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Relation of Excessive Accumulation of Calcium and Calcium-dependent Apoptotic Cell Death in the Organotin-exposed Olfactory System

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Summary

AIM : To analyze the dynamic state of intracellular calcium and the mechanism for induction of apoptosis in olfactory neurons and astrocyte-like RCR-1 cells induced by the exposure to tributyltin (TBTC).

METHODS : Astrocyte-like RCR-1 cells and primary cultures of olfactory neurons were cultured in a serum-free DMEM medium and exposed to TBTC. The intracellular calcium of olfactory neurons or astrocyte-like RCR-1 cells was labeled using the calcium specific fluorescent dye Fluo3-AM, and examined before and after administration of TBTC, utilizing confocal laser scanning microscope (CLSM). Apoptotic cells were further analyzed by flow cytometry.

RESULTS: Intracellular concentration of calcium increased within 5 min (as noted by fluorescent intensity) of exposure to TBTC and cytosolic calcium was transported to the nucleus in olfactory neurons or RCR-1 cells. Finally, caspase-dependent apoptosis occurred.

CONCLUSION : The TBTC-induced significant increase in calcium may be associated with caspase-dependent cell death. This calcium is a critical messenger in the apoptotic pathway. Further investigation is warranted to examine the TBTC-induced calcium-dependent apoptotic pathway and the apoptotic signaling messenger for olfactory neurons and astrocyte cells.

Introduction

It is well known that olfactory lesions induced by exposure to tributyltin (TBTC) are common occurrences especially in the form of industrial injury[1, 2] ; similarly published studies conclude that trialkyltins such as TBTC provoke behavioral toxicity[3], disorders of memory and learning and olfactory disorders[1, 2], with the central nervous system as the primary target. However, TBTC specifically has not yet been confirmed to be neurotoxic and is therefore not a definitive causative factor in olfactory lesions.

In a previous study, we found that exposure to TBTC induced a significant change in the balance of trace elements in brain tissues and accumulation of excessive calcium in the olfactory bulb and olfactory epithelium followed by pathological cell death of granular neurons, of the olfactory bulb and epithelium[1, 2, 4, 7]. Moreover, in rats, after administration of a single intraperitoneal injection of TBTC, DNase was activated and such activity affected the concentration of calcium in a dose-dependent manner in the olfactory bulb[8].

However, the mechanism of apoptosis induction after the exposure of TBTC and the dynamic state of intracellular calcium in these conditions remains unclear. In this study, we have therefore undertaken establishment of an *in vitro* experimentation system to analyze detailed apoptotic mechanism present in the olfactory bulb. We predicted that the movement of intracellular calcium might contribute to apoptosis.

Materials and Methods

1. Cell culture and treatment

Approval for this study was obtained from the Institutional Committee for the Care and Use of Animals at the University of Shizuoka. Cultured cells from the olfactory bulb of three-week-old Wistar-derived male rats [Japan SLC Inc.] were used for this study. Rats were guillotined and removal of the olfactory bulb was accomplished as per previous literature[8]. The olfactory bulb was chemically digested with papain at 37℃ for 40 min and the resulting cell suspension was centrifuged at 170 × *g* for 4 min at room temperature. Supernatant consisting of the merged solutions and debris was removed and the cellular pellet was re-suspended in Dulbecco's modified medium (DMEM) supplemented with 10 % heat-inactivated fetal calf serum (FCS). The resulting cell suspension was seeded in 3.5-cm culture dishes in 5.0 % CO_2 at 37℃. To inhibit the multiplication of glial cells, 1 μM cytosine β-D-arabinofuranoside was added to the culture

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dishes on the second day. Olfactory neurons were cultured for 14 days.

A rat astrocyte-like cell line (RCR-1 cells) was obtained from Health Science Research Resources Bank. 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 then detached by exposure to 0.05 % trypsin and washed with DMEM supplemented with 10 % FCS. The detached RCR-1 cells were seeded in 3.5-cm culture dishes at 3.0×10^5 cells per dish for calcium imaging and 5.0×10^5 cells per dish for assay of Flow cytometry. Both the olfactory neuron and RCR-1 cells were exposed to 10^{-6} M TBTC for calcium imaging.

2. Imaging

The movement and the location of calcium in olfactory neuron and RCR-1 cells were observed using fluorescent Ca^{2+} probe, Fluo3-AM. Loading was performed by incubating cells in Hepes ringer buffer (118 mM NaCl, 5.5 mM D-glucose, 2 mM L-glutamic acid, 2 % 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 Fluo3-AM for 30 min at 37℃. A confocal laser-scanning microscope LSM 510 (Carl Zeiss, Jena, Germany) was utilized for these procedures. After plateau was reached, 535 nm emission fluorescent images were obtained after excitation at 488 nm. A 40 × oil-immersion objective was used. To clarify the location of intracellular TBTC in the olfactory neurons after the exposure to 10^{-6} M TBTC, such location was facilitated using morin with CLSM. The position of the nuclei was confirmed by CLSM after nuclei staining with Hoechst33258. To clarify the movement and location of intracellular calcium ($[Ca^{2+}]i$) in the olfactory neurons and RCR-1 cells after exposure to 10^{-6} M TBTC, we conducted CLSM with Fluo-3-AM. Analysis was accomplished using Zeiss LSM Software. In addition, dose-dependent effect of TBTC on $[Ca^{2+}]i$ levels in RCR-1 cells were analyzed utilizing CLSM software.

3. Flow cytometry analysis of activated caspase-3

The type of cell death was analyzed by double staining with fluorescein-labeled inhibitor of caspase-3 (FLICA) and propidium iodide (PI), in which FLICA binds to the early apoptotic cells, while PI labels late apoptotic and necrotic cells membrane damage. Staining was performed according to the kit instructions provided by manufacturer (carboxy-fluorescein FLICA Assay Kit, B-Brige International, San Jose, CA, USA). After labeling with FLICA, all samples were analyzed by flow cytometry (BD FACSCant Ⅱ, BD Biosciences Immunocytometry Systems, San Jose, CA) to detect the type of cell death. The percentage of apoptotic (FLICA+/PI±) and necrotic (FLICA−/PI+) cells was determined using BD FACSDiVa software.

Results

In olfactory neurons, the location of intracellular TBTC accumulation (at 60 min after administration) was mostly around the nuclear membrane (Fig. 1). Within 5 min of exposure to TBTC, Ca^{2+} of cytosol was remarkably transported to the nucleus (Fig. 2-A, B). This nuclear calcium ($[Ca^{2+}]n$) increased 2.5 fold as compared to concentrations before exposure to TBTC (Fig. 2-C). The movement and location of calcium in TBTC-treated RCR-1 cells was similar to the results of the olfactory neurons (Fig. 3). As a result, the elevation of $[Ca^{2+}]i$ in RCR-1 cells was approximately 50 percent as compared to pre-treatment. $[Ca^{2+}]i$ increased 1.3 fold at 5 min after the exposure to 10^{-5} M TBTC, and 1.5 fold after exposure to $10^{-6\sim-8}$ M. TBTC-induced $[Ca^{2+}]i$ levels were also increased in RCR-1 cells in a dose-independent manner (Fig. 4).

The non-apoptotic cells showed neither FLICA nor PI fluorescence (Fig. 5-A) with the highest concentration (~ 90 %) in the control cells. Sixty min after exposure to TBTC, a distinct subpopulation of cells with increased FLICA but unchanged PI fluorescence was apparent (Fig. 5-B). Apoptotic cells accounted for 68 % of the cell population at that time.

Discussion

In this study, TBTC was taken into olfactory neurons or RCR-1 cells immediately, and accumulated around the nuclear membrane at approximately 60 minutes post-administration. In human skin fibroblasts (SF-YT), visualization of the intracellular distribution of organotin compounds reveals that dibutyltin, triethyltin, tributyltin, and triphenyltin, but not monomethyltin, monobutyltin, and dimethyltin, are selectively distributed to the region of the Golgi apparatus and endoplasmic reticulum (ER)[3, 4]. Similarly, we report there that in olfactory neurons and astrocytes, TBTC was distributed to the region around the Golgi apparatus and ER around the nuclear membrane. The intracellular distribution of organotin compounds is probably due to their liposolubility or their affinity for the intracellular lipids and lipophilic proteins[10].

Primary culture of olfactory bulb neurons and astrocyte-like cells (RCR-1) revealed increased $[Ca^{2+}]i$ within 5 min after exposure to TBTC; indeed, the Ca^{2+} of cytosol immediately transferred to the nucleus. This phenomenon shows for the first time that mechanism for TBTC uptake into the cell may be due to concomitant development of high levels calcium. Even at administration of 10^{-8} M concentration of TBTC, $[Ca^{2+}]i$ increased significantly. In our previous

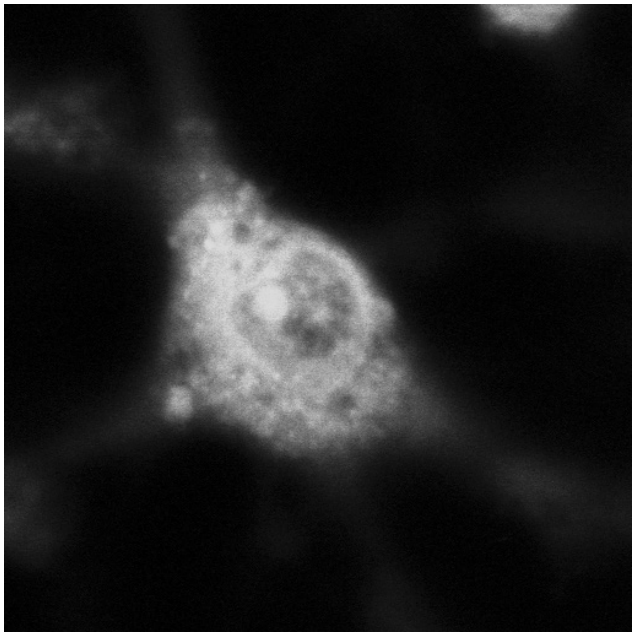

Fig. 1 Intracellular distribution of tributyltin (TBTC) in primary neurons as seem by confocal laser scanning microscopy (CLSM). Primary neurons were incubated for 60 min with 1μM tributyltin chloride (10^{-6} M TBTC), washed, incubated with 100 μg/mL morin for 5 min, and washed again. Fluorescence intensity of intracellular TBTC in primary neurons: Green fluorescence- ex, 405 nm; em, 495 nm.

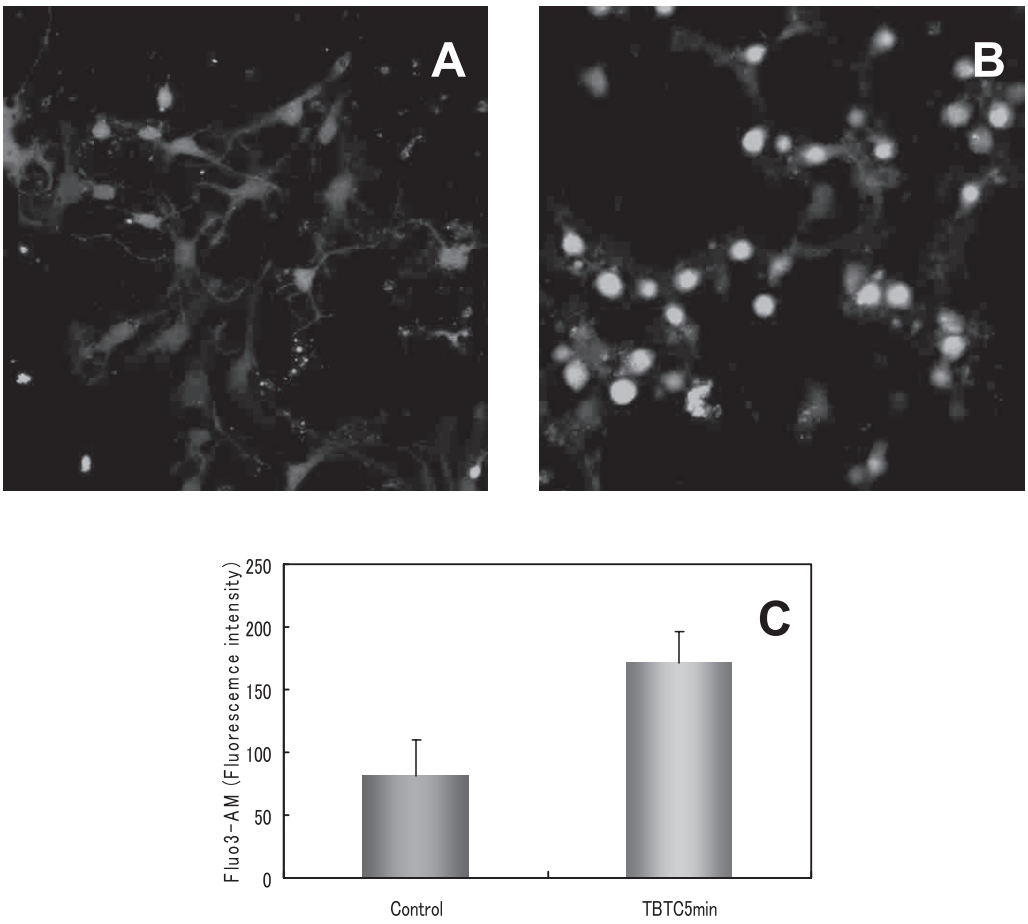

Fig. 2 Intracellular distribution of Ca^{2+} in primary neurons by CLSM (× 200).
$[Ca^{2+}]i$ distribution in primary neurons was analyzed utilizing Fluo3-AM staining. Images were quantitatively analyzed for changes in fluorescence intensity within regions of interests (ROI): (A) control, (B) TBTC (10^{-6} M) for 5 min. (C) Averaged $[Ca^{2+}]n$ levels are shown as the mean ± SD, n = 20.

study, we investigated a mechanism for increased transport of extracellular Ca^{2+} to the tissues. In particular, the tributyltin-induced significant increase in olfactory calcium may be caused by a considerable increase in cAMP production mediated by activation of adenyl cyclase and increased transport of extracellular Ca^{2+} mediated by cAMP-activated channels[1, 2, 4–6, 11, 12].

Examination of cell death of RCR-1 cells exposed to TBTC

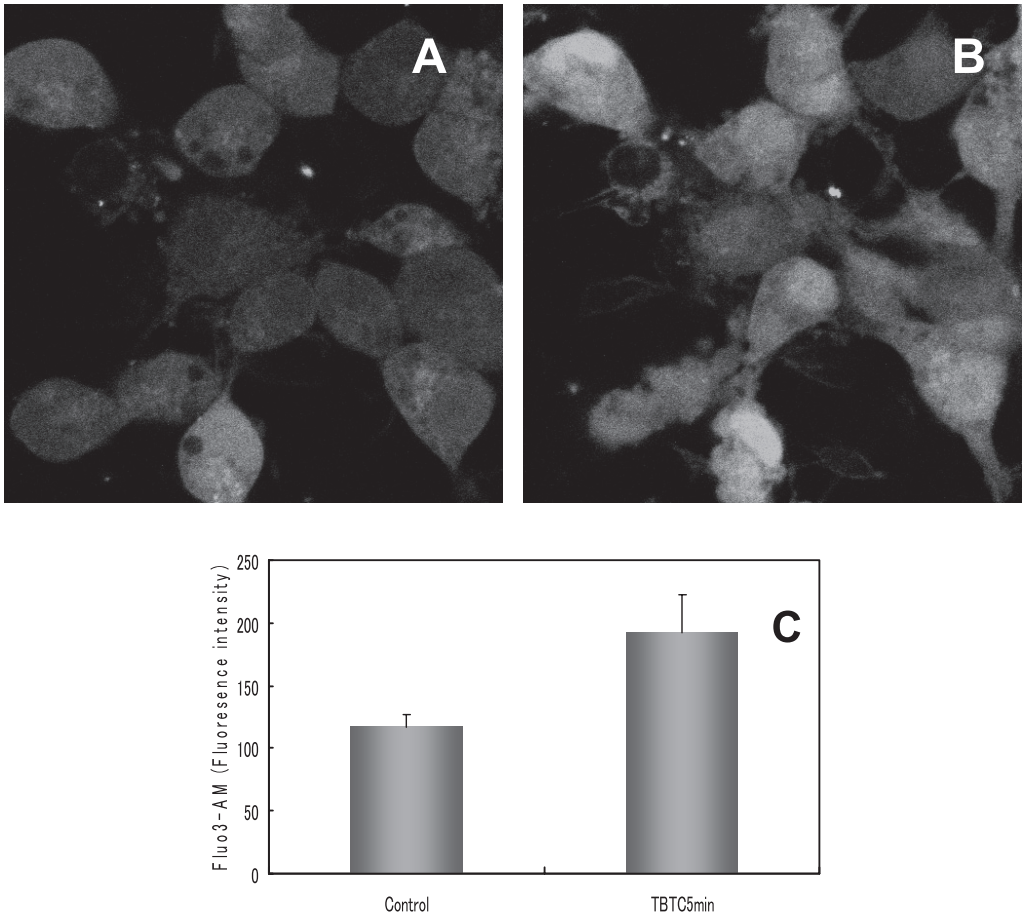

Fig. 3 Intracellular distribution of Ca^{2+} in RCR-1 cells by CLSM.
RCR-1 cells were analyzed by LCSM (× 200) with Fluo3-AM staining. (A) Control, (B) TBTC (10^{-6} M) for 5 min. Images were quantitatively analyzed for changes in fluorescence intensity within regions of interests (ROI). (C) Averaged $[Ca^{2+}]$n levels are shown as the mean ± SD, n = 5.

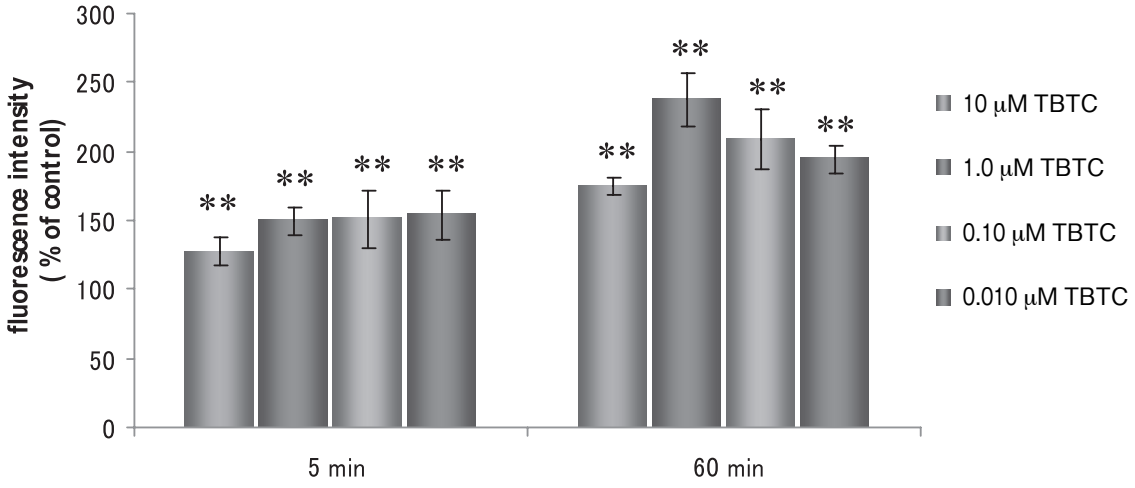

Fig. 4 Dose-dependent effect of TBTC on intracellular Ca^{2+} levels in RCR-1 cells.
$[Ca^{2+}]$i was labeled by using fluorescent dye (Fluo3-AM) and detected utilizing CLSM. RCR-1 cells were treated at 37℃ for 1h with the indicated concentrations of TBTC. TBTC induced a $[Ca^{2+}]$i increase in RCR-1 in a dose-independent manner. Averaged $[Ca^{2+}]$i levels are shown (mean ± SD, n = 20), ** $P < 0.01$ vs. control.

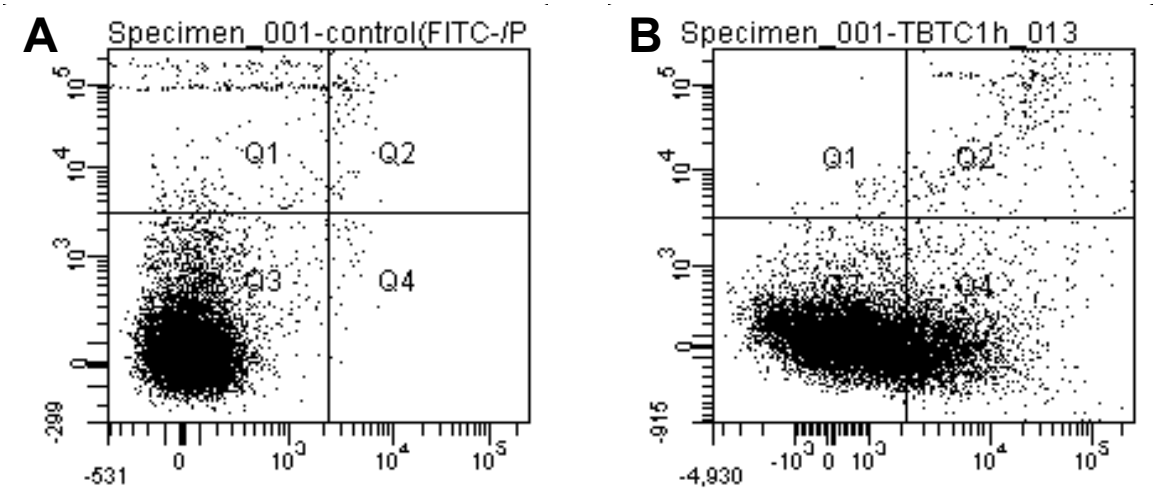

Fig. 5 Cell staining analysis by Flow cytometry.
(A : Control cells. B : Cells exposed to TBTC for 60 min.) RCR-1 cytograms analyzed for viability and apoptosis, necrosis. Scattergrams of flow measurements representing the cells stained with FAM-VAD-FMK (green fluorescence) and PI (red fluorescence). Cell status as revealed by green versus red fluorescence. Q-1 : necrosis ; Q-2 : late apoptotic cells ; Q-3 : live cells ; Q-4 : early apoptotic cells.

revealed that 68 % of the cell population death was induced by caspase-3 at 60 min post-exposure to TBTC. Excessive accumulation of calcium occurred earlier than caspase-3 activation. Thus, the cause of the caspase-3 activation is thought to be the TBTC-induced excessive increase of $[Ca^{2+}]i$. TBTC triggers apoptosis but not necrosis in RCR-1 cells. Excessive accumulation of calcium plays a critical role in initiating the apoptotic cell death pathway.

In vivo, we previously demonstrated that the Ca^{2+}-required DNase-activation occurred concomitantly with excessive accumulation of calcium in the olfactory system[8]. DNase is localized in the ER, and is released and transported to the nucleus by ER stress (i.e., impairment of ER structure and function)[13]. However, the relationship between caspase-3 and DNase-remains unknown. In other words, the calcium-induced cell death pathway activates caspase-3, and independently mediates DNase-activation. In caspase-dependent cell death, the excessive increase of $[Ca^{2+}]i$ may be associated with calpain activation[14] and ER stress[15]. The first pathway links calpain activation to consequent increases in intracellular calcium. However, in the second pathway, apoptosis induced by ER stress depends on activation of caspase-3 via caspase-12[16, 17]. Further study is needed to fully understand these pathways.

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