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Reactive oxygen species and Ca^{2+} are involved in cadmium-induced cell killing in yeast cells

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Draft

Abstract

Cadmium (Cd) is one of the most toxic heavy metals of great environmental concern and its toxicity has been investigated in a variety of cells. In this study, we elucidated the toxic effects of cadmium in yeast cells. Our results showed that Cd^{2+} (0.05–5.0 mmol L^{-1}) significantly inhibited yeast cell growth, and the inhibitory effect was positively correlated with Cd^{2+} concentrations. Cd^{2+} caused loss of cell viability in a concentration- and duration- dependent manner in yeast cells. Intracellular reactive oxygen species (ROS) and Ca^{2+} levels increased in yeast cells after exposed to 5.0 mmol L^{-1} cadmium for 6 h. Cd^{2+} -caused cell viability loss was blocked by antioxidants (0.5 mmol L^{-1} ascorbic acid (ASA) or 500 $\text{U} \cdot \text{mL}^{-1}$ catalase (CAT)) or Ca^{2+} antagonists (0.5 mmol L^{-1} ethylene glycol tetraacetic acid (EGTA) or 0.5 mmol L^{-1} LaCl_3). Moreover, a collapse of mitochondrial membrane potential ($\Delta\Psi_m$) was observed in Cd^{2+} -treated yeast cells. These results indicated that cadmium-induced yeast cell killing was associated with the elevation of intracellular ROS and Ca^{2+} levels and also the loss of $\Delta\Psi_m$.

Keywords: Cadmium, yeast, cell killing, reactive oxygen species, Ca^{2+} .

18 Introduction

19 Cadmium (Cd^{2+}), one of the toxic heavy metals, has become a major pollutant
20 worldwide mainly due to anthropogenic activities such as application of phosphate
21 fertilizers, disposal of household and industrial wastes (di Toppi and Gabbrielli 1999;
22 DalCorso 2008). Cd^{2+} can rapidly enter the food chain, causing toxicity in both animals
23 and plants (di Toppi and Gabbrielli 1999). Once in the cells, Cd^{2+} inhibits DNA
24 replication and repair, causes chromatin condensation, and disrupts cell cycle
25 progression (Bjerregaard 2007; Sun et al. 2013). Furthermore, excessive Cd^{2+} usually
26 triggers reactive oxygen species (ROS) bursts in the cytoplasm (Gallego et al. 2012;
27 Chmielowska-Bak et al. 2014). ROS accumulation results in oxidative stress within
28 cells, including harmfully changing protein structures, destroying phospholipids, and
29 eventually leading to cell death (Gallego et al. 2012).

30 Previous studies have indicated that cell death in yeast could be induced by series
31 of abiotic stresses, such as UV-B, hydrogen peroxide (H_2O_2), hyperosmotic stress and
32 aluminum (Al^{3+}) (Madeo et al. 1999; Del et al. 2002; Ribeiro et al. 2006; Zheng et al.
33 2007). It has been reported that the increase of intracellular Cd^{2+} concentration resulted
34 in ROS overproduction in yeast cells (Brennan and Schiestl 1996; Perrone et al. 2008).
35 To date, the cellular mechanisms of Cd^{2+} toxicity in yeast are far from being completely
36 elucidated. ROS and calcium ions (Ca^{2+}) are largely recognized as important signaling
37 messengers involved in cell killing of animals and plants (Zheng et al. 2007; Brookes et
38 al. 2004; Nargund et al. 2008; De Michele et al. 2009; Yi et al. 2012). However, for
39 yeast cells, it is not clear whether Cd^{2+} -induced cytotoxicity is associated with
40 accumulation of intracellular ROS and Ca^{2+} , and how ROS and Ca^{2+} signaling involve
41 in Cd^{2+} -induced toxicity.

42 In this study, we examined the effects of cadmium on the alterations of cell
43 physiology in *Saccharomyces cerevisiae*, which was considered as an ideal system to

research the regulatory mechanisms of cell death because of its advantages such as well-understood genetics, rapid growth, and not being pathogenic (Carmona-Gutierrez et al. 2010; Matuo et al. 2012; Wu et al. 2013). The object of this study was to explore the role of ROS and Ca^{2+} in the signaling events leading to cell death, and elucidate the possible mechanisms that explain these evidences.

Materials and methods

Strains and growth conditions

Saccharomyces cerevisiae yeast cells were maintained on YEPD agar slants (1% yeast extract, 2% peptone, 2% glucose, and 2% agar, pH 5.0) at 4 °C. After being refreshed in YEPD liquid medium at 30 °C and 200 rpm for 24 h on a rotary shaker (Boxun Technologies Inc., Shanghai, China), the yeast cells were used in the following experiments.

Measurement of cell growth

Saccharomyces cerevisiae cells were cultured in a 100 mL flask containing 50 mL YEPD liquid medium supplemented with different CdCl_2 concentrations (0.05–5.0 mmol L^{-1}), and shaken at 30 °C and 200 rpm for 24 h on a rotary shaker (Boxun Technologies Inc., Shanghai, China). Cells incubated in YEPD broth without CdCl_2 were used as control. Cell growth was detected by measuring the optical density of the cultures at 600 nm ($\text{OD}_{600\text{nm}}$) using a spectrophotometer. $\text{OD}_{600\text{nm}}$ values were determined every two hours over the 24-h period. The inhibition rate (%) of cell growth was calculated using the following formula: Inhibition rate (%) = $(1 - \text{OD}_{600\text{nm}}$ value of treated cells/ $\text{OD}_{600\text{nm}}$ value of control cells) $\times 100\%$.

Determination of cell viability

For cadmium treatment, yeast cells were incubated in YEPD liquid culture containing different concentrations of CdCl_2 (0.05–5.0 mmol L^{-1}). For other combination treatments, selected antagonists including 0.5 mmol L^{-1} AsA (ascorbic acid), 500 $\text{U}\cdot\text{mL}^{-1}$ CAT (catalase), 0.5 mmol L^{-1} Ca^{2+} chelator EGTA (ethylene glycol tetraacetic acid), and 0.5 mmol L^{-1} Ca^{2+} channel inhibitor LaCl_3 were respectively added to YEPD liquid medium in the presence of 0.5 or 5.0 mmol L^{-1} CdCl_2 . After 6 h of treatment, cell viability was measured by methylene blue staining method as described by Wu et al. (2013). The stained cells were examined by a Leica inverted fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Detection of ROS and Ca^{2+} levels, and mitochondrial membrane potential ($\Delta\Psi_m$)

Intracellular ROS levels in yeast cells were detected using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) according to the methods described by Wu et al. (2013). After exposure to chemicals, yeast cells were incubated in DCFH-DA at a final concentration of 5 $\mu\text{mol}\cdot\text{L}^{-1}$ at 30 °C for 30 min in the dark. For determination of intracellular Ca^{2+} levels, a fluorescent calcium indicator Fluo-3 acetomethoxyester (Fluo-3 AM) was used. After treatment, yeast cells were incubated in PBS, pH 7.4, at 30 °C for 50 min with 5 $\mu\text{mol}\cdot\text{L}^{-1}$ Fluo-3 AM. Mitochondrial $\Delta\Psi_m$ was detected as described by Shen et al. (2014) with some modification. The cells were incubated with RH-123 (10 $\mu\text{g}\cdot\text{mL}^{-1}$ final concentration) at 30 °C for 30 min in the dark, and then re-suspended in PBS. The levels of intracellular ROS and Ca^{2+} , and $\Delta\Psi_m$ were analyzed using a fluorescence-activated cell sorter (FACS) Calibur (Becton Dickinson) and a Leica inverted fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany) with 488 nm excitation and 515 nm bandpass filter (I3). Fifty thousand cells were measured per sample.

Statistical analysis

Data were calculated as the means of results from at least three independent experiments. The data were subjected to analysis of variance (ANOVA). Significant differences between the means were determined by Duncan's multiple range test.

Results

Effect of cadmium on yeast cell growth

Optical density at 600 nm was measured to investigate the effect of cadmium exposure on yeast cell growth. The results showed that a negative correlation existed between the cell density and cadmium concentration. At the concentration of 0.05 mmol L⁻¹, cadmium exposure had no significant effect on the cell density (Fig. 1a). The cell density decreased with increasing cadmium concentration in a range of 0.25–5.0 mmol L⁻¹ (Fig. 1a). No increase in cell density was observed in yeast cells after exposure to 5.0 mmol L⁻¹ cadmium (Fig. 1a), and the inhibition rate at 24 h reached about 90% (Fig. 1b). These results indicated that cadmium could inhibit cell growth, and the inhibitory effect was positively correlated with cadmium concentrations.

Cadmium induced cell killing

As shown in Fig. 2, cadmium induced yeast cell killing after exposure to 0.05–5.0 mmol L⁻¹ cadmium for 3–24 h, and the cell killing rate increased with increasing cadmium concentration and exposure time. When cells were exposed to 0.05 mmol L⁻¹ cadmium for 3–24 h, no significant cell killing was observed. There was also no obvious cell killing when yeast cells were exposed to 0.25 mmol L⁻¹ cadmium for 3–6 h, but for the longer term (9–24 h), cell killing rate increased significantly as compared to the control group. However, exposure to 5.0 mmol L⁻¹ cadmium for a short term (3 h) caused significant cell killing, the killing rate reached about 60% after 24 h exposure. These results indicated that higher concentrations of cadmium or long-term exposure

time could induce cytotoxicity in yeast cells, and the toxic effect occurred basically in a time- and dose- dependent manner.

Cadmium induced intracellular ROS elevation

As shown in Fig. 3, intracellular ROS levels increased after cadmium exposure, and significant differences were observed between the control and treatment group after exposure to 5.0 mmol L⁻¹ cadmium for 6 h. When ROS scavengers (0.5 mmol L⁻¹ AsA or 500 U·mL⁻¹ CAT) were used simultaneously with 0.5 mmol L⁻¹ or 5.0 mmol L⁻¹ cadmium, cell killing induced by cadmium was effectively blocked. These results showed a positive relationship between intracellular ROS levels and cell killing, suggesting an important role of ROS in cadmium-induced yeast cell killing.

Cadmium induced intracellular Ca²⁺ elevation

To assess whether intracellular Ca²⁺ accumulation regulates cadmium-induced cell killing, the fluorescence intensity of Fluo-3 AM in yeast cells was investigated in two independent experiments. The results showed that the relative fluorescence intensity of intracellular Ca²⁺ obviously increased in yeast cells after exposure to 5.0 mmol L⁻¹ cadmium for 6 h (Fig. 4i). There was a 2.3-fold increase in Ca²⁺ levels in 5.0 mM cadmium treatment group as compared to the control (Fig. 4i). When yeast cells were incubated in 0.05 mmol L⁻¹ or 5.0 mmol L⁻¹ cadmium simultaneously with 0.5 mmol L⁻¹ EGTA (Ca²⁺ chelator) or 0.5 mmol L⁻¹ LaCl₃ (a calcium channel blocker), cadmium-induced cell killing was effectively blocked (Fig. 4iii), associated with a significant decrease in Flou-3 AM fluorescence signal of yeast cells (Fig. 4ii). These results indicated that cadmium-induced cell killing was associated with increased intracellular Ca²⁺ levels.

149 **Cadmium decreased mitochondrial membrane potential ($\Delta\Psi_m$)**

150 Since mitochondria are the major site of ATP production and mitochondrial
151 membrane potential is the driving force of ATP synthesis, we examined the
152 mitochondrial membrane potential ($\Delta\Psi_m$). Our results showed that after exposure to 5.0
153 mmol L⁻¹ cadmium for 6 h, the relative fluorescence intensity within yeast cells was
154 58.5% lower as compared to the control (Fig. 5i). The same trend was observed in the
155 fluorescence microscope assay (Fig. 5ii). The results therefore indicated that cadmium
156 could induce mitochondrial dysfunction in these yeast cells.

157

158 **Discussion**

159 Cadmium is a well-known human carcinogen. A series of adverse health effects,
160 such as bone fracture, renal dysfunction, hypertension, arteriosclerosis, growth
161 inhibition and chronic diseases of old age can happen after a prolonged exposure of
162 cadmium (Krivosheev et al. 2012). However, the exact mechanism of cadmium-induced
163 toxicity is not clear. As a sensitive and repeatable system (Wu et al. 2013), yeast cells
164 were used to discover the potential mechanisms underlying cadmium toxicity in the
165 present study.

166 In this study, we found that a high concentration of cadmium could markedly
167 inhibit cell growth and cause cell killing (Figs 1, 2). Increasing evidences indicated that
168 cadmium-induced cytotoxicity was due to ROS induction and oxidative stress (Chen et
169 al. 2011; Yang et al. 2008). In yeast cells, it was reported that excessive ROS
170 production could lead to free radical attack of membrane phospholipids (Ott et al. 2007),
171 modify proteins and DNA, activate related signaling pathways (Chen et al. 2011), and
172 eventually induce cell death (Perrone et al. 2008). Our results showed that the level of
173 intracellular ROS significantly increased after 6 h of 5.0 mmol L⁻¹ cadmium exposure
174 (Fig. 3), which is consistent with the earlier report in human neuroblastoma and rat

175 pheochromocytoma exposed to cadmium (Chen et al. 2011; Xu et al. 2011). To
176 determine whether ROS played a role in the cadmium-induced cell death, we also
177 applied antioxidants AsA and CAT to the yeast cells followed by analysis of cell killing
178 rate. Our results clearly showed that both ASA and CAT could significantly inhibit ROS
179 production and correspondingly reduce the rate of cadmium-induced yeast cell killing
180 (Fig. 3), indicating that intracellular ROS burst was required for cadmium-induced cell
181 killing. Similar results were also observed in the arsenite-induced yeast cell death (Wu
182 et al. 2013).

183 Ca^{2+} is widely considered as a central regulating signal in cell death of animals and
184 plants (Berridge et al. 2003; Clapham 2007; Sun et al. 2012). In this study, cadmium
185 induced a marked Ca^{2+} elevation within yeast cells (Fig. 4). This result was consistent
186 with Wu et al. (2013), who observed increased Ca^{2+} accumulation in arsenite-stressed
187 yeast cells. In our study, when yeast cells were incubated with cadmium (0.5 and 5.0
188 mmol L^{-1}) in the presence of either a Ca^{2+} chelator (EGTA) or a Ca^{2+} channel blocker
189 (LaCl_3) that could suppress intracellular Ca^{2+} elevation, cadmium-induced cell killing
190 markedly decreased (Fig. 4). These results indicated that cadmium-caused cell killing
191 was associated with intracellular Ca^{2+} elevation. ROS-triggered Ca^{2+} influx was
192 previously reported in various types of cells (Xu et al. 2011, 2013; Sun et al. 2012; Yan
193 et al. 2012). We speculated that high level of intracellular ROS evoked by cadmium
194 might activate the plasma membrane Ca^{2+} channel, leading to extracellular Ca^{2+} influx
195 and thus intracellular Ca^{2+} elevation.

196 Numerous studies have shown that mitochondrial function has a role in regulating
197 cell death (Sun et al. 2012; Wu et al. 2013). To confirm the role of mitochondrial
198 dysfunction in the cadmium-induced yeast cell death, we examined mitochondrial
199 membrane potential ($\Delta\Psi\text{m}$). Our results revealed that the decrease of $\Delta\Psi\text{m}$ did occur
200 when yeast cells were exposed to cadmium for 6 h (Fig. 5). These results were in accord

with previous studies, in which cadmium could induce significant inhibition of mitochondrial function, increased ROS production, and eventual death in human and animal cells (Lee et al. 2005; Oh and Lim 2006). It was speculated that an elevated ROS could destroy the construction of mitochondrial membrane, leading to mitochondrial membrane potential loss, related signaling activation, and eventual cell killing. However, the exact interactions among ROS, Ca^{2+} , and mitochondrial dysfunction during cadmium-induced cell death remain unclear and require further studies.

In conclusion, we identified the toxic effect of cadmium on yeast cells. Cadmium treatment can elevate intracellular ROS and Ca^{2+} accumulation, and promote mitochondrial dysfunction (Fig. 6). ROS and Ca^{2+} are two important signals regulating cell killing in yeast.

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318 **Figure legends**

320 **Fig.1** (a) Growth curves of yeast cells in liquid YEPD medium containing different

321 concentrations of cadmium. OD values were determined at 2-h intervals over 24-h

322 period. (b) Inhibition rate was calculated as 1 minus the relative OD₆₀₀ (the OD₆₀₀ value

323 of treated cells divided by that of untreated cells).

325 **Fig.2** Viability assays of yeast cells exposed to cadmium. a and b indicate significant

326 differences (^a $P < 0.05$, ^b $P < 0.01$) between the control and cadmium treatment groups.

328 **Fig.3** (i) DCFH-DA fluorescence intensity, indicating the ROS level in yeast cells

329 measured by flow cytometric analysis after 6 h of cadmium (5.0 mmol L⁻¹) exposure.

330 The asterisk indicates significant difference between control and cadmium treatment. (ii)

331 The effect of ASA and CAT on cadmium-induced ROS accumulation in yeast cells. The
332 green fluorescence of DCFH-DA within yeast cells was detected using fluorescence
333 microscopy. (a and a') control; (b and b') cadmium treatment; (c and c') combination
334 treatment of 5.0 mmol L⁻¹ cadmium and 0.5 mmol L⁻¹ AsA; (d and d') combination
335 treatment of 5.0 mmol L⁻¹ cadmium and 500 U·mL⁻¹ CAT. (iii) The effect of ASA or
336 CAT on cadmium-induced cell killing rate. Yeast cells were exposed to 0.5 or 5.0 mmol
337 L⁻¹ cadmium in the presence of 0.5 mmol L⁻¹ AsA or 500 U·mL⁻¹ CAT. The letters a and
338 b indicate the significant difference (^a*P*<0.05, ^b*P*<0.01) between the control and
339 cadmium treatment groups; c and d indicate the significant difference (^c*P*<0.05, ^d*P*<0.01)
340 between the cadmium treatment groups and combination treatment groups.

341

342 **Fig. 4** Fluo-3 AM fluorescence intensity, indicating intracellular Ca²⁺ level, measured
343 by flow cytometric analysis after 6 h of cadmium (5.0 mmol L⁻¹) exposure. The asterisk
344 indicates significant difference between control and cadmium treatment. (ii) The effect
345 of EGTA and LaCl₃ on cadmium-induced Ca²⁺ accumulation in yeast cells. The green
346 fluorescence of Fluo-3 AM within yeast cells was detected using fluorescence
347 microscopy. (a and a') control; (b and b') cadmium treatment; (c and c') combination
348 treatment of 5.0 mmol L⁻¹ cadmium and 0.5 mmol L⁻¹ EGTA; (d and d') combination
349 treatment of 5.0 mmol L⁻¹ cadmium and 0.5 mmol L⁻¹ LaCl₃. (iii) The effect of EGTA
350 or LaCl₃ on cadmium-induced cell killing rate. Yeast cells were exposed to 0.5 or 5.0
351 mmol L⁻¹ cadmium in the presence of 0.5 mmol L⁻¹ EGTA or 0.5 mmol L⁻¹ LaCl₃. The
352 letters a and b indicate the significant difference (^a*P*<0.05, ^b*P*<0.01) between the control
353 and cadmium treatment groups; c and d indicate the significant difference (^c*P*<0.05,
354 ^d*P*<0.01) between the cadmium treatment groups and combination treatment groups.

355

356 **Fig. 5** RH-123 fluorescence intensity, indicating the mitochondrial membrane potential

357 ($\Delta\Psi_m$), measured by flow cytometric analysis after 6 h of cadmium (5.0 mmol L^{-1})
358 exposure. The asterisk indicates significant difference between control and cadmium
359 treatment. (ii) The green fluorescence of RH-123 within yeast cells was detected using
360 fluorescence microscopy after 6 h of cadmium exposure. (a and a') control; (b and b')
361 5.0 mmol L^{-1} cadmium treatment.

362

363 **Fig. 6** A schematic model showing the signal regulations of ROS and Ca^{2+} , as well as
364 mitochondrial dysfunction during cadmium-induced yeast cell death.

365

Draft

60x22mm (300 x 300 DPI)

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70x51mm (300 x 300 DPI)

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