Homologous Recombination is Activated at Early Time Points Following Exposure to Cobalt Chloride Induced Hypoxic Conditions in *Saccharomyces cerevisiae*

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Abstract DNA repair functions are essential for the maintenance of genetic integrity and are regulated in response to both environmental and chemical stressors in mammalian and yeast cells in culture. The inhibitory effect of limited O₂ availability on DNA repair functions in general and on homologous recombination (HR) in particular, correlates with increased chromosomal abnormalities in hypoxic cancer cells. Given the above, we have investigated the effects of CoCl₂,—a hypoxia mimetic agent on HR and genetic aberrations in *Saccharomyces cerevisiae*. Our studies demonstrate that both acute and chronic exposure to CoCl₂ activated HR and increased genetic aberrations in *S. cerevisiae* D7 cells. At early time points following addition of CoCl₂ to the growth media, cells were briefly arrested in the G1-S boundary concomitant with a transient increase in Rad52-GFP foci formation and induction of low levels of DNA damage. The mode of action of CoCl₂ is thus similar to that of DNA synthesis inhibitors, the later are known to induce HR and cause G1-S arrest. We propose that the activation of HR in the presence of the hypoxia mimetic agent may be attributed to the replication stress and/or DNA damage induced by the stressor.

Keywords *Saccharomyces cerevisiae* · Hypoxia mimetic · Homologous recombination · DNA damage · G1-S arrest

Intracellular DNA double-strand breaks (DSB) generated by exposure to ionizing radiation, chemotherapeutic drugs and reactive oxygen species, as well as during V(D)J recombination and immunoglobulin class switching are repaired by homologous recombination (HR) and non-homologous end joining (NHEJ). Failure to repair DSBs results in various chromosomal abnormalities that may ultimately lead to neoplastic transformation [1]. In *S. cerevisiae*, HR is the major pathway for DNA double strand break repair (DSBR) and is carried out by members of the RAD52 epistasis group that include RAD52, RAD50, RAD51, RAD54, RAD55, RAD57, RAD59, MRE11 and XRS2 and requires extensive DNA sequence homology between the DNA molecules involved in recombination. HR is initiated by DSBs followed by 5' to 3' resection of the ends. The 3' ends thus formed are recombinogenic that bind Rad51 in an ATP dependent reaction and invades homologous template to initiate DNA replication by strand displacement. The inhibitory effect of RPA on Rad51 binding to DNA is overcome by Rad52. Broken ends are then joined by ligation leading to the formation of two Holliday junctions that are subsequently resolved to yield either crossover or non-crossover products [2]. DSBR by NHEJ requires little or no sequence homology and is carried out by the MRX complex along with Yku80, Yku70, DNA Pol4 and DNA ligase IV [3]. While DSB repair by HR is error-proof, that by NHEJ is an error-prone process.

DNA replication and repair functions are regulated in response to environmental stress conditions in *S. cerevisiae*. Hypoxic stress results in transient down-regulation in the expression of MCB and SCB regulated genes required

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for DNA replication and repair [4]. Exposure to environmental stress conditions and to DNA damaging agents increases the expression of genes involved in base excision repair [5] and in HR [6] respectively. In spite of the above gene expression data, not much is known about the influence of environmental stress on the functionality of individual DNA repair pathways in *S. cerevisiae*. DSB repair functions are also regulated in the extreme hypoxic condition present in the tumor microenvironment. Limited O₂ availability and exposure to CoCl₂ inhibits HR without having any effect on NHEJ in several cancer cell lines. It has hence been postulated that preferential down-regulation of HR functions leading to low fidelity DSB repair by NHEJ alone, could lead to the accumulation of chromosomal abnormalities in hypoxic cancer cells [7 and references therein]. The effect of hypoxia on DSB repair is further complicated by the suggestion that acute and chronic hypoxia may lead to different tumor biology that might directly affect tumor therapy [8]. In addition, studies on the effect of hypoxia on cell proliferation and cell cycle progression using mammalian cells in culture have indicated that oxygen availability reversibly arrests DNA replication [9] by directly regulating Ribonucleotide reductase (RNR) function [10] resulting in cell cycle arrest either at metaphase or just before S phase [11].

Given the above, we have investigated the effect of CoCl₂—a hypoxia mimetic agent, to study the effects of hypoxic stress on HR functions, genetic aberrations and cell cycle progression in *S. cerevisiae*. CoCl₂ is a well-established hypoxia mimetic agent that has been shown to adversely affect O₂ delivery and utilization in whole animals [12]. It also induces hypoxia-specific gene expression in mammalian [13] and yeast cells [14] in culture. Furthermore the yeast system has been widely accepted as a model for the study of anti-cancer therapeutic agents [15].

Materials and Methods

Yeast Strains and Growth Conditions

Yeast strains used in this study are listed in Table 1. All standard yeast techniques were as described in [16]. Wherever indicated, CoCl₂ was added to a final concentration of 0.75 mM to the media. At a concentration of 0.75 mM, CoCl₂ induced hypoxia-specific Ole1p expression without having any effect on cell viability [17].

Rate of Mitotic Recombination

Rates of mitotic recombination were determined by fluctuation analysis. Single colonies of D7 cells [18] were inoculated in 20 × 3 ml YPD with and without 0.75 mM

CoCl₂ and allowed to grow for 48 h at 30°C. Approximately 750 cells from each culture (based on OD_{600 nm}) were plated on YPD plates and the number of red, pink, red-white, pink-white and red-pink twin sector colonies was counted following incubation at 28°C for 6 days followed by incubation at 4°C for 2 days [19]. For acute exposure to CoCl₂, 20 × 3 ml log phase cultures grown from single colonies were exposed to 0.75 mM CoCl₂ for 1 h, plating and subsequent incubation were as above. The experiments were performed twice and combined results from both the experiments have been shown. Since the nature of the experiment does not allow calculation of recombination rates by standard methods [20, 21], we have expressed recombination rates as the ratio of total recombinants obtained: total number of cells plated and have not performed further statistical analysis on the results.

Rad-52 Foci Formation

Log phase ATCC 201388 *RAD52-GFP* cells (OD_{600 nm} = 0.8) were either exposed to 0.75 mM CoCl₂ or left untreated in SD media; 50 µl aliquots were withdrawn at definite intervals of time and incubated with 2 µg/ml DAPI for 15 min, washed with PBS and resuspended in 50 µl SD media. 10 µl DAPI stained cells was then fixed by mixing with equal volume of 2% low melting agarose in SD media followed by incubation at 30°C for 30 min before microscopy [22]. Cells exposed to CoCl₂ were stained and fixed in the presence of 0.75 mM CoCl₂. Rad52-GFP and DAPI stained images were acquired at 100× magnification with a digital camera (mbf biosciences CX 9000) mounted on an Olympus microscope BX51 using 430–480 nm and 290–400 nm filters respectively for excitation and analyzed by Photoframe software from mbf.

Cell Cycle Progression by Flow Cytometry

Alpha-factor (Sigma) was added to a final concentration of 5 µg/ml to 2 × 15 ml BY4741 cultures in YPD (0.2 OD_{600 nm}) and incubated at 30°C for 2 h. Following incubation, cells were precipitated, washed and released into SD media containing appropriate supplements with and without 0.75 mM CoCl₂ and incubated at 30°C with shaking. Aliquots taken out every 30 min were analyzed by flow cytometry as described earlier [17].

Comet Assay

Comet assays were performed essentially as described in [23, 24] with minor modifications. Cell pellet from a log phase 3 ml culture of BY4741 cells (OD_{600 nm} = 0.8) either exposed to stressors or without exposure was incubated with 1 mg/ml Lyticase (Sigma) in 1 M Sorbitol at

Table 1 Yeast strains used in this study

Strains	Genotype	Source
BY4741	<i>Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	Dr. A. Bachhawat IMTECH, Chandigarh India
D7	<i>Mata ade2-40 ilv1-92 trp5-12</i> <i>Matα ade2-119 ilv1-92 trp5-27</i>	Dr. Madhubala INMAS, Delhi, India
ATCC 201388	<i>MATa his3Δ1leu2Δ0 met15Δ0 ura3Δ0</i>	Invitrogen
<i>RAD52-GFP</i>	<i>RAD52-GFP-HIS3MX6</i>	

30°C for 1 h in a total volume of 100 µl. Half of the cells were used for slide preparation as described in [24]. Cells were then lysed by incubation in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris base, 1% Triton X-100, 100 mM DMSO and 0.1% SDS) at room temperature for 2 h. The slides were then rinsed in electrophoresis buffer (300 mM NaOH, 1 mM EDTA) and left in the same buffer for another 30 min. Following electrophoresis at approximately 25 volts and 300 mA for 45 min the slides were neutralized in 0.4 M Tris–HCl (7.5) for 15 min, stained with ethidium bromide in the same buffer, air dried briefly and stored at 4°C overnight. Comets were visualized by fluorescence microscopy (Olympus BX51) at 100× magnification using a 380–405 nm filter for excitation. Microscopic images were captured by a digital camera (mbf biosciences CX 9000) and comets were scored and analyzed by the cometScore15 software. A total of 50 individual cells were analyzed by the cometScore15 software for each exposure condition and the Olive moment distribution for each exposure condition is shown.

Results and Discussion

Exposure to CoCl₂ Increased HR and Genetic Aberrations in *S. cerevisiae*

We first investigated the overall effect of CoCl₂ exposure on HR by scoring for pink and red twin sectored colonies in *S. cerevisiae* D7 cells following both acute and chronic exposure to the stressor. Acute exposure to 0.75 mM CoCl₂ for 1 h induced an increase in the number of red-pink twin sectored colonies indicative of increased homologous recombination between the centromere and the *ade2* locus in D7 cells. Genetic aberrations as estimated by the appearance of red, pink, red-white and pink-white D7 colonies also increased considerably under similar conditions. Chronic exposure to the stressor for 48 h further increased the rates of both HR and genetic aberrations (Table 2) in D7 cells. The increased rates of the HR and genetic aberrations following acute exposure to CoCl₂ and the exposure-time dependent further increase in the same

following chronic exposure to the stressor indicated that, CoCl₂ is recombinogenic and mutagenic in *S. cerevisiae* D7 cells.

Acute Exposure to CoCl₂ Induced Rad52-GFP Foci Formation

The increase in HR events in the presence of CoCl₂ led us to examine if the stressor influenced Rad52 foci formation. Rad52-GFP foci formation was induced within 30 min of addition of CoCl₂ to cells expressing GFP-tagged Rad52; foci intensity and the number of cells showing foci decreased sharply thereafter (Fig. 1 and Table 3) and returned to control levels within 2 h (data not shown). Our results thus demonstrate that, CoCl₂ transiently induced formation of recombination centers at early time points following addition to the growth media, which support our conclusion from the previous experiment that CoCl₂ induced HR in *S. cerevisiae*.

Exposure to CoCl₂ Caused DNA Damage and Induced Transient G1–S Arrest

Since Rad52 foci formation is induced by DSBs [25] caused either by a direct effect of stressors (e.g., γ-irradiation) on the genomic DNA or as a consequence of its inhibitory effect on DNA replication (replication stress) leading to the accumulation of replication intermediates [25], we investigated if exposure to CoCl₂ caused DNA damage and/or inhibited DNA replication. We did not observe any DNA damage when genomic DNA isolated from cells exposed to CoCl₂ was examined by gel electrophoresis under normal and alkaline conditions (data not shown). However, examination of cells by the comet assay demonstrated that exposure to CoCl₂ for 30 min induced DNA damage in BY4741 cells (Fig. 2A, B) which was significantly higher [P (t -test) < 0.05] than cells not exposed to the stressor. Given that DNA damage following CoCl₂ exposure could be detected by the comet assay and was undetectable by gel electrophoresis, we concluded that the hypoxia mimetic agent caused low levels of DNA

Table 2 CoCl₂ induced mitotic recombination and genetic aberrations in *S. cerevisiae* D7 cells

Growth media	Colonies	R	P	R–W	P–W	R–P	HR rate $\times 10^{-4}$	TA rate $\times 10^{-4}$
YPD (Control)	23,745	0	2	0	2	0	0	1.7
YPD + CoCl ₂ acute exposure (1 h)	22,534	0	0	2	6	2	0.9	3.5
YPD + CoCl ₂ chronic exposure (48 h)	21,324	1	5	0	5	4	1.9	5.1

R, P, R–W, P–W and R–P red, pink, red–white, pink–white and red–pink twin sector colonies, MR Mitotic recombination events (red–pink twin sector colonies). TA Total aberrations (red, pink, red–white and pink–white sector colonies) arising from point mutations, mitotic gene conversion, deletion and aneuploidy. Rate Number of events per cell division

Fig. 1 CoCl₂ induces Rad52 foci formation: Log phase ATCC 201388 *RAD52-GFP* cells in SD media containing appropriate supplements were exposed to CoCl₂ for intervals of time as indicated in the figure and prepared for microscopy as described in “materials and methods” section. Rad52-GFP foci formation was examined by fluorescence microscopy. Experiments were done four times and representative data from a single experiment are shown. Foci have been marked by arrowheads. Side panel magnification of nuclei

Table 3 CoCl₂ induced transient Rad52-GFP foci formation

Time of exposure to CoCl ₂ /min	0	30	60
% of cells forming Rad52-GFP foci	0 ^a	50.1 $\pm$ 0.1 [*]	5.8 $\pm$ 7.8 ^b

^{*} *P* (*t*-test) < 0.05 compared to both ^a and ^b

damage. The similar time course of induction of Rad52-GFP foci formation and that of DNA damage suggested that the later induced Rad52-GFP foci formation.

To determine if CoCl₂ induced replication stress we investigated if the stressor inhibited cell cycle progression in addition to its activating effect on HR (Fig. 1 and Table 3). Compounds that directly or indirectly inhibit

DNA synthesis (aphidicolin and hydroxyurea respectively) are known to induce S-phase arrest and activate HR in mammalian cells in culture [26]. Our results demonstrate that cell cycle progression was transiently inhibited in the G1–S boundary at early time points following exposure to CoCl₂ (Fig. 2C). The effects of CoCl₂ on cell cycle progression and HR were thus similar to the effect of DNA synthesis inhibitors. It was however less severe than the effect of either aphidicolin or hydroxyurea [26].

In conclusion, both acute and chronic exposure to CoCl₂ induced HR and genetic aberrations in *S. cerevisiae* cells. Concomitant with the increase in HR following acute exposure to the stressor, we observed a transient increase in Rad52 foci formation. Furthermore CoCl₂ exposure induced low levels of DNA damage and transiently

Fig. 2 CoCl_2 induces DNA damage and inhibits DNA replication. (A) CoCl_2 induced DNA damage: Log phase BY4741 cells were either exposed to 0.1 mM H_2O_2 , 0.1% MMS and 0.75 mM CoCl_2 for 30 min or left unexposed (control) before proceeding for the comet assay. H_2O_2 and MMS were used as positive controls for the experiment. 50 individual comets were examined for each condition and the distribution of the Olive tail moment for each exposure condition is shown. (B) Same as (A) except that the data has been plotted in logarithmic scale to depict the low values of olive moment. (C) Cell cycle progression in the presence and absence of CoCl_2 : The experiment was performed thrice and representative data from a single experiment is shown

arrested cells in the G1–S boundary within the same time frame as it induced Rad52 foci formation and HR. The activation of HR and inhibition of cell cycle progression in the presence of CoCl_2 suggest that the mode of action of the stressor is similar to the effect of DNA synthesis inhibitors [26]. The induction of HR in the presence of CoCl_2 may thus be attributed to the effect of replication stress and/or DNA damage induced by the stressor. Finally, the notion that selective inhibition of HR in hypoxic cancer cells leads to increased chromosomal abnormalities does not hold true in *S. cerevisiae* exposed to similar conditions since genetic aberrations increased in the later in spite of increased HR activity. The above suggests that HR may not be the only DNA repair pathway responsible for the maintenance of genetic integrity under hypoxic stress.

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