TRIBUTYLTIN INDUCES APOPTOSIS IN R2C RAT LEYDIG CELLS VIA OXIDATIVE STRESS AND CASPASE-3 ACTIVATION BY DISTURBANCE OF Ca^{2+}

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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 recognized as an endocrine disrupter At low concentrations (less than 500 nM), TBT effectively inhibit macromolecules such as DNA synthesis and disrupt mitochondrial energy metabolism[1]. At higher concentrations (1–5 μM), this is known to induce apoptosis[2]. The mechanistic studies have linked a TBT-induced sustained increase in the cytosolic-free Ca^{2+} concentration to a subsequent endonuclease activation and DNA fragmentation[3].

Many of the chemical and physical treatments capable of inducing apoptosis are associated with oxidative stress, suggesting an active role for reactive oxygen species (ROS) in cell death[4]. An important intracellular source of ROS is mitochondria. TBT compounds are well known to disturb mitochondrial activity inhibiting ATP synthesis[5] and 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 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[6]. However, mechanistic information of TBT-induced apoptotic process was still not well elicited. In the present study, we investigated the apoptotic pathway elicited by TBT in the rat Leydig cell line, R2C.

Methods and Materials

R2C cells were obtained from the American Type Culture Collection. Quantitative DNA fragmentation assay was carried out according to the method of Sellins and Cohen[7]. Cells were treated with TBT for 24 h and then stained with Propidium iodide (PI). The fluorescence of individual nuclei was measured using FACScan flow cytometry (Becton-Dickinson, Korea).

R2C cells were loaded with Fura-2/AM in buffer or DCF-DH in dimethysulfoxide for 30 min at room temperature. The cells were then washed and then the intercellular Ca^{2+} and ROS were measured using FACScan flow cytometry.

R2C cells were pretreated with the Ca^{2+} chelator, 1,1-bis(2-aminophenoxy)ethane-*N,N,N,N*9-tetraacetic acid (BAPTA) and then treated with TBT for 24 h. Cytochrome c release from mitochondria to cytosol was measured by western blotting.

All experiments were repeated at least three times. Student's t-test was used to assess the statistical significance of differences. A confidence level of < 0.05 was considered significant.

Results and Discussion

In the present study, we confirmed the appearance of apoptotic process in rat Leydig cell line, R2C by morphological, biochemical and 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 is the rise in intracellular Ca^{2+} concentration in the hepatoma cell[8]. We found that TBT also is able to increase the Ca^{2+}, at low concentrations (Fig. 2). 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[9] due to stimulation of electron flux along the electron transport chain. Indeed, TBT induced generation of ROS in a time-dependent manner (Fig. 2). To correlate Ca^{2+} and mitochondria in ROS release, R2C were pretreated with the Ca^{2+} chelator, 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[10]. Previously study demonstrated that TBT also is able to induce the release of cytochrome *c*[6]. 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 caspase-3 during DNA fragmentation, knowing that a link between caspase-3 and release of cytochrome *c* by TBT. The caspase-3 was activated by TBT in R2C cells with dose-dependent 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, caspases are activated, cleaving defined target proteins and leading to an irreversible apoptotic damage of the cell. The influx of Ca^{2+} may be caused by disruption of membrane or cytoskeletal functioning.

Acknowledgments
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Fig. 1

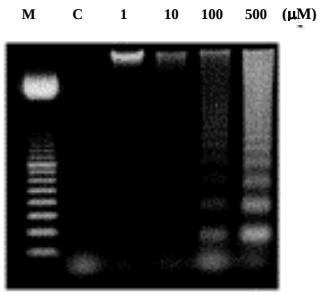

Fig. 2

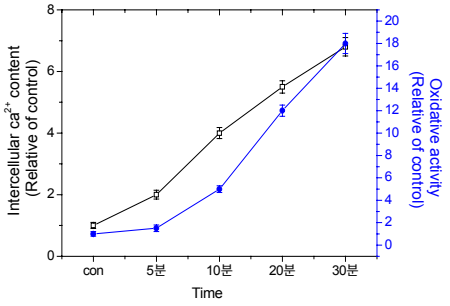

Fig.1. Detection of internucleosomal DNA fragmentation of R2C after 24 h of treatment with TBT using gel electrophoresis.

Fig. 2. TBT induces a dose-dependent increase in the intercellular Ca^{2+} and ROS in R2C Cells. The cells were loaded with Fura-2/AM or DCF-DH and then were incubated with TBT. The intercellular Ca^{2+} (–□–) and ROS (–■–) were measured using FACScan flow cytometry. Each value represents the mean ± SD of three experiments.

Fig. 3

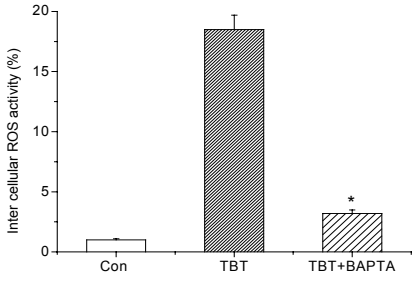

Fig. 4

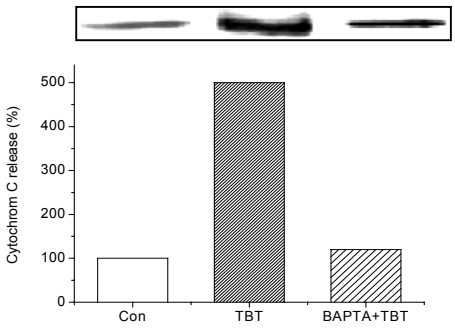

Fig. 3. BAPTA prevent ROS production in R2C treated with TBT. R2C cells were first treated for 30 min with 10 μM BAPTA, and then 500 μM TBT was added. Oxidative activity was measured 15 min after TBT treatment. Each value represents mean ± SD of three experiments. **p*, 0.05 vs cells treated with TBT.

Fig. 4. TBT induce a rapid cytochrome *c* release. R2C cells were first treated for 30 min with 10 μM BAPTA, and then 500 μM TBT was added. The presence of cytochrome *c* (12 kDa) in cytoplasmic extracts was measured by Western blot with an anti-cytochrome *c* antibody.

Fig. 5

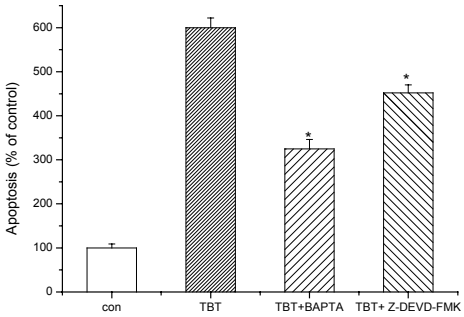

Fig. 5. TBT-induced apoptosis reduced by BAPTA and Z-DEVD FMK. R2C cells were first treated for 30 min with BAPTA (10 μM) or Z-DEVD FMK (20 μM), and then 500 μM TBT was added. Flow cytometry (PI staining) was used to quantify apoptosis. The amount of apoptotic nuclei is indicated as percentage of relative control. Values are means ± SD of three experiments. **p*, 0.05 vs cells treated with TBT.

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