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Effects of Tributyltin Chloride on L-DOPA-Induced Cytotoxicity in PC12 Cells

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Tributyltin chloride (TBTC) at concentrations of 0.5-1.0 μM inhibits dopamine biosynthesis in PC12 cells. In this study, the effects of TBTC on L-3,4-dihydroxyphenylalanine (L-DOPA)-induced cytotoxicity in PC12 cells were investigated. TBTC at concentrations up to 1.0 μM neither affected cell viability, nor induced apoptosis after 24 or 48 h in PC12 cells. However, TBTC at concentrations higher than 2.0 μM caused cytotoxicity through an apoptotic process. In addition, exposure of PC12 cells to non-cytotoxic (0.5 and 1.0 μM) or cytotoxic (2.0 μM) concentrations of TBTC in combination with L-DOPA (20, 50 and 100 μM) resulted in a significant increase in cell loss and the percentage of apoptotic cells after 24 or 48 h compared with TBTC or L-DOPA alone. The enhancing effects of TBTC on L-DOPA-induced cytotoxicity were concentration- and treatment time-dependent. These data demonstrate that TBTC enhances L-DOPA-induced cytotoxicity in PC 12 cells.

Key words: Tributyltin chloride, L-3,4-Dihydroxyphenylalanine (L-DOPA), Cytotoxicity, Apoptosis, PC12 cells

INTRODUCTION

Organotin (trialkyltin) compounds such as tributyltin chloride (TBTC) and trimethyltin chloride are mainly used as biocides in crop protection, in anti-fouling paints for large ships and in fabrics (Fent, 1996). However, TBTC has received much attention as an endocrine-disrupting chemical, because of its environmental and health hazards (Mizuhashi *et al.*, 2000).

Organotins have been used extensively as a research tool because of their potent pro-apoptotic action on various types of cells, including thymocytes (Gennari *et al.*, 2000), hepatocytes (Reader *et al.*, 1999), lymphocytes (Stridh *et al.*, 1998), hippocampal slice cultures (Mizuhashi *et al.*, 2000) and PC12 cells (Jenkins and Barone, 2004). Organotins may also play an important role in the pathogenesis of Parkinson's disease (Mailman and Lewis, 1987) and may induce epilepsy and amnesia (Tsunashima *et al.*, 1998). However, few studies have shown the neurotoxic effect of TBTC on the central nervous system.

Parkinson's disease is characterized by the degeneration of dopaminergic neurons in the substantia nigra and dopamine depletion in the striatum (Agid, 1991). L-3,4-Dihydroxyphenylalanine (L-DOPA), which is the natural precursor of dopamine and often used in the treatment of Parkinson's disease, has demonstrated toxicity towards cell lines including PC12 cells (Migheli *et al.*, 1999; Lee *et al.*, 2003), cultured sympathetic neurons (Boyce *et al.*, 1990), and striatal neurons (Cheng *et al.*, 1996). L-DOPA, through auto- and enzymatic oxidation, can generate a variety of cytotoxic oxygen radical species including superoxide, hydrogen peroxide, semiquinones and quinones (Basma *et al.*, 1995; Migheli *et al.*, 1999). Therefore, oxidative stress is considered a mediator of L-DOPA-induced apoptosis, and oxidative stress to dopaminergic neurons is believed to be one of the causes of neurodegeneration in Parkinson's disease.

PC12 rat pheochromocytoma cells display the properties of adrenal chromaffin cells (Greene and Tischler, 1976) and have been widely used as *in vitro* models to investigate the dopaminergic neurotoxicity of various compounds (Basma *et al.*, 1995; Migheli *et al.*, 1999; Lee *et al.*, 2003).

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In this study, the effects of L-DOPA exposure, alone or in combination with TBTC, on PC12 cells were investigated to determine whether TBTC could, in turn, worsen L-DOPA cytotoxicity.

MATERIALS AND METHODS

Materials

TBTC, L-DOPA, RNase A and propidium iodide were purchased from Aldrich Chemical Co. (St. Louis, Mo, U.S.A.). The *in situ* cell death detection kit (deoxynucleotidyl-transferase dUTP nick-end labeling, TUNEL) was from Boehringer Mannheim (Mannheim, Germany). All sera, antibiotics and RPMI 1640 for cell culture were obtained from Gibco (Grand Island, NY, U.S.A.). All other chemicals were of reagent grade.

Cell culture

PC12 cells were routinely maintained in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, plus 100 units/mL penicillin and 100 μl/mL streptomycin at 37°C (Greene and Tischler, 1976). PC12 cells (ca. $1x10^5$ cells/cm^2) in culture were incubated in the absence or presence of TBTC (0.5-2.0 μM) in conjunction with increasing concentrations of L-DOPA (20-100 μM) for 24 or 48 h.

Assessment of cell viability

Cell viability was determined by the conventional MTT assay with a slight modification (Mosman, 1983), which is based on the conversion of tetrazolium salt into an insoluble formazan product by various dehydrogenases in mitochondria. Briefly, treated cells were incubated with MTT solution (final concentration, 1 μg/mL) for 4 h, followed by the addition of 0.8 M HCl in isopropanol and mixed thoroughly; the absorbance was then measured at 570 nm with a Bauty Diagnostic Microplate Reader (Molecular Devices, CA, U.S.A.).

TUNEL assay for apoptotic DNA fragmentation

The *in situ* cell death detection kit (Boehringer Mannheim, Mannheim) was utilized to detect DNA fragmentation, as an indicator of apoptosis. TUNEL staining was performed as described previously (Lee *et al.*, 2003).

Flow cytometric analysis of apoptotic cells

Detection of DNA fragmentation was assessed by flow cytometry with propidium iodide as a fluorescent indicator (Nicoletti *et al.*, 1991). PC12 cells were harvested by centrifugation, washed in phosphate-buffered saline, and fixed in 70% ethanol for 30 min at 4°C. Before staining with propidium iodide (50 mg/mL), the fixed cells were centrifuged and washed again. The cellular DNA content was measured using a FACS vantage fluorescence-activated flow cytometer (Becton Dickinson, San Jose, CA, U.S.A.).

Statistical analysis

All data were expressed as mean±SEM of at least four experiments. Statistical analysis was performed using ANOVA followed by Tukey's test.

RESULTS

Effects of TBTC on L-DOPA-induced cytotoxicity

When PC12 cells were treated with 0.5 to 2.5 μM TBTC for 24 or 48 h, there was a concentration- and treatment time-dependent decrease in cell viability, as measured by the MTT assay (Fig. 1). TBTC at concentrations up to 1.0 μM did not significantly reduce the cell viability. However, at concentrations higher than 2.0 μM, TBTC induced

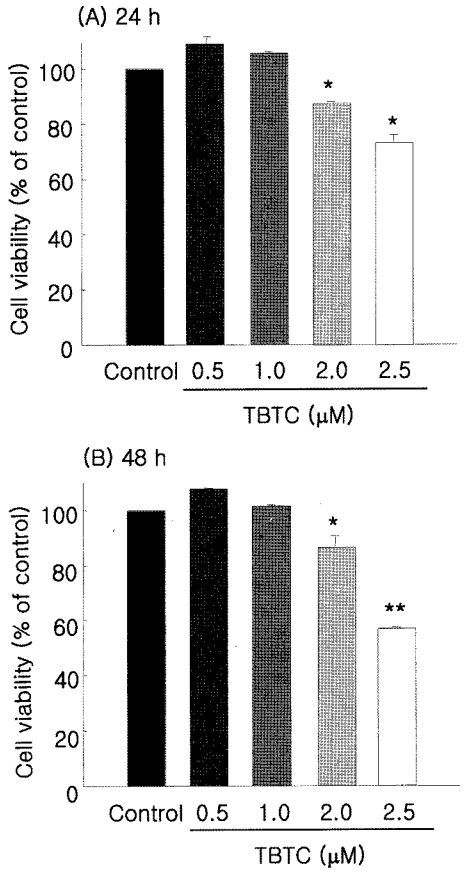

Fig. 1. Effects of tributyltin chloride (TBTC) on PC12 cell viability. PC12 cells were exposed for 24 h (A) or 48 h (B) to different concentrations of TBTC (0.5, 1.0, 2.0 and 2.5 μM). Cell viability was assessed using MTT methods, in which viable cells convert the soluble dye MTT to insoluble blue formazan crystals. The results represent the mean±SEM of five experiments. *, $p<0.05$; **, $p<0.001$ compared to control (ANOVA followed by Tukey's test).

cytotoxicity in PC12 cells (Fig. 1).

Twenty to 100 μM L-DOPA did not significantly decrease cell viability after 24 h in PC12 cells (Fig. 2A). However, after 48 h, L-DOPA at concentrations higher than 50 μM displayed cytotoxicity (Fig. 2B). Next, the effects of TBTC on L-DOPA-induced cytotoxicity in PC12 cells were investigated. Non-cytotoxic concentrations of TBTC (0.5 and 1.0 μM) in combination with L-DOPA (20, 50 and 100 μM) for 24 h slightly decreased cell viability (Fig. 2A). However, a significant decrease in cell viability was observed when PC12 cells were exposed to TBTC (0.5 and 1.0 μM) plus L-DOPA (20, 50 and 100 μM) for 48 h (Fig. 4). Exposure of PC12 cells to a cytotoxic concentration of TBTC (2.0 μM) in combination with L-DOPA (20, 50 and 100 μM) also resulted in a marked decrease in cell viability compared with TBTC or L-DOPA alone (Fig. 2A and 2B).

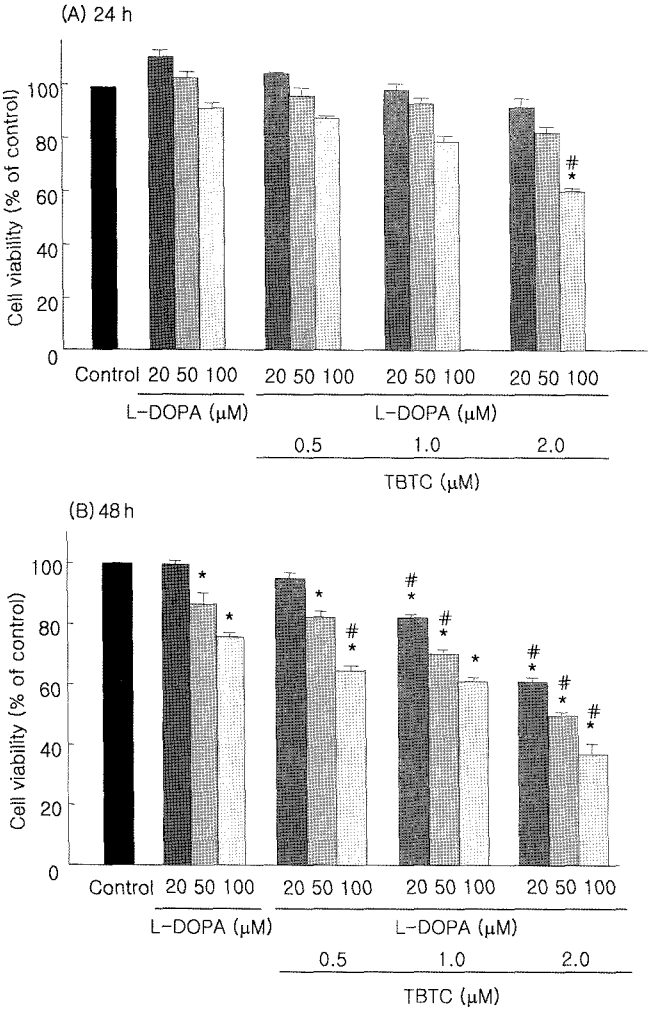

Fig. 2. Effects of TBTC on L-DOPA-induced decreases in PC12 cell viability. PC12 cells in culture were incubated in the absence or presence of L-DOPA (20, 50 and 100 μM) in conjunction with TBTC (0.5, 1.0 and 2.0 μM) for 24 h (A) or 48 h (B). Cell viability was assessed using MTT methods, and the results represent the mean±SEM of five experiments. *, $p<0.05$ compared to control; #, $p<0.05$ compared to the corresponding L-DOPA concentrations (ANOVA followed by Tukey's test).

Interaction of TBTC with L-DOPA-induced apoptosis

In control PC12 cells, apoptotic cells were not detected by either the TUNEL technique (Fig. 3) or flow cytometry (Fig. 4). In addition, neither TBTC (0.5 and 1.0 μM) nor L-DOPA (20 μM) induced apoptotic nuclear changes at 24 h or 48 h. However, TBTC at a non-cytotoxic concentration (0.5 μM) in combination with L-DOPA at a non-cytotoxic (20 μM; data not shown) or cytotoxic (50 μM; Fig. 3) concentration after 48 h resulted in a marked induction of apoptotic nuclear changes compared with TBTC or L-DOPA alone; these changes included highly condensed chromatin and extensive membrane blebbing. TBTC (2.0

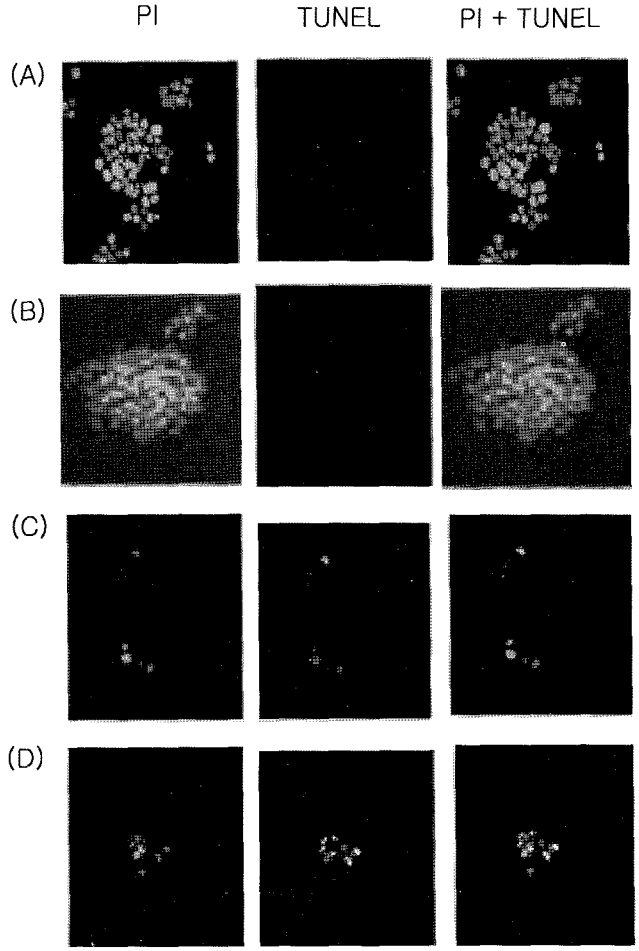

Fig. 3. Enhancing effects of TBTC on L-DOPA-induced apoptosis in PC12 cells as determined by *in situ* TUNEL staining. Fluorescence micrographs of untreated PC12 cells (A) and apoptotic PC12 cells (green or yellow-green cells) after 48-h exposure to 0.5 μM TBTC (B), 50 μM L-DOPA (C) and 0.5 μM TBTC + 50 μM L-DOPA (D). Propidium iodide was used to counterstain the cells. Apoptotic nuclei are those with green or yellow-green fluorescence.

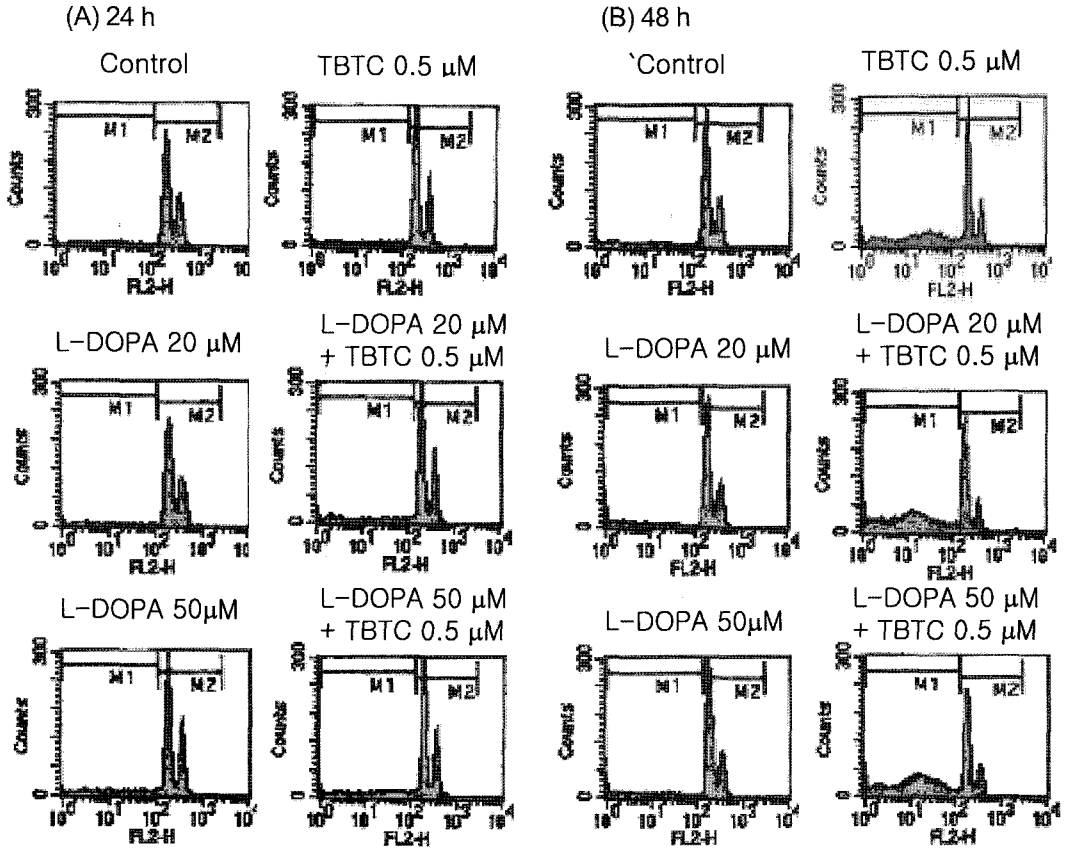

Fig. 4. Flow cytometry histograms of control PC12 cells and PC12 cells after 24-h and 48-h exposure to TBTC (0.5 μM) alone or in combination with L-DOPA (20 and 50 μM). After incubation, the cells were harvested and stained with propidium iodide. Relative DNA content was analyzed by flow cytometry. X-axis, DNA content; Y-axis, number of cells.

μM), L-DOPA (50 μM) and TBTC (2.0 μM) plus L-DOPA (20 and 50 μM) after 48 h also induced apoptotic cell death, as revealed by TUNEL staining (data not shown).

In addition, a 24 or 48-h exposure of PC12 cells to 0.5 μM TBTC in combination with 20 μM L-DOPA revealed 7-9% or 15-23% apoptotic cells, respectively (Fig. 4). TBTC (0.5 μM) in combination with L-DOPA (50 μM) further increased the percentage of apoptotic cells to 13-22% or 29-33% after 24 or 48 h, respectively (Fig. 4). Furthermore, TBTC at a cytotoxic concentration (2.0 μM) in combination with L-DOPA at non-cytotoxic (20 μM) or cytotoxic (50 μM) concentrations markedly increased the percentage of apoptotic cells after 48 h compared to TBTC or L-DOPA alone, as determined by flow cytometry (data not shown). TBTC (0.5-2.0 μM) plus L-DOPA (20-50 μM) synergistically increased the percentage of apoptotic cells in a concentration- and time-dependent manner compared to TBTC or L-DOPA alone.

DISCUSSION

TBTC at 2.0 μM induces the release of pro-apoptotic factors, cytochrome *c*, and subsequently, activates caspases (Reader *et al.*, 1999). TBTC at 1.0-2.5 μM triggers apoptosis in trout hepatocytes through a step involving Ca^{2+} efflux (Nicoletti *et al.*, 1991). TBTC at 3.0 μM also induces apoptosis by disturbing intracellular Ca^{2+} levels and mitochondrial activity, causing oxidative stress and activation of caspases in rat thymocytes (Gennari *et al.*, 2000). In this study, 2.0 μM TBTC, but not 0.5 to 1.0 μM, also exhibited cell cytotoxicity through an apoptotic process in PC12 cells. These data demonstrate that TBTC at relatively high concentrations (1.0-3.0 μM) induces oxidative stress-mediated apoptosis.

Treatment with L-DOPA (20-50 μM) increases dopamine content approximately 2.0- to 2.5-fold for 24 h in PC12 cells (Migheli *et al.*, 1999; Yin *et al.*, 2004), however, L-DOPA at the same concentrations only increases dopamine content 1.2- to 1.4-fold after 48 h; this attenuation is attributed to oxidative stress (Yin *et al.*, 2004). L-DOPA generates reactive oxygen free radicals, leading to apoptotic oxidative stress mediated by these semiquinone and quinone derivatives (Basma *et al.*, 1995; Migheli *et al.*, 1999). In addition, isoquinoline derivatives, such as tetrahydropapaveroline, are detected in the urine, brain and blood of patients receiving

therapeutic L-DOPA (Sandler *et al.*, 1973). Furthermore, such isoquinoline compounds, including tetrahydropapaveroline, berberine and hydrastine, inhibit dopamine biosynthesis and aggravate L-DOPA-induced cytotoxicity in PC12 cells (Lee *et al.*, 2003; Yin *et al.*, 2004). Some metals such as iron, manganese, zinc and copper also induce cytotoxic effects through oxidative stress in PC12 cells (Cheng *et al.*, 1994; Migheli *et al.*, 1999; Wang *et al.*, 1999). For example, L-DOPA with manganese added to the media is more cytotoxic in PC12 cells than L-DOPA alone (Migheli *et al.*, 1999). Likewise, L-DOPA also causes oxidative DNA cleavage in the presence of copper (Cheng *et al.*, 1994). These results suggest that the coexistence of L-DOPA and some metals and isoquinoline derivatives, which induce oxidative stress, may produce harmful effects in dopaminergic cells.

TBTC at low concentrations (0.5-1.0 μM) is reported to decrease dopamine content by inhibiting tyrosine hydroxylase activity and its gene expression in PC12 cells (IC_{50} value for dopamine biosynthesis, 0.72 μM) (Kim *et al.*, 2002). The increase in dopamine levels induced by L-DOPA (20-50 μM) in PC12 cells was also partially inhibited when L-DOPA was administered with 0.5-2.0 μM TBTC (Kim *et al.*, 2002). In this experiment, TBTC at 0.5-2.0 μM also significantly enhanced L-DOPA (20-50 μM)-induced cytotoxicity in PC12 cells.

In conclusion, TBTC at non-cytotoxic (0.5-1.0 μM) and cytotoxic (2.0 μM) concentrations enhances L-DOPA-induced cytotoxicity in PC12 cells. These results suggest that Parkinsonian patients who receive long-term L-DOPA therapy are potentially at risk from exogenous neurotoxins such as TBTC. The *in vivo* neurobiological toxicity and mechanisms induced by organotin derivatives remain to be fully elucidated.

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