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Original article

Analysis of Relation between an Increase in Intracellular Calcium and Cell Death Mechanism in RCR-1 Cells Exposed to Tributyltin Chloride

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Summary

Tributyltin compound is a toxic organotin compound that produces injury to the central nervous systems of mammals as the main target. In *in vitro* studies, we examined the effect of tributyltin chloride (TBTC) on RCR-1 cells (a rat astrocytoma cell line). After exposure to 1 μM TBTC, RCR-1 cells induced apoptosis, such as caspase-3 activation, calpain activation and mitochondrial cytochrome c release. TBTC-induced apoptosis was suppressed when RCR-1 cells were pretreated with BAPTA-AM, an intracellular calcium chelator. Furthermore, significant activation of calpain was associated with calcium-dependent enzyme action and an increase of intracellular calcium ($[Ca^{2+}]_i$) was confirmed in TBTC-exposed RCR-1 cells. On the other hand, calpain activation was suppressed by BAPTA-AM, an intracellular calcium chelator. JC-1 assays were used to evaluate mitochondrial function, since a strong expression of cytochrome *c* by TBTC suggested mitochondrial involvement; cytochrome *c* release and the loss of mitochondrial function occurred within 10 min of TBTC exposure. These results indicate the presence of a TBTC-induced calcium-dependent apoptotic pathway in RCR-1 cells. In conclusion, the results are thought to contribute greatly to the elucidation of TBTC-induced cell death mechanism of astrocytes and neurons of the central nervous system.

Introduction

Tributyltin compounds have been used widely as antiseptics for fishing nets and paint used on the bottoms of ships[1, 2)]. It is well known that tributyltin compounds cause behavioral toxicity[3)] and olfactory disorders[4, 5)] with the central nervous system as the primary target. In particular, it was reported that olfactory disturbance was seen in workers employed in the manufacturing of butyltin compounds for the industry[6)]. However, the mechanism of the olfactory disturbance induced by tributyltin compounds remained to be unclear.

TBTC transfer to the brain may be related to changes in blood-brain barrier (BBB) permeability. The BBB provides trophic support for neurons in the brain, and protects the brain environment from potentially toxic substances or neurotropic virus found in the blood[7)]. Astrocytes are a type of glial cells in the central nervous system, support function of the neural network[8)] and also play a critical role in the modulation of tight junction structure and/or regulation of the BBB[9)].

The increase in $[Ca^{2+}]_I$ and cytotoxicity induced by tributyltin chloride (TBTC) has been reported in the various experimental systems that utilize SY-SK5Y and SK-N-MC cells[10)], PC12 cells[11)], hepatocyte cells[12)] and lymphocytes[13)]. The evidence indicates that excessive calcium induces apoptosis, which is accompanied by activation of calcium-dependent calpain and endonuclease and involves caspase activation. However, its molecular mechanism in astrocytes is not fully understood. Calpain is a calcium-dependent protease characterized by high substrate specificity for spectrin and several apoptosis-related protease[14)], and

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is activated in neuronal apoptosis, which is more likely to occur in brain diseases such as cerebral ischemia and brain injury[15].

It has been known that organotin compounds can cause mitochondrial respiratory dysfunction and inhibition of oxidative phosphorylation[16, 17]. This dysfunction also were hypothesized to serve as a starting point in cell death induction[18]. Past studies in apoptosis have shown that the mitochondrial membrane potential collapse and the release of cytochrome c from mitochondria play a key role in the processes of caspases activation[19].

The aim of the current study was to investigate the dynamic state of $[Ca^{2+}]_I$ and the mechanism for induction of apoptosis induced by exposure to TBTC in astrocyte-like RCR-1 cells. To clarify the relationship between TBTC-induced the increase in $[Ca^{2+}]_I$ and apoptosis, measurement of intracellular calcium concentration, induction of the mitochondrial permeability transition, cytochrome *c* release, and caspase-3 and calpain activation were specifically studied.

Materials and Methods

1. Materials

Doulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were from Gibco-Invitrogen (Paisley, UK). Fura2-AM was from Dojindo Laboratories (Kumamoto, Japan). Carboxyfluorescein FLICA Apoptosis Detection kit caspase Assay was from Immunochemistry Technologies, LLC (Bloomington, MN). Calpain substrate-Ⅱ (Suc-LY-Tyr-AMC), and Fluorogenic was purchased from CALIBIOCHEM (San Diego, CA). The BCA™ Protein assay kit was purchased from PIERCE (Rockford, IL, USA). JC-1 dye and anti-β-actin were from Sigma-Aldrich, Inc (St. Louis, MO). Mitochondria- Cytosol Fraction Kit was from BioVision (Mountain View, CA). Anti-cytochrome *c* was from Santa Cruz (San Diego, CA). Block Ace was from Dainippon Sumitomo Pharma Co., Ltd. (Osaka, Japan). Hybond-P membrane and ECL Western blotting kit were from Amersham-Pharmacya Biotech, Ltd (Buckinghamshire, UK). TBTC and all other chemicals were from Wako Pure Chemical Industries, Ltd (Osaka, Japan).

2. Cell culture

Rat astrocyte-like cell line RCR-1 cells were obtained from the Health Science Research Resources Bank (JCRB0129.1). RCR-1 cells were cultured in DMEM supplemented with 10% FCS in 25 cm^2 culture flasks in a humidified atmosphere of 5.0% CO_2 at 37℃. RCR-1 cells were detached by exposure to 0.05% trypsin and washed with DMEM. The detached RCR-1 cells were seeded in 3.5 cm culture dishes or 96-well plates for the various experiments. TBTC was dissolved in absolute ethanol and then diluted to 1 μM in the culture medium with a final ethanol concentration of 0.1% (that is ineffective by itself).

3. Flow cytometry analysis of activated caspase-3

The type of cell death was determined by double staining with a fluorescein-labeled inhibitor of caspase-3 (FLICA) and propidium iodide (PI), in which FLICA binds to early apoptotic cells, while PI labels late apoptotic and necrotic cell membrane damage. After exposure to 1 μM TBTC for 60 min, the RCR-1 cells were adjusted to a density of 1.0×10^6 cells/mL and washed in PBS. Staining was performed according to kit instructions provided by the manufacturer (Carboxyfluorescein FLICA Assay Kit, B-Brige International, San Jose, CA). After labeling with FLICA, all samples were analyzed directly using a BD FACSCant flow cytometer with excitation at 488 nm wavelength argon laser beam and emission at 520 nm (BD Biosciences Immunocytometry Systems, San Jose, CA) to determine apoptosis. In addition, PI for necrosis was detected by excitation at 546 nm and emission at 637 nm.

In experiments studying the effect of $[Ca^{2+}]_i$ on TBTC-exposed PCR-1 cells, the cell cultures were preincubated with 50 μM BAPTA-AM (1, 2-bis-(o-Aminophenoxy)-ethane-N, N, -N', N'-tetra-acetic acid tetra-acetoxy-Methyl ester) for 30 min before 1 μM TBTC exposure. The percentage of apoptotic (FLICA-positive) and necrotic (PI-positive) cells was determined using BD FACSDiVa software.

4. Measurement of intracellular calcium

The time-course of the effect TBTC of on the $[Ca^{2+}]_i$ in the RCR-1 cells was measured with the fluorescent Ca^{2+} probe, Fura2-AM[21]. Loading was attained by incubating 3.0×10^5 cells per dish in Hepes ringer buffer (118 mM NaCl, 5.5 mM D-glucose, 2 mM L-glutamic acid, 2.0% MEM, 10 mM Hepes, 1 mM NaH_2PO_4, 4.7 mM KCl, 1.13 mM $MgCl_2$, 1.25 mM $CaCl_2$, 0.1 mM EGTA, pH 7.4) containing 5.1 μM Fura2-AM for 30 min at 37℃. After loading, RCR-1 cells were then washed twice in DMEM medium, and resuspended in this buffer at room temperature to remove extracellular fura-2 AM. After exposure to 1 μM TBTC, RCR-1 cells were collected at 1-minute intervals for up to 60 min. In experiments studying the effect of the $[Ca^{2+}]_i$ on TBTC-exposed PCR-1 cells, the cell cultures were treated with 50 μM BAPTA-AM for 30 min before 1 μM TBTC exposure. The Fura2-AM dye was followed (excitation at 340 and 380 nm; emission at 510 nm) using a plate reader Flex Station 3 (Molecular Devices, Sunnyvale, CA), and

$[Ca_{2+}]_i$ levels were plotted as the fluorescent ratio (340 nm/380 nm).

5. Measurement of calpain activity

Calpain activation was measured by fluorometric assay using fluorometric calpain substrate (Suc-LY-Tyr-AMC). RCR-1 cells were seeded at a density of 3.0×10^5 cells per well in a 96-well plate and preincubated in KRH buffer (115 mM NaCl, 25 mM Hepes, 1 mM NaH_2PO_4, 5 mM KCl, 1.2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.2% BSA, pH 7.4) containing 50 μM Suc-LY-Tyr-AMC in a 5.0% CO_2 humidified incubator at 37℃ for 30 min. In experiments studying the effect of chelating the effect of chelating $[Ca_{2+}]_i$ on TBTC-exposed PCR-1 cells, the cell cultures were treated with 50 μM BAPTA-AM for 30 min before 1 μM TBTC exposure. After TBTC exposure, fluorescence was detected using a plate reader Flex Station 3 with a 380 nm excitation and a 480 nm emission filter.

6. Mitochondrial function assay

The reduction of mitochondrial membrane potential ($\Delta\psi m$) in the RCR-1 cell was analyzed usi ng JC-1 dye, according to the method of Reers *et al.*[20]. JC-1 was chosen for use here as a dye selectively enters the mitochondria and, depending on the membrane potential, forms J-aggregates that are associated with a large shift in emission spectra. In particular, a loss of mitochondrial membrane potential is shifted in fluorescence from red to green. In this study, the RCR-1 cells were plated at 1.0×10^6 cells/mL in culture flasks and then cultured with 1 μM TBTC at 37℃ for 0 (untreated controls that received medium containing≦0.1% ethanol), 5, 10 or 30 min. The cells were then resuspended in PBS and incubated with 10 μg JC-1 dye/mL at 37℃ for 15 min. Both red and green fluorescence emissions were then analyzed during flow cytometry using an excitation wavelength of 488 nm and emission wavelength of 530 nm (for green fluorescence) or 585 nm (for red fluorescence).

7. Western-blot analysis of cytochrome *c*

Proteins related to apoptosis were analyzed using Western blot analysis of suitable subcellular fractions. RCR-1 cells were placed at 8.0×10^6 cells/mL in culture flasks and then cultured with 1 μM TBTC at 37℃ for 0 (untreated controls that received medium containing≦0.1% ethanol), 5, 10, 30, 60 min. The RCR-1 cells were then collected, washed with PBS, and cytosolic fractions without mitochondria were then extracted using a Mitochondria-Cytosol Fraction Kit, according to manufacturer's instructions. Protein concentrations in the resultant extracts were assessed using the BCA™ assay kit. For the detection of cytochrome *c* in each sample, ten μg of protein in the cytosolic fraction without mitochondria was separated over 15% SDS-PAGE and then transferred electrophoretically to polyvinylidene difluoride (PVDF). PVDF membranes were blocked with Block Ace. The resulting membranes were washed with TBS-Tween 3 times for 5 min, and then immunoblotted with rabbit polyclonal anti-cytochrome *c* (1:500), or anti-β-actin (1:5,000) for 60 min at room temperature. PVDF membranes were washed 3 times for 10 min in Tris Buffered Saline-Tween (0.1%) and then the signals were detected by use of an ECL Western blotting kit. β-actin was the internal standard to verify uniformity of protein loading and transfer. The fluorometric intensity of each immunoreactive band was ultimately estimated using Imaging Software (ImageQuant; Amersham Place, Little Chalfont, Buckinghamshire).

8. Statistics

All statistical analyses were performed using one-way analysis of variance (ANOVA) followed by post-hoc testing when appropriate using Fisher's LSD or Kruskal Wallis test followed by the Mann Whitney U test using SPSS 11.5J for Windows (SPSS Japan Inc., Tokyo). Differences were considered statistically significant at p-values < 0.05.

Results

1. Detection of cell death in RCR-1 cells

The morphology of cell death was analyzed using flow cytometry with PI and FLICA, which binds covalently to active caspase-3[22]. When the percentages of viable, apoptotic, and necrotic cells were calculated (Table 1), the results showed that an insignificant amount (0.3%) of the necrotic cell populations after 60 min of the TBTC exposure.

Table 1 Analysis of cell death using flow cytometry in RCR-1

Cell nunber (%)	Control	TBTC	BAPTA-AM+TBTC	ANOVA
Viable	97 ± 1.6	29 ± 9**	73 ± 3.9**	$p < 0.0001$
FLICA-positive	0.3 ± 0.1	70.8 ± 9.1**	24.3 ± 4.9**	$p < 0.0001$
PI-positive	1.9 ± 1.6	0.3 ± 0.3	2.7 ± 0.8	NS

Three different morphology of RCR-1 cells analysed for viability and apoptosis, necrosis. RCR-1 cells were treated with 1 μM TBTC for 60 min. Cells were also pretreated with 50 μM BAPTA-AM for 30 min and then exposed to TBTC for 60 min. The percentage of viable, FLICA-positive and PI-positive cells was determined using BD FACSDiVa software. Values are mean ± SD (n=5). **: $p < 0.01$, compared to the control.

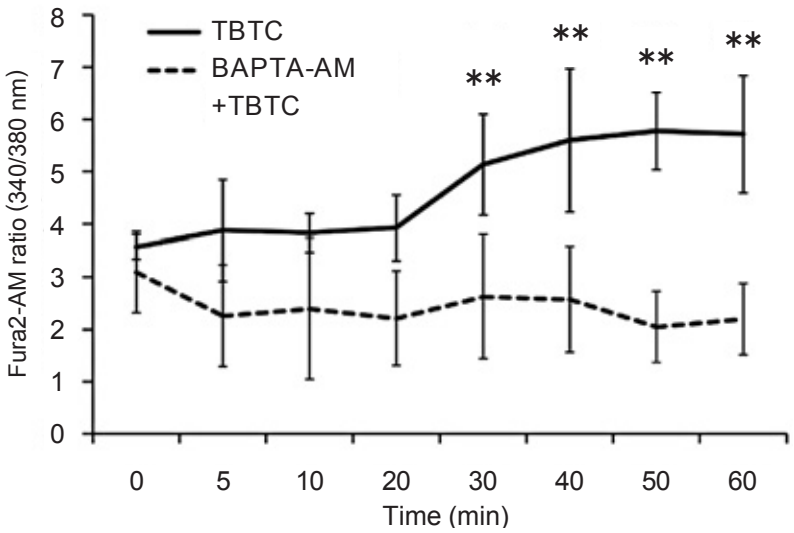

Fig. 1 Effect of TBTC on intracellular calcium levels in RCR-1 cells.

The time-course of the effect of TBTC on the intracellular calcium in the RCR-1 cells was measured with fura2-AM. RCR-1 cells exposed to 1 μM TBTC for the indicated times. Increases in intracellular calcium are expressed as the ratio of fluorescence intensity of fura2-AM over baseline (340 nm/380 nm). Results shown are the mean ± SD (n = 5). Value statistically different at $^{**}p < 0.01$ compared to the control (0 min).

In contrast, the number of apoptotic cell populations was 70.8% after 60 min of TBTC exposure at which time, the 29 % of the cells were viable. When RCR-1 cells were pretreated with BAPT-AM, the cell population in the induction of apoptosis was suppressed to a level of 24.3% after 60 min of TBTC exposure. In contrast, the number of viable cells was 73% in the TBTC exposed group.

2. Measurement of intracellular calcium

The ratio of changes over the time-course in the $[Ca^{2+}]_i$ in RCR-1 cells was analyzed using Fura2-AM (Fig. 1). After TBTC stimulation, the fluorescence value of $[Ca^{2+}]_i$ in RCR-1 cells tended to increase during the experimental periods. Additionally, the $[Ca^{2+}]_i$ did not also cause any significant change throught the chelation of $[Ca^{2+}]_i$ with BAPTA-AM before 1 μM TBTC exposure. Control experiments showed that the addition of an equal volume of ethanol (same volume as TBTC solution) did not induce an increase in the $[Ca^{2+}]_i$ in RCR-1 cells (data not shown).

3. Measurement of calpain activity

Calpain activation for the involvement of caspase-3 activation pathway or increase of $[Ca^{2+}]_i$ is known to play a role in the regulation of apoptosis[13-15]. To investigate whether TBTC induced increases in $[Ca^{2+}]_i$ are associated with changes in calpain activation in a time-dependent manner, calpain activation was examined using Suc-LY-Tyr-AMC, a fluorescent substrate. Calpain was significantly activated

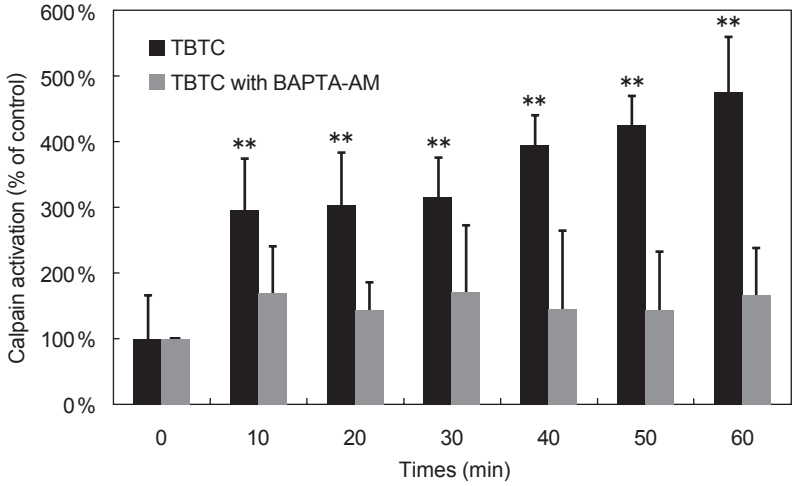

Fig. 2 Time-course of the effect of TBTC on activation of Calpain in RCR-1.

RCR-1 cells exposed to 1 μM TBTC for the indicated times. Activation of calpain was determined using specific fluorescent substrates Suc-LY-Tyr-AMC. Fluorescence was detected using a plate reader. Cells were pre-treated with vehicle, 50 μM BAPTA-AM 30 min prior to the challenge with 1 μM TBTC. One hour after the treatment with TBTC was begun, calpain activities were determined using a plate reader. Date are expressed as percentage of values found at 0 hr (control). Results shown are the mean ± SD (n = 5 separate cell populations examined per treatment). Value statistically different at $^{**}p < 0.01$ compared to control (0 min).

for 10 min after TBTC exposure as compared to control (Fig. 2). At any given time point, RCR-1 cells showed greater calpain activity than control cells, and such differences increased over time. However, the addition of BAPTA-AM to RCR-1 cells markedly suppressed calpain activity.

4. Analysis of Mitochondrial Function

There appeared to be a strong relationship between the induced changes in the ability of the treated RCR-1 cells to maintain membrane potential (s) and release cytochrome *c* of the treated RCR-1 cells. As shown in representative flow cytograms with JC-1 dye for monitoring change in mitochondrial membrane potential (s) (Fig. 3), the cell populations in the region designated "A" represented cells that had maintained normal potentials while those in the region "B" had a decrease in membrane potential (s). It was readily seen that there was a significant decrease in mitochondrial membrane potential in cells within the first 5 min after the start of the TBTC exposure. Western blotting was used to analyze the expression of cytochrome *c*, proteins that have each been shown to be associated with apoptosis. As shown in Figure 4, there was a marked increase in expression of cytochrome *c* between 10 and 60 min after the start of the TBTC exposure. These changes appear to mirror the period in which the maximal drops in mitochondrial cytochrome *c* release occurred (see above).

Discussion

In our previous study, we found that exposure to TBTC induced a significant change in the levels of trace elements in brain tissues and accumulation of excessive calcium in the hippocampus and olfactory bulb, followed by pathological cell death[5, 23]. Moreover, in rats, behavioral toxicity, disorders of memory and learning and olfactory disorders have been reported following TBTC exposure[3, 4, 6]. Organotin-induced increase in $[Ca_{2+}]_i$ and apoptosis has been reported to be elevated in several cell lines in *in vitro*[10–13]. Because a few information for toxicity revelation mechanism

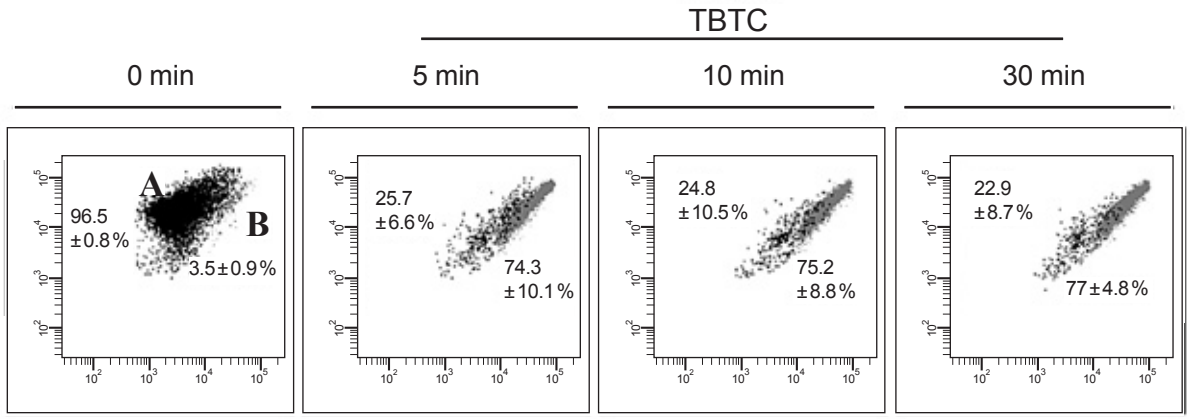

Fig. 3 Analysis of mitochondrial membrane potential.

Mitochondrial membrane potential was analyzed using a flow cytometry with JC-1. Red fluorescence intensity indicated cells with stable $\Delta\phi m$, while green fluorescence intensity indicated cells with low $\Delta\phi m$. A shift from red to green denotes dissipation of potential. The cell populations in region designated "A" represented cells that had maintained normal potentials; those in the region designated "B" had a decrease in membrane potential (s). The percentage of mitochondrial membrane potential depolarized cells in region (B) is indicated. While the images shown are a representative cytogram from these assays, the percentage results shown in each are the mean ± SD (n = 5 separate cell populations examined per treatment shown).

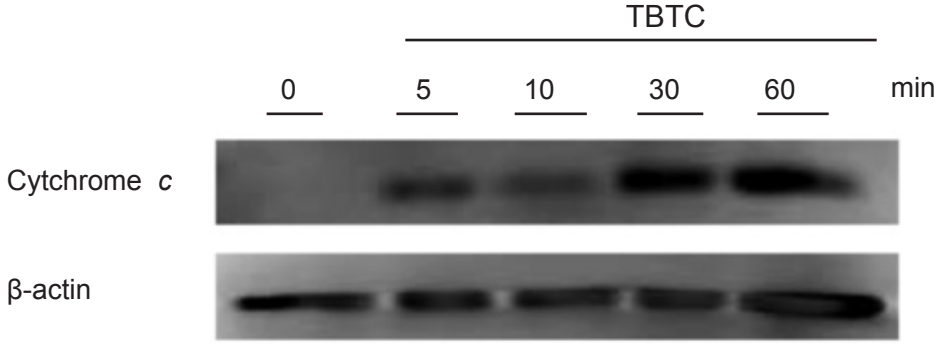

Fig. 4 Western blot analyses of time-dependent changes in cytochrome *c*.

Cytosolic fractions without mitochondria (10 μg protein/mL) from TBTC-exposed RCR-1 cells were separated over a 15% SDS-PAGE gel, and then transferred to PVDF membranes. The membranes were immunoblotted with rabbit polyclonal anti-cytochrome *c* (1:500), and the signals were detected by use of an ECL system. β-actin was employed as an internal standard to verify uniformity of protein loading and transfer. Representative immunoblot; each band indicates cytochrome *c* protein and β-actin protein.

caused by TBTC, we elucidated the relation between dynamic change of $[Ca^{2+}]_i$ and the mechanism for induction of apoptosis induced by exposure to TBTC in astrocyte-like RCR-1 cells. After exposure to 1 μM TBTC for 60 min, cell death detection utilizing flow cytometry showed that TBTC induced apoptosis but not necrosis in RCR-1 cells. At this time point, intracellular calcium chelator BAPTA-AM, reduced apoptosis by caspase-3 activation. These results suggest that calcium concentration rise plays a major role in TBTC-induced cell death. Next, the change of $[Ca^{2+}]_i$ in RCR-1 cells were observed using Fura2-AM. After TBTC stimulation, the $[Ca^{2+}]_i$ tended to increase in RCR-1 cells during the experimental periods. Our study demonstrates that although strong activation of calpain was observed in TBTC-exposed RCR-1 cells, calpain activation was significantly suppressed in the process of removing an intracellular calcium by means of a chelate, with BAPTA-AM. In caspases-dependent apoptosis, the excessive increase of intracellular calcium may be associated with calpain activation. Calpain can induce activation of caspase-3 via caspase-12, and can also activate caspase-3 directly[24, 25]. Moreover, caspase-3 and calpain can synergistically attack several common or related cytoskeletal, cytosolic and nuclear substrates[26]. These findings therefore suggest that calpains play an important role in inducing activation of caspase-3 in RCR-1 cells.

On other hands, it is well known that TBTC-induced apoptosis is accompanied by induction of the mitochondrial membrane potential and cytochrome *c* release from mitochondria[23]. In this study, induction of the mitochondrial membrane potential and cytochrome *c* release from mitochondria in RCR-1 cells were confirmed by TBTC exposure.

In conclusion, the cell death of RCR-1 cells induced by exposure to TBTC is thought to occur by at least two apoptotic events: mitochondrial dysfunction, such as membrane potential collapse associated with the release of cytochrome *c* from mitochondria and caspases activation; and the disturbance of calcium homeostasis associated with an increase in $[Ca^{2+}]_i$ and caspases cascade via activation of calpain. Thus, the results are thought to contribute greatly to the elucidation of the organotin-induced cell-death mechanism of astrocytes and neurons of the central nervous system.

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