a significant resistance to

osmotic stress, which may explain why we observed only a little increase in the resistance to osmotic stress in mitotically arrested cells.

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