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Mechanisms of Tributyltin-induced Leydig Cell Apoptosis

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유기주석화합물이 웅성생식세포주에 미치는 영향

이경진, 김덕송, 라명석*, 위성욱, 임욱빈, 최흥식, 이종빈※**

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요 약

본 연구는 환경 호르몬으로 분류된 67종 중의 하나인, 선저 도료나 어망, 어구 및 방오페인트 재료로 사용되어지고 유기주석화합물(tributyltin)을 사용하여 설치류의 웅성생식세포에서 세포자연사를 일으키는 작용기작을 조사하였다. 먼저 흰쥐의 레이딕 세포주인 R2C에 유기주석화합물을 농도별(1~500 nM)로 처리한 후 DNA fragment현상을 전기영동법을 통하여 조사하였다. 그 결과 유기주석화합물을 처리한 군들에서 대조군에 비하여 세포자연사현상이 농도 의존적으로 증가하였다. 유기주석화합물이 세포 내 칼슘이온(Ca^{2+}) 및 유해 산소종(reactive oxygen species)에 미치는 영향을 조사해본 결과 유기주석화합물 처리 시 세포 내 칼슘이온 및 유해 산소종이 시간에 의존적으로 크게 증가하였다. 또한 칼슘 킬레이터인 BAPTA를 전 처리한 경우 유기주석화합물에 의해 유도된 칼슘이온 및 유해 산소종이 대조군에 비해 유의성 있게 감소하였다. 이러한 세포자연사 과정이 미토콘드리아의 cytochrome c 방출에 의한 과정인지를 확인하기 위해 세포질 내 cytochrome c 양을 western blot법을 사용하여 확인해 본 결과 유기주석화합물 처리 시간 및 농도에 따라 증가하며, 이 또한 BAPTA를 전처리 한 경우 대조군에 비하여 유의성 있게 감소하였다. 또한 유기주석화합물이 세포자연사 유발 시 caspase-3 효소 활성과의 관계를 확인하기 위해 ELISA법을 사용하여 확인해 본 결과 유기주석화합물 처리 농도에 의존적으로 증가하였으며, caspase-3 효소 억제자로 잘 알려진 Z-DEVD FMK을 전 처리한 경우 유기주석화합물을 처리한 군에 비해 세포자연사율이 크게 감소하였다. 이러한 결과들을 종합해 볼 때 유기주석화합물은 세포 내 칼슘이온의 증가를 일으키고, 그로 인하여 세포질 내 유해산소종 및 cytochrome c의 양이 증가함으로써 세포자연사 다음 단계인 caspase 효소 활성의 증가를 통하여 흰쥐의 레이딕 세포주인 R2C의 세포자연사를 일으킬 것이라 추론할 수 있다.

Key words : tributyltin, reactive oxygen species, intracellular Ca^{2+}, caspase activity, R2C

INTRODUCTION

Organotin compounds such as tributyltin (TBT) are widely used as agricultural biocides, and for antifoul-

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ing 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.*, 1997). 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 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 the rat leydig cell line, R2C.

MATERIALS AND METHODS

Chemicals and solutions

TBT, Fura-2/AM and propidium iodide (PI) was 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, 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.

Cell Culture and treatment

Rat leydig cell line (R2C) 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 penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO_2 humidified incubator. The cells were incubated with different concentrations of TBT dissolved in ethanol absolute (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, 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.

Apoptosis detection

DNA fragmentation assay was carried out according to the method of Miller *et al.* (1988). Detection of apoptosis by flow cytometry with PI as fluorescence indicator was done essentially (Nicoletti *et al.*, 1976). Briefly, after incubation with the test compounds, 5×10^6 cells/ml 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) at room temperature, 0.5 ml PI was then added (5 mg/ml, in PBS) and the fluorescence of individual nuclei was measured using FACScan flow cytometry (Becton-Dickinson, Korea).

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).

Determination of Ca^{2+} level

R2C (5×10^6 cells/ml) 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).

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.

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.

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 demon-

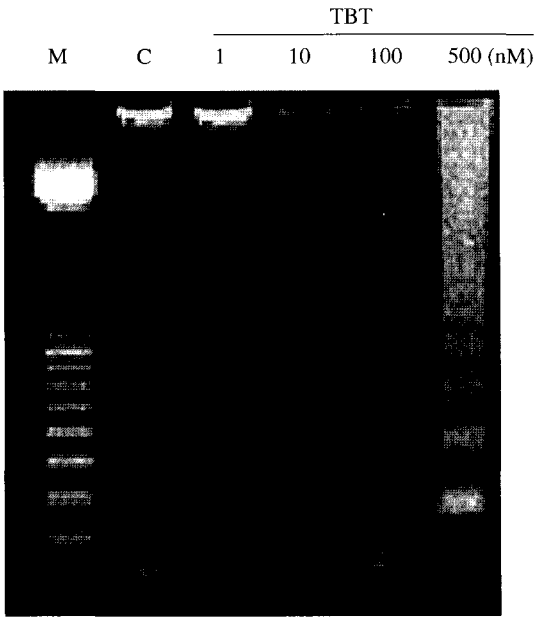

Fig. 1. Detection of internucleosomal DNA fragmentation of R2C after 24 h of treatment with TBT using gel electrophoresis. Abbreviation : C; control, TBT; tributyltin.

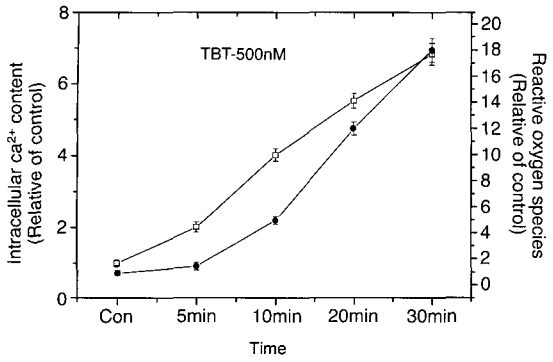

Fig. 2. TBT induces a dose-dependent increase in the intercellular Ca^{2+} and ROS in R2C Cells. The cells were loaded with Fura-2/AM or DCF-DH and then were incubated with TBT (500 nM). Intracellular Ca^{2+} (- □ -) and ROS (- ● -) were measured using FACScan flow cytometry. Each value represents the mean ± SD of three experiments. Abbreviation : Con; control, TBT; tributyltin.

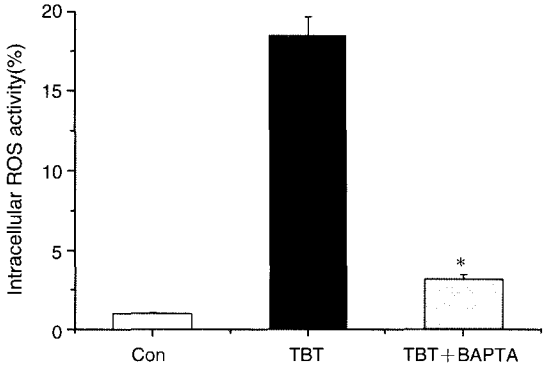

Fig. 3. BAPTA prevents ROS production in R2C treated with TBT. R2C cells were first treated for 30 min with 10 μM BAPTA and then 500 nM TBT was added. ROS was measured 15 min after TBT treatment. Each value represents the mean ± S.D. of three experiments. *p, 0.05 versus cells treated with TBT. Abbreviation : Con; control, TBT; tributyltin.

strate 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 (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 (Chacon and Acosta, 1991). due to stimulation of electron flux along the electron transport chain. Indeed, TBT induced generation of ROS in a time-dependent manner (Fig. 2B). 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 (Stridh *et al.*, 1998). Previously study demonstrated that TBT also is 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 in release of cytochrome c by TBT. 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

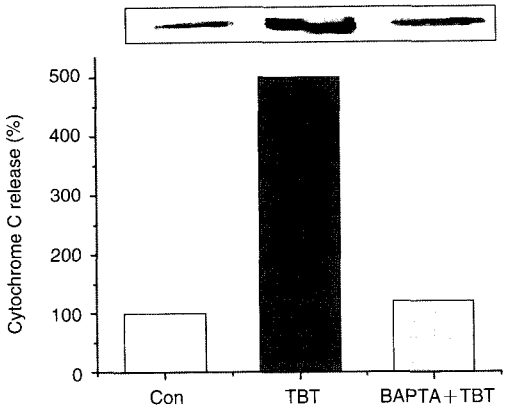

Fig. 4. TBT induces a rapid cytochrome c release. R2C cells were first treated for 30 min with 10 μM BAPTA and then 500 nM TBT was added for 5 min. The presence of cytochrom c (12 kDa) in cytoplasmic extract was measured by western blot with an anti-cytochrome c antibody. Abbreviation : Con; control,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

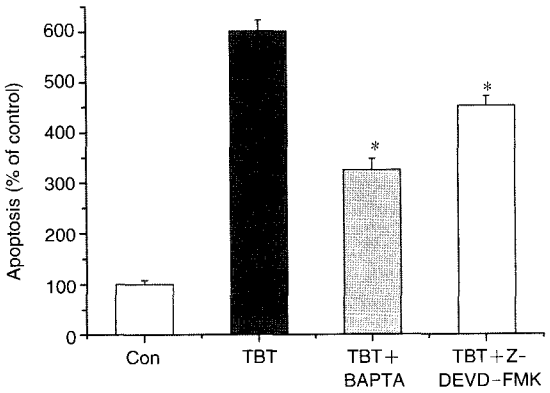

Fig. 5. TBT-induced apoptosis reduced by BAPTA and Z-DEVD FMK. R2C cells were first treated for 30 min with BAPTA (10 μM) or Z-DEVD FMK (20 μM), and then 500 nM TBT was added. Flow cytometry (PI staining) was used to quantify apoptosis. The amount of apoptotic nuclei is indicated as percentage of relative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

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, during DNA fragmentation, a link between release of cytochrome c by TBT and activation of caspase-3. This results indicated that caspase-03 might have a crucial role in TBT-induced apoptosis in R2C cells. 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 function.

ABSTRACT

Tributyltin (TBT) used world-wide in antifouling paints for ships is a widespread environmental pollutant and cause reproductive organs atrophy in rodents. 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 DNA fragmentation induced by TBT in the rat leydig cell line, R2C. 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.

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