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Tributyltin Induces Apoptosis in R2C via Oxidative Stress and Caspase-3 Activation by Disturbance of Ca^{2+}

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Abstract - Tributyltin (TBT) used world-wide in antifouling paints for ships is a widespread environmental pollutant. At low doses, antiproliferative modes of action have been shown to be involved, whereas at higher doses apoptosis seems to be the mechanism of toxicity in reproductive organs by TBT. In this study, we investigated that the mechanisms underlying apoptosis induced by TBT in R2C cell. Effects of TBT on intracellular Ca^{2+} level and reactive oxygen species (ROS) were investigated in R2C cells by fluorescence detector. TBT significantly induced intracellular Ca^{2+} level in a time-dependent manner. The rise in intracellular Ca^{2+} level was followed by a time-dependent generation of reactive oxygen species (ROS) at the cytosol level. Simultaneously, TBT induced the release of cytochrome c from the mitochondrial membrane into the cytosol. Furthermore, ROS production and the release of cytochrome c were reduced by BAPTA, an intracellular Ca^{2+} chelator, indicating the important role of Ca^{2+} in R2C during these early intracellular events. In addition, Z-DEVD FMK, a caspase-3 inhibitor, decreased apoptosis by TBT. Taken together, the present results indicated that the apoptotic pathway by TBT might start with an increase in intracellular Ca^{2+} level, continues with release of ROS and cytochrome c from mitochondria, activation of caspases, and finally results in DNA fragmentation.

Key words : TBT, R2C, ROS, intracellular Ca^{2+}, caspase activity

INTRODUCTION

Organotin compounds such as tributyltin (TBT) are widely used as agricultural biocides, and for antifouling paint of ship bottoms and of fishing nets. TBT is also recognized as an endocrine disrupter. At low concentrations (less than 500 nM), TBT effectively inhibit DNA synthesis and disrupt mitochondrial energy metabolism (Snoeij *et al.* 1986). At higher concentrations (1~5 μM), TBT is known to induce apoptosis (Gennari *et al.* 1997). The mechanistic studies have already linked a TBT-induced sustained increase in the cytosolic-free Ca^{2+} concentration to a subsequent endonuclease activation and DNA fragmentation (Chow *et al.* 1992). Many of the chemical and physical treatments capable of inducing apoptosis are also associated with oxidative stress, suggesting an active role for reactive oxygen species (ROS) in cell death (Buttke and Sandstom 1994). An important intracellular source of ROS is mitochondria. TBT compounds are well known to disturb mitochondrial activity inhibiting ATP synthesis (Marinovich *et al.* 1990). It has been suggested that TBT could also affect oxidative phosphorylation of mitochondria and demonstrated that alterations of Ca^{2+} homeostasis precede TBT-induced ROS production at the mitochondrial level in murine

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keratinocytes. Furthermore, targeting of mitochondria by TBT has been shown capable of releasing proapoptotic factors, such as cytochrome c, which is considered a primary event in the induction of DNA fragmentation (Kroemer 1997). However, mechanistic information of TBT-induced apoptotic process was still not well elicted. In the present study, we investigated the apoptotic pathway elicited by TBT in R2C cell.

MATERIALS AND METHODS

1. Chemicals

TBT, Fura-2/AM and propidium iodide (PI) were obtained from Sigma-Aldrich Chemical Co., Inc. (Milwaukee, WI) and 6-carboxy-29, 79-dichlorodihydrofluorescein diacetate di (acetomethyl ester) (DCFH) and 1, 1-bis (2-aminophenoxy) ethane-N, N, N, N9-tetraacetic acid (BAPTA) were obtained from Molecular Probes (Eugene, OR). Z-DEVD-FMK (mainly a caspase 3 inhibitor) was purchased from Calbiochem (La Jolla, CA) and dissolved in DMSO to obtain the final concentration of 20 mM.

2. Cell culture and treatment

R2C cell was purchased from the American Type Culture Collection and maintained in RPMI-1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin at 37°C in a 5% CO_2 humidified incubator. The cells were incubated with different concentrations of TBT dissolved in ethanol (EtOH) or with EtOH as vehicle control (the final EtOH concentration was 0.1%, which is ineffective by itself). For measurement of intracellular Ca^{2+} and oxidative activity, The R2C cells were incubated with different concentrations of TBT. To calculate the release of cytochrome c, R2C cells were treated with 3 mM of TBT for 5 min. To detect apoptosis, the cells were treated with 500 nM of TBT for 10 min and then were washed and incubated overnight at 37°C without compounds. When necessary, cells were pretreated 30 min with BAPTA (10 μM, in DMSO) or 30 min with Z-DEVD-FMK (20 μM, in DMSO) before incubation with TBT.

3. Apoptosis detection

DNA fragmentation assay was carried out according to the method of Miller *et al.* (1988).

Detection of apoptosis by flow cytometry with propidium iodide (PI) as fluorescence indicator was done essentially (Nicoletti *et al.* 1991). Briefly, after incubation with the test compounds, 5×10^6 cells ml^{-1} were centrifuged and resuspended in 1 ml PBS. Of this suspension, 200 μl was incubated for 30 min with 0.5 ml RNase (0.5 mg ml^{-1}) at room temperature, 0.5 ml PI was then added (5 mg ml^{-1}, in PBS) and the fluorescence of individual nuclei was measured using FACScan flow cytometry (Becton-Dickinson, Korea).

4. Intracellular production of reactive oxygen species

The fluorescent probe dichlorodihydrofluorescein diacetate was used to monitor the intracellular generation of reactive oxygen species by H_2O_2 (Benov *et al.* 1998).

5. Determination of Ca^{2+} level

R2C cells (5×10^6 cells ml^{-1}) were loaded with 4 mM Fura-2/AM in buffer (Krebs-Henseleit buffer supplemented with 2% BSA) for 30 min at room temperature. Cells were then washed and resuspended in buffer A without Fura-2/AM for another 15 min, to allow complete hydrolysis of Fura-2/AM using FACScan flow cytometry (Becton-Dickinson, Korea).

6. Cytochrome c release

R2C cells were washed once in 5 ml ice-cold phosphate buffered saline. Cells were then centrifuged and the pellet was resuspended in 200 μl of ice-cold buffer (20 mM Hepes, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 0.1 mM PMSF) supplemented with protease inhibitors (1 : 50 protease inhibitor cocktail, Sigma). After being kept on ice for 15 min, cells were lysed by passing 15 times through a G22 needle. After centrifugation in a microcentrifuge for 5 min at 4°C, the supernatants were further centrifuged at 4×10^4 g for 30 min at 4°C in a table top ultracentrifuge.

7.Western blotting

Protein extracts (20 μg) were loaded onto a 18% SDS-polyacrylamide gel and electrophoresed at 120 V and then transferred to PVDF membranes at 250 mA for 1 h. Membranes were blocked in 5% nonfat dried milk and then incubated with a rabbit anti-cytochrome c polyclonal IgG antibody (1 : 1000 diluted in 5% nonfat dried milk, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C, followed by an incubation with an anti-rabbit IgG alkaline phosphate (AP)-conjugate antibody (1 : 50,000 diluted in 5% nonfat dried milk, Sigma) for 1 h at room temperature, and visualized by CDP-star (NEN). The Western blot image was acquired with a Nikon video camera module (Nikon, Melville, NY). The optical density of the bands was calculated, and peak area of a given band was analyzed by means of the Image 1.61.

8. Statistics

Statistical analysis was determined by Student's t test. Each experiment was performed at least three times, with representative results shown.

RESULTS AND DISCUSSION

In the present study, we confirmed the appearance of apoptotic process in rat leydig cell line R2C by molecular biological Techniques (Fig. 1). We demonstrate the important role of Ca^{2+} and mitochondria during apoptosis induced by TBT in R2C. Previously, it has been shown that one of the early events in the apoptotic cell death induced by TBT was the rise in intracellular Ca^{2+} concentration in the hepatoma cell (Aw *et al.* 1990). We have found that TBT also is able to increase the Ca^{2+}, at low concentrations (Fig. 2A). So, we hypothesized that a disturbance of the Ca^{2+} homeostasis may initiate TBT-induced oxidative stress in R2C as well. High cytoplasmic Ca^{2+} levels can cause an increased mitochondrial Ca^{2+} uptake and disruption of mitochondrial Ca^{2+} equilibrium, which results in ROS formation due to stimulation of electron flux along the electron transport chain (Chacon and Acosta 1991). Indeed, TBT induced generation of ROS in a time-dependent manner

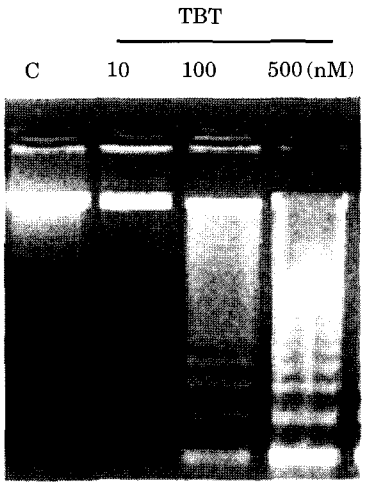

Fig. 1. Detection of internucleosomal DNA fragmentation of R2C after 24 h of treatment with TBT using gel electrophoresis. Abbreviation : C, control; TBT, tributyltin.

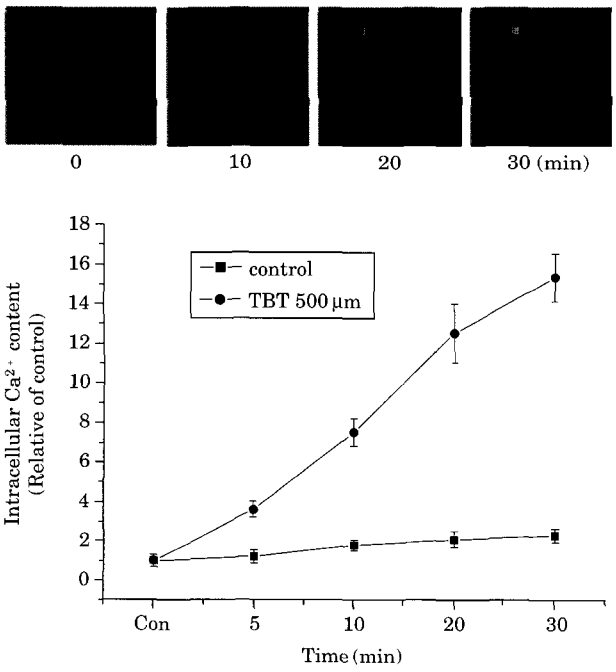

Fig. 2. TBT induced a dose-dependent increase in the intercellular Ca^{2+} in R2C Cells. The cells were loaded with Fura-2 and then were incubated with TBT (500 nM). Intracellular Ca^{2+} were measured using fluorescence microscopy. Each value represents the mean±SD of three experiments. Abbreviation : Con, control; TBT, tributyltin.

(Fig. 2B). To correlate Ca^{2+} and mitochondria in ROS release, R2C were pretreated with the Ca^{2+} chelator,

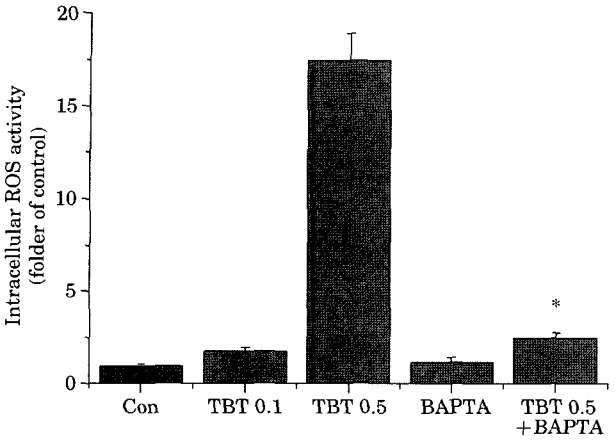

Fig. 3. BAPTA prevented ROS production in R2C treated with TBT. R2C cells were first treated for 30 min with 10 μM BAPTA and then TBT was added. ROS was measured 15 min after TBT treatment. Each value represents the mean±S.D. of three experiments. *p, 0.05 versus cells treated with TBT. Abbreviation : Con, control; TBT, tributyltin.

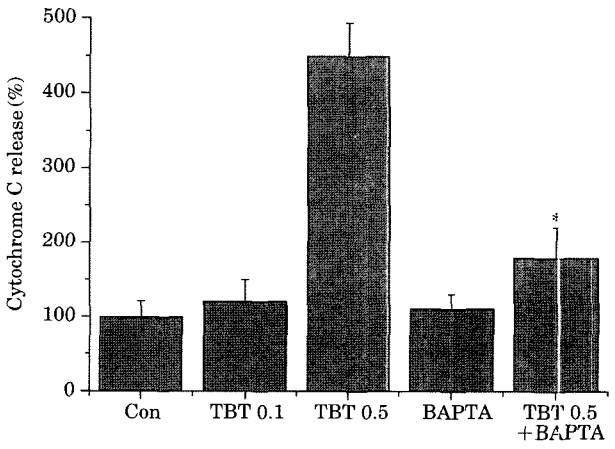

Fig. 4. TBT induces a rapid cytochrome c release. R2C cells were first treated for 30 min with 10 μM BAPTA and then 500 nM TBT was added for 5 min. The presence of cytochrom c (12 kDa) in cytoplasmic extract was measured by western blot with an anti-cytochrome c antibody. Abbreviation : Con, control; TBT, tributyltin.

BAPTA. BAPTA treatments significantly reduced TBT-induced production of ROS in R2C (Fig. 3). The presence of this inhibitor resulted in a significant Ca^{2+} reduction of TBT-induced oxidative activity, indicating that the uptake of Ca^{2+} at the mitochondrial level is necessary for the generation of ROS induced by TBT.

It is known that mitochondrial cytochrome c release from the inner membrane into the cytosol is a common early event in the induction of apoptosis by multiple agents and that cytochrome c release is linked to caspase activation and subsequent DNA fragmentation (Stridh *et al.* 1998). Previous study demonstrated that TBT also was able to induce the release of cytochrome c. The addition of BAPTA to the cells before the TBT modulated cytochrome c release, suggesting that the increase of intracellular Ca^{2+}, ROS release, and transport of cytochrome c into the cytosol are early and functionally correlated events in the pathway leading to DNA fragmentation induced by TBT. Our results, based on the blocking of increase of intracellular Ca^{2+} in the presence of BAPTA, led us to the proposal that ROS production precedes cytochrome c release (Fig. 4).The subsequent step was performed to evaluate the possible involvement of caspases during DNA fragmentation, knowing that a link between release of cytochrome c and activation of caspase-3 by TBT. Caspase-3 was activated by TBT in

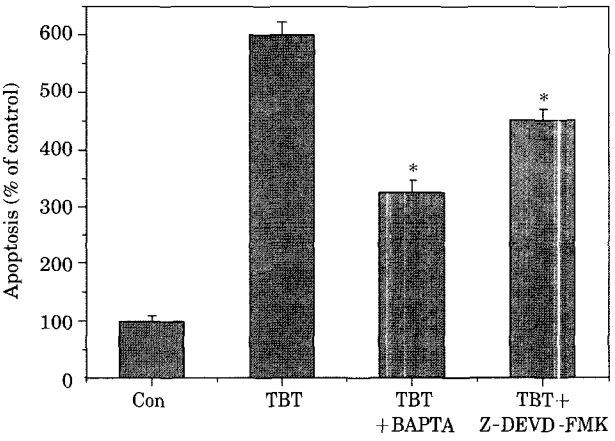

Fig. 5. TBT-induced apoptosis reduced by BAPTA and Z-DEVD FMK. R2C cells were first treated for 30 min with BAPTA (10 μM) or Z-DEVD FMK (20 μM), and then 500 nM TBT was added. Flow cytometry (propidium iodide stained) was used to quantify apoptosis. The amount of apoptotic nuclei is indicated as percentage of relative control. Values are mean±S.D. of three experiments. *p, 0.05 versus cells treated with TBT. Abbreviation : Con, control; TBT, tributyltin.

R2C cells with dose-dependt manner (data not shown). Furthermore, we used Z-DEVD-FMK to inhibit caspase -3 activation, in order to characterize the apoptotic pathway activated by TBT. As the results, apoptosis induced

by TBT was down-regulated by a 30-min pretreatment of the cells with Z-DEVD-FMK (Fig. 5). Present data indicate that inhibition of caspase-3 reduced the extent of TBT-induced apoptosis, confirming also for this compound the crucial role of the caspase family in the activation of apoptotic cell death. Thus, we conclude that TBT initiate an increase of Ca^{2+}, causing the generation of ROS and release of cytochrome c by mitochondria.

As a result, actived caspases-3 was leaded to an irreversible apoptotic damage of the R2C cell. and the influx of Ca^{2+} may be caused by disruption of membrane or cytoskeletal function.

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REFERENCES

Aw TY, P Nicotera, L Manzo and S Orrenius. 1990. Tributyltin stimulates apoptosis in rat thymocytes. Arch. Biochem. Biophys. 283:46-50.

Benov L, L Sztejnberg and I Fridovich. 1998. Critical evaluation of the use of hydroethidine as a measure of superoxide anion radical. Free Radic. Biol. Med. 25:826-831.

Buttke MT and PA Sandstrom. 1994. Oxidative stress as a mediator of apoptosis. Immunol. Today. 15:7-10.

Chacon E and D Acosta. 1991. Mitochondrial regulation of superoxide by Ca^{2+}: An altered mechanism for the cardiotoxicity of doxorubicin. Toxicol. Appl. Pharmacol. 107:117-128.

Chow SC, GEN Kaas, MJ McCabe and S Orrenius.1992. Tributyltin increases cytosolic free Ca^{2+} concentration in thymocytes by mobilizing intracellular Ca^{2+}, activating a Ca^{2+} entry pathway, and inhibiting Ca^{2+} efflux. Arch. Biochem. Biophys. 298:143-149.

Gennari A, M Potters, W Seinen and RHH Pieters. 1997. Organotin-induced apoptosis as observed *in vitro* is not relevant for induction of thymus atrophy at antiproliferative doses. Toxicol. Appl. Pharmacol. 147:259-266.

Kroemer G. 1997. Mitochondrial implication in apoptosis. Towards an endosymbiotic hypothesis of apoptosis evolution. Cell Death Differ. 4:443-456.

Marinovich M, B Viviani and CL Galli. 1990. Reversibility of TBT-induced protein synthesis inhibition after ATP recovery in HEL30 cells. Toxicol. Lett. 52:311-317.

Miller SA, DD Dykes and HF Polesky. 1988. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 16:121-125.

Nicoletti I, G Migliorati, MC Pagliacci, F Grignani and C Riccardi. 1991. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J. Immunol. Methods. 139:271-279.

Snoeij NJ, PM Punt, AH Penninks and W Seinen. 1986. Effects of tri-*n*-butyltin chloride on energy metabolism, macromolecular synthesis, precursor uptake and cyclic AMP production in isolated rat thymocytes. Biochem. Biophys. Acta 852:234-243.

Stridh H, M Kimland, DP Jones, S Orrenius and MB Hampton. 1998. Cytochrome *c* release and caspase activation in hydrogen peroxide- and tributyltin-induced apoptosis. FEBS Lett. 429:351-355.

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