Letter

**Tributyltin induces Yca1p-dependent cell death of yeast Saccharomyces cerevisiae**

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(Received May 15, 2009; Accepted May 25, 2009)

**ABSTRACT** — Tributyltin chloride (TBT), an environmental pollutant, is toxic to a variety of eukaryotic and prokaryotic organisms. Although it has been reported that TBT induces apoptotic cell death in mammalian, the action of TBT on eukaryotic microorganisms has not yet been fully investigated. In this study we examined the mechanism involved in cell death caused by TBT exposure in *Saccharomyces cerevisiae*. The median lethal concentration of TBT was 10 µM for the parent strain BY4741 and 3 µM for the pdr5Δ mutant defective in a major multidrug transporter, respectively. Fluorescence microscopic observations revealed nuclear condensation and chromatin fragmentation in cells treated with TBT indicating that cells underwent an apoptosis-like cell death. TBT-induced cell death was suppressed by deletion of the *ycal* gene encoding a homologue of the mammalian caspase. In parallel, reactive oxygen species (ROS) were produced by TBT. These results suggest that TBT induces apoptosis-like cell death in yeast via an Yca1p-dependent pathway possibly downstream of the ROS production. This is the first report on TBT-induced apoptotic cell death in yeast.

**Key words:** Tributyltin, Apoptosis, Reactive oxygen species, Pdr5, Yca1, *Saccharomyces cerevisiae*

**INTRODUCTION**

Tributyltin chloride (TBT) is a widespread environmental pollutant. TBT has several uses, such as wood preservation and antifouling paints for marine vessels. It is also used as a biocide in cooling system and as an organometallic chemical in diverse applications (Antizar-Ladislao, 2008). The use and production are now strictly regulated, but TBT and its degradation products will persist in aquatic sediments causing widespread contamination of the environment (Iwata et al., 1995; Tanabe et al., 1998). TBT is toxic to mammalian species, causing diseases in various organs as well as in the nervous, endocrine and immune systems (Antizar-Ladislao, 2008). Many studies have demonstrated that TBT induced apoptosis, programmed cell death, on a variety of cell types (such as thymocytes, neuronal cells and hepatocytes) (Aw et al., 1990; Thompson et al., 1996; Grondin et al., 2007). Apoptotic cell death is triggered by extrinsic inducers and mediated by a change in the intracellular calcium level followed by production of reactive oxygen species (ROS). A group of caspases (cysteine proteases) is strictly associated to the apoptosis pathway via the regulation of mitochondrial function. The details of signaling pathway for TBT-dependent apoptosis are now under investigation (Gennari et al., 2000).

In order to evaluate the fate relative to the TBT pollution in the contaminated environment, the examination of the interaction (such as bioaccumulation, toxicity and detoxification) of this compound in the microbial populations is necessary (White et al., 1999; Gadd, 2000). TBT is harmful to eukaryotic microorganisms such as fungi; studies were performed with yeast such as marine yeast *Debaryomyces Hansenii* (Oliver et al., 1989), *Rhodotorula ferulica* (Veiga et al., 1997), *Saccharomyces cerevisiae* (Masia et al., 1998; Golin et al., 2000) and *Candida maltosa* (White and Tobin, 2004). In these papers, TBT-induced cellular K⁺ leakage as well as a defect in ATP

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production has been reported, but the effect of TBT on the cell viability, as studied in mammalian cells, has not been investigated. Difference in the effect of TBT on cell growth and cell death has not been characterized in yeast. Apoptosis-like cell death is known in *S. cerevisiae* (Mazzone and Falcone, 2008). Based on the reverse genetics, *S. cerevisiae* will be an organism helpful for understanding cell toxicity induced by TBT exposure. In this study we examined TBT-induced cell death of *S. cerevisiae*.

**MATERIALS AND METHODS**

**Yeast strains and growth medium**

*S. cerevisiae* strains used in this study were the laboratory strain BY4741 (*MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*) and its isogenic knockout mutants: *pdr5Δ* mutant (*pdr5Δ::kanMX*) and *ycalΔ* mutant (*ycal::kanMX*), gifted by Dr. Yoshinori Ohsumi, Tokyo Institute of Technology. Yeast cells were grown aerobically at 30°C in YPD medium (1% Bacto-yeast extract, 2% Bacto-peptone, and 2% dextrose).

**Cell death assessment**

Yeast cells were grown to the mid-exponential phase in YPD medium. The cells were harvested and then resuspended in YPD medium and treated with tributyltin chloride (SUPELCO, Sigma-Aldrich, St. Louis, MO, USA) at various concentrations. As control, cells were treated with dimethylsulfoxide (DMSO) at 0.1% in similar conditions. After 2 hr of incubation at 30°C, cells were harvested, plated on YPD agar and incubated 2 days at 30°C. The survival rates were calculated based on the number of colonies observed after TBT treatment in relation with the number of colonies obtained with DMSO treatment as control. All assays were repeated three times at each point and the data were averaged. The lethal TBT concentration inducing 50% of cell death (LC50) was calculated based on these results.

**Fluorescence microscopic observation of nuclear condensation, chromatin fragmentation and ROS production**

To detect chromatin condensation and DNA fragmentation, samples were stained with 4',6-diamidino-2-phenylindole (DAPI). Yeast cells were treated with 0.1% DMSO or 10 μM TBT for 2 hr, fixed with 3.7% formaldehyde for 30 min, and washed twice with phosphate buffered saline (PBS). After incubation with DAPI (1 μg/ml) for 10 min, cells were washed with PBS. Fluorescence was examined by fluorescent microscopy (DMRXA, Leica, Wetzlar, Germany). The detection of DNA strand breaks was performed by terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL) test using *in situ* Cell Death Detection Kit, AP (Roche Diagnostics, Rotkreuz, Switzerland) according to the manufacturer’s instructions. Cells were prepared on the slide glass as described by Fahrenkrog et al. (2004). After TUNEL reaction, the incorporation of fluorescein was detected by anti-fluorescein antibody conjugated with alkaline phosphatase and stained with BM purple AP substrate precipitating (Roche Diagnostics). To detect ROS production, cells were harvested at mid-exponential phase and were incubated in YPD with 10 μg/ml 2',7'-dichlorofluorescin diacetate (H2DCFH-DA; Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 30°C. The cells were then rinsed once with YPD and resuspended in YPD containing 5 μM of TBT. After incubation for 30 min at 30°C, cells were harvested, washed twice with 1ml of PBS. ROS production was observed by fluorescent microscopy and the fluorescent intensity was measured by spectrofluorimeter (FL2500, Hitachi, Tokyo, Japan) at the excitation wavelengths of 475 nm and emission wavelengths of 525 nm, respectively.

**RESULTS AND DISCUSSION**

Fig. 1 shows the effect of TBT on cell viability (index for cell death) in *S. cerevisiae*. LC50 of the parent strain BY4741 was estimated to be about 10 μM. Pdr5p is a major multidrug transporter among members of the ATP-binding cassette transporter superfamily (Balzi et al., 1994). It has been reported that Pdr5p is involved in the TBT sensitivity of the growth of *S. cerevisiae* (Golin et al., 2000). TBT-induced cell death was also affected by the function of Pdr5p; LC50 of TBT in the *pdr5Δ* mutant was 3 μM. Thus, the accumulation of TBT into *pdr5Δ* mutant cells caused a higher percentage of cell death. It should be mentioned that the median inhibitory concentration of TBT (IC50) recorded for cell growth was 1 μM in BY4741 and 0.2 μM in *pdr5Δ* mutant, respectively (data not shown). Cell growth was completely arrested by 5 μM of TBT in the parent strain and 1 μM in the *pdr5Δ* mutant, but only few cell death were observed at these concentrations (Fig. 1).

To assess the hallmarks of the cell death induced by TBT exposure in *S. cerevisiae*, we examined the alterations of the DNA. First, we observed nuclei fragmentation by DAPI staining. As shown in Fig. 2A, whereas the nuclei of the control cells appeared to be rounded (a single round spot) (Fig. 2A, b), most of the cells treated with 10 μM of TBT showed abnormal nuclear morphology: the appearance of multiple DAPI-stained regions...
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Fig. 1. Effect of TBT on cell survival of S. cerevisiae.
S. cerevisiae cells (open circles, parent; closed circles, pdr5Δ) were harvested at mid-exponential phase, treated with TBT at various concentrations indicated for 2 hr, spread on YPD agar plate and then incubated at 30°C for 2 days. Survival rates were calculated as the number of colonies compared with those treated with DMSO as control. n = 3.

Fig. 2. Fluorescence microscopic observation of apoptosis-linked cellular incidents.
Effect of TBT on fragmentation of nuclei (A) and chromatin (B). The parent cells were treated with DMSO (A, a and b; B, a) or 10 μM of TBT (A, c and d; B, b) for 2 hr and DAPI staining (A) and TUNEL (B) were performed as described in Materials and Methods. A: a and c, phase contrast; B: b and d, fluorescence. Effect of TBT on ROS production (C). Cells were incubated with H2DCFH-DA and treated with DMSO (C, a and b) or 5 μM of TBT (C, c and d). ROS production was detected by fluorescent microscopy and fluorescent intensity was quantified by fluorimeter (e).
within a single cell (Fig. 2A, d). An increased in chromatin fragmentation occurred in parallel with the increased number of dead cell detected by TUNEL assays (Fig. 2B). TUNEL positive cells were abundant in the group treated with 10 μM of TBT during 2 hr (Fig. 2B, b). In the control group, most of the untreated cells were not stained by TUNEL (Fig. 2B, a). These results suggest that TBT induced apoptosis in yeast.

Caspases are a group of cysteine proteases responsible for apoptosis in mammalian cells. Yca1p is so far the only mammalian caspase homologue identified in S. cerevisiae, which is induced by several intrinsic and extrinsic factors (Mazzoni and Falcone, 2008). Recently it has been reported that formic acid induces Yca1p-independent apoptosis pathway in S. cerevisiae (Du et al., 2008); a new caspase, different from Yca1p, might be involved in S. cerevisiae. To assess whether TBT-induced cell death is Yca1p-dependent or not, we compared the rate of cell death caused by TBT exposure in the parent strain and in the yca1Δ mutant strain under different concentrations of TBT from 7.5 μM to 60 μM (Fig. 3). The results indicated that cell death induced by TBT was suppressed by deletion of the yca1 gene at all the concentrations tested, suggesting the involvement of Yca1p caspase signaling pathway in apoptosis triggered by TBT.

ROS play a pivotal role in induction of apoptotic cell death in mammalian and yeast cells. The high production level of ROS is regarded as an important marker of apoptosis. An elevation of ROS production was observed during TBT-induced apoptosis in several mammalian cells (Gennari et al., 2000; Nakatsu et al., 2007). We examined TBT-induced ROS production using H₂DCFH-DA staining and fluorescence microscopy (Fig. 2C). An intensive green fluorescence was observed in the parent cells treated with 5 μM of TBT (Fig. 2C, d), signaling the production of ROS, whereas few ROS were detected in the control groups (Fig. 2C, b). ROS production was confirmed by quantification of the specific fluorescence in the cell suspension using a fluorimeter. Fluorescence increased in TBT-treated cells compared with the non-treated cells (Fig. 2C, e). These results indicate that TBT exposure induces apoptotic cell death in yeast as well as various mammalian cells via the Yca1p caspase-dependent pathway probably downstream of ROS production. Apoptotic cell death has been extensively studied in mammalian cells. However, the signaling pathway of the apoptotic cell death induced by TBT exposure was not well defined. Our finding in TBT-induced apoptosis-like cell death of S. cerevisiae will be valuable for better understanding the precise molecular process triggered by TBT based on reverse genetics.

ACKNOWLEDGMENTS

We thank Dr. Gersende Maugars, Ehime University, for helpful discussion and M. Hyodo and R. Umeyama, Ehime University, for their technical assistance. This work was supported by Global COE Program “Interdisciplinary Studies on Environmental Chemistry” (to Ehime University) by the Ministry of Education, Culture, Sports, Cultural Affairs, Sports, Science and Technology, Japan.

Fig. 3. Role of Yca1p in TBT-induced cell death. S. cerevisiae cells (open bars, parent; closed bars, yca1Δ) were harvested at mid-exponential phase, treated with TBT at concentrations of 7.5, 15, 30 or 60 μM for 2 hr, spread on YPD agar plate and then incubated at 30°C for 2 days. Survival rates were calculated as the number of colonies compared with those treated with DMSO as control. n = 3.

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Science, and Technology (MEXT), Japan.

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