Concentration Dependence of the Mechanisms of Tributyltin-Induced Apoptosis

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Tributyltin chloride (TBT), an endocrine-disrupting chemical, has been used as a heat stabilizer, agricultural pesticide, and component of antifouling paints. In this study, we investigated the concentration dependence of the mechanisms of tributyltin cytotoxicity in PC12 cells. Exposure of PC12 cells to both 500nM and 2μM tributyltin increased the number of cells showing nuclear fragmentation, a typical apoptotic feature, and activated caspase-3. The peak Ca2+ concentration in 2μM tributyltin–treated cells was higher than that in 500nM tributyltin–treated cells. The intracellular Ca2+ increase induced by 2μM tributyltin was mediated by Ca2+ release from both inositol 1,4,5-trisphosphate receptor and ryanodine receptor, while the Ca2+ increase induced by 500nM tributyltin was mediated through the voltage-dependent calcium channel (VDCC). Next, we investigated whether the mechanisms leading to cell death after Ca2+ increase were different. Reactive oxygen species (ROS) were involved only in 2μM tributyltin–induced cell death, while c-jun N-terminal kinase (JNK) mediated only 500nM tributyltin–induced toxicity. Thus, caspase-dependent apoptosis caused by 2μM tributyltin was mediated by a large Ca2+ increase via inositol 1,4,5-trisphosphate receptor and ryanodine receptor, followed by generation of ROS. Apoptosis caused by 500nM tributyltin was mediated by a moderate Ca2+ increase through the VDCC, followed by phosphorylation of JNK. These results suggest that apoptosis by TBT is induced via distinct pathways depending on the TBT concentration, and we showed a rare example that upstream mechanisms of apoptosis are distinct depending on strength of toxic insult.

Key Words: tributyltin; calcium; apoptosis; reactive oxygen species; c-jun N-terminal kinase; caspase.

Organotin compounds have been widely used as heat stabilizers for polyvinyl chloride plastics and wood preservatives, as well as agricultural pesticides. Among them, tributyltin chloride (TBT) has been most widely used in antifouling paints for marine vessels even though its residues represent an environmental and health hazard. Recently, the use of TBT in antifouling paints has been prohibited (van Wezel et al., 2004) due to its severe impact on aquatic ecosystems, but TBT and its degradation products, dibutyltin and monobutyltin, will persist in marine sediments for some time, causing widespread contamination of the marine environment (Iwata et al., 1995; Tanabe et al., 1998). Human exposure to organotin compounds arises from drinking water that has been contaminated with industrial effluents and through leaching of the compounds from polyvinyl chloride water pipes (Snoeij et al., 1987). Tsuda et al. (1995) reported that the daily intake of TBT in Japan was 2.2–6.9 μg, and Whalen et al. (1999) reported the presence of butyltin compounds, including TBT, at concentrations between 50 and 400nM in human blood. TBT is toxic to various organs, including neurons (O’Callaghan et al., 1988), hepatocytes (Jürkiewicz et al., 2004), and sex organs (Heidrich et al., 2001), but the mechanism of its cytotoxicity has not been established.

Intracellular Ca2+ is well recognized as a second messenger. Under normal conditions, the concentration of intracellular Ca2+ is maintained at 10–100nM. However, sustained Ca2+ release from endoplasmic reticulum (ER) or Ca2+ influx through the voltage-dependent calcium channel (VDCC) causes the collapse of Ca2+-homeostasis. Disruption of Ca2+-homeostasis activates various Ca2+-dependent enzymes and induces cell death. Ca2+ is thought to play a role in TBT-induced cell death because Ca2+ chelation alleviates TBT toxicity (Nakatsu et al., 2006b; Yu et al., 2000). Viviani et al. (1995) have reported that the degree of Ca2+ overload caused by TBT is concentration dependent in PC12, but it is not clear whether the subsequent signaling pathway is also different depending on the intracellular Ca2+ concentration.

Here, we investigated the concentration dependence of the mechanisms leading to TBT-induced cell death in PC12 cells. It has been reported that TBT affected dopamine metabolism and decreased dopamine contents (Kim et al., 2002; Tsunoda et al., 2004). Thus, TBT seems to show the toxicity in the dopaminergic neurons. PC12 cells are well used as a model of dopaminergic neurons because PC12 has been reported to synthesize dopamine. Moreover, Viviani et al. (1995) reported that TBT induced apoptosis in PC12 cells. Therefore, we selected PC12 cells to investigate concentration dependence of the mechanisms of tributyltin-induced apoptosis.
Interestingly, we found that TBT induced apoptosis via distinct mechanism depending on its concentration.

MATERIALS AND METHODS

Materials. TBT, nifedipine, α-tocopherol, and xestospongin C were purchased from Wako (Osaka, Japan). A caspase-3 substrate (Ac-DEVD-AMC), a caspase-3 inhibitor (Ac-DEVD-CMK), U-73122, PD98059, and SB202190 were purchased from Calbiochem (Darmstadt, Germany). SP600125 was purchased from Biomer BioLab (Plymouth Meeting, PA). Fetal calf serum (FCS) and horse serum (HS) were purchased from JRH Laboratories (Lenexa, KS). Fura-2-acetoxymethylester and Hoechst 33258 were purchased from Dojindo (Kumamoto, Japan). Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Nissui Pharmaceutical (Tokyo, Japan). Dantrolene and catalase were purchased from Sigma (St Louis, MO). Primary antibodies and horseradish peroxidase–conjugated anti-rabbit antibody were purchased from Cell Signaling Technology (Beverly, MA).

Cell culture. We used rat pheochromocytoma PC12 cells as model of catecholaminergic cells, and they were grown in DMEM containing 10% FCS and 5% HS by referring to previous reports (Jung et al., 2007; Nishina et al., 2007; Pellegrino and Stork, 2006). When the cells were subconfluent, they were placed in collagen-coated multiwell cell culture plates or culture dishes at a density of 1 × 10^5 cells/cm² throughout all assays. The medium was replaced every 2 days, and experiments were performed within 30 passages.

Drug treatment. Inhibitors and TBT were dissolved in dimethylsulfoxide (DMSO). TBT was added to the culture medium (including serum) 30 min after the addition of various inhibitors. Concentrations of inhibitors were determined based on previous reports (Ferreiro et al., 2004; Sperling et al., 2003; Yamamoto et al., 2006). The concentration of DMSO was 0.1 or 0.2%.

Lactate dehydrogenase assay. As an index of cell death, lactate dehydrogenase (LDH) release into the extracellular medium was measured using the Cytotox-one homogenous membrane integrity assay (Promega, Madison, WI), according to the manufacturer’s protocol. Briefly, PC12 cells were plated in multiwell plates, and the assay buffer containing resazurin was added. The plates were incubated for 10 min in the dark for conversion of resazurin to resorufin by LDH. The reaction was stopped by adding stopping solution. The maximum LDH release was measured after addition of the lysis buffer (9% Triton X-100). The fluorescence was measured with an excitation wavelength of 544 nm and an emission wavelength of 590 nm. The percent cytotoxicity was calculated as the percent release of LDH induced by an experimental treatment relative to the maximum LDH release. Inhibitor alone (xestospongin C, dantrolene, nifedipine, U-73122, PD98059, SB202190, α-tocopherol, catalase, and Ac-DEVD-CMK) did not affect the cell viability.

Caspase-3 activity. Caspase-3 activity was measured with a caspase-3 assay kit (Promega) according to the manufacturer’s protocol. Briefly, after exposure to TBT, cell cultures were harvested with a cell scraper in lysis buffer. The cells were centrifuged at 12,000 rpm for 30 min. The supernatant was transferred to a 96-well microplate, and caspase assay buffer containing 10 mM diithiothreitol and 50 μM Ac-DEVD-7-amino-4-methylcoumarin (AMC), a caspase-3 substrate, was added. After incubation for 30 min at 37°C in the dark, caspase activity was measured with a fluorescence microplate reader (excitation 355 nm and emission 460 nm). Caspase-3 activity was expressed as AMC content per milligram of protein.

Hoechst 33258 staining. Cultured cells were stained with Hoechst 33258 to examine nuclear morphological changes. Cells were fixed with neutral formaldehyde for 30 min and then stained with 10 μg/ml Hoechst 33258 for 30 min. Cells were visualized and photographed under ultraviolet illumination using a fluorescence microscope.

Measurement of intracellular Ca^{2+}. We measured intracellular Ca^{2+} as described in our previous report (Nakatsu et al., 2006b). PC12 cells were plated on glass coverslips with a silicon rubber wall (Flexiplant, Heraeus Bio-technology, Hanau, Germany) and incubated overnight at 37°C to allow adhesion. Then, the cells were loaded with 5 μM fura-2 for 30 min in HEPES-buffered salt solution (HBSS) containing 125 mM NaCl, 5 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 1.2 mM CaCl₂, 6 mM glucose, and 25 mM HEPES. They were washed three times with HBSS and then were placed on a fluorescence image microscope. TBT was applied to HBSS in the presence of inhibitors 1 min after measurement was started. Changes in Ca^{2+} concentration were evaluated by means of fluorescence intensity measurements with excitation at 340 and 380 nm and an emission wavelength of 510 nm. The video image output was digitized by an ECLIPSE TE2000-U (Nikon, Tokyo, Japan). Data are expressed as the fluorescence ratio at the two wavelengths. All points represent the means of 30 cells.

Measurement of reactive oxygen species. Reactive oxygen species (ROS) was measured according to a reported method (Lee et al., 2005), using the dye, 2’,7’-dichlorodihydrofluorescein diacetate (DCFH-DA). DCFH-DA is oxidized to 2’,7’-dichlorofluorescein (DCF) by hydrogen peroxide and DCF generates fluorescence. Briefly, PC12 cells were plated at a density of 10^5 cells per 35-mm dish. After having been exposed to TBT, cells were incubated with 50 μM DCFH for 30 min at 37°C and washed with phosphate-buffered saline (PBS). Cell suspensions were centrifuged at 1000 rpm for 3 min. The medium was removed, and the cells were dissolved in 1% Triton X-100. The fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a fluorescence microplate reader. The fluorescence (arbitrary units) was normalized with respect to the amount of protein. The amount of protein was measured by Bradford method.

Western blotting. After TBT treatment, cells were washed with PBS buffer and lysed in TNE buffer containing 50 mM Tris-HCl, 1% NP-40, 20 mM ethylenediaminetetraacetic acid, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, and protease inhibitor cocktail (1:200). The mixture was centrifuged at 13,500 rpm for 3 min, and then the supernatant was transferred to an Eppendorf tube. The supernatant was added to the sample buffer containing 125 mM Tris-HCl, 4% sodium dodecyl sulfate (SDS), 10% sucrose, 0.004% bromophenol blue, and 10% mercaptoethanol and then denatured at 95°C for 3 min. The same amount of protein (30 μg) was separated by 10% SDS polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with blocking buffer containing 3% skim milk for 1 h and then incubated with primary antibody (1:1000) overnight and secondary antibody (1:2000) for 1 h. The protein was detected with an enhanced chemiluminescence detection system.

Statistics. Reproducibility of results was confirmed by at least two different sets of experiments. Data shown in figures are from a representative set of experiments. Data are expressed as mean ± SEM. Statistical evaluation of the data was performed by ANOVA followed by Newman-Keul’s test. A value of p < 0.05 was considered to be indicative of significance.

RESULTS

Cytotoxicity of TBT in PC12 Cells

Firstly, we investigated the cytotoxicity of TBT to PC12 cells by measuring released LDH activity, which represents leakage of the enzyme from dead cells. Application of 0.1% DMSO did not affect released LDH activity over the course of 24 h (data not shown). Exposure of 50 mM TBT for 24 h did not affect cell viability (data not shown). PC12 cells were exposed to 500 mM, 2 μM, and 5 μM TBT for various times. Exposure to 500 mM TBT for 24 h induced about 80% cell death (Fig. 1A). TBT (2 μM) for 6 h and TBT (5 μM) for 1 h had a similar effect (Figs. 1B and 1C).
Detection of Apoptosis

To examine whether cell death caused by TBT is apoptosis, we investigated the effect of caspase-3 inhibitor on TBT toxicity. Caspase-3 inhibitor reduced both 500nM TBT– and 2µM TBT–induced cell deaths by about 50% (Figs. 2A and 2B), but it did not have cytoprotective effect on 5µM TBT toxicity (Fig. 2C). These results indicate that 500nM and 2µM TBT induced caspase-dependent cell death but that cell death caused by 5µM TBT was not caspase dependent. Many previous reports have demonstrated that caspase-dependent cell death is apoptosis. Therefore, we confirmed that 2µM TBT– and 500nM TBT–induced PC12 cell death is apoptotic. We examined whether TBT activated caspase-3. Both 500nM and 2µM TBT increased caspase-3 activity at 1 h after exposure (Figs. 2D and 2E). Morphological changes in the nuclei after exposure of cells to TBT were examined by Hoechst 33258 staining. Nuclear condensation, an apoptotic feature, was recognized in cells treated with 2µM and 500nM TBT (Figs. 3B and 3D), while few control cells showed this feature (Figs. 3A and 3C).

Involvement of Intracellular Ca²⁺ Increase in TBT Cytotoxicity

Next, we investigated the pathway of TBT-induced cell death. Because it has been reported that Ca²⁺ chelators decreased TBT toxicity (Nakatsu et al., 2006b; Yu et al., 2000), we investigated involvement of intracellular Ca²⁺ in 500nM TBT– and 2µM TBT–induced cell deaths. Consistent with previous report, TBT increased intracellular Ca²⁺ depending on its concentration (Fig. 5A). We investigated whether 50nM TBT, nontoxic levels, changed intracellular Ca²⁺ concentration and it induced Ca²⁺ increase slightly (data not shown). It is known that Ca²⁺ is released from IP₃ receptor and ryanodine receptor on ER, or it may enter the cells from the medium through VDCCs. We used pharmacological inhibitors to investigate the involvement of their receptors. IP₃ receptor antagonist, xestospongin C (3µM), and ryanodine receptor antagonist, dantrolene (40µM), decreased 2µM TBT toxicity but VDCC inhibitor, nifedipine (10µM), did not (Figs. 4A–C). Xestospongin C and dantrolene also reduced 2µM TBT–induced Ca²⁺ increase (Figs. 5B and 5C). To test the
involvement of phospholipase C (PLC), which produces IP₃, we examined the effect of a PLC inhibitor, U-73122, on 2µM TBT–induced cell death and Ca²⁺ increase. PLC activation appeared to be required for both phenomena (Figs. 4D and 5D). On the other hand, application of nifedipine decreased 500nM TBT–induced cell death, but other inhibitors have no effect (Figs. 4E–H). Nifedipine also decreased Ca²⁺ increase of 500nM TBT (Fig. 5E).

Involvement of ROS in TBT Cytotoxicity

Because it is known that an increase of intracellular Ca²⁺ generates ROS, we examined the involvement of ROS in TBT cytotoxicity. α-Tocopherol (100µM) and catalase (1000 U/ml) rescued the cells from cytotoxicity due to 2µM TBT (Fig. 6A) but not 500nM TBT (Fig. 6B). Next, we attempted to detect ROS generated by 2µM TBT by using DCFH. Exposure to 2µM TBT for 1 h increased the generation of ROS, and the effect was sustained up to 6 h (Fig. 6C). On the other hand, exposure to 500nM TBT did not increase the level of intracellular ROS (Fig. 6D). These results suggest that ROS is involved only in cytotoxicity induced by 2µM TBT. Further, we examined whether inhibition of calcium release from ER would reduce ROS generation. Xestospongin C inhibited ROS generated by 2µM TBT, and dantrolene slightly, though not significantly, decreased ROS (Fig. 6E), suggesting that intracellular Ca²⁺ increase via IP₃ receptor plays an important role in ROS generation.

Involvement of Mitogen-Activated Protein Kinases in TBT Cytotoxicity

Because several lines of evidence suggest that phosphorylation of mitogen-activated protein kinases (MAPKs) leads to cell death (Chen et al., 1996; Ma et al., 1999), the involvement of MAPKs in TBT cytotoxicity was examined using PD98059 (a mitogen-extracellular signal-regulated kinase [MEK] inhibitor, 10µM), SP600125 (a c-jun-N-terminal kinase [JNK] inhibitor, 10µM), and SB202190 (a p38 MAPK inhibitor, 10µM). None of these inhibitors affected the cytotoxicity due to 2µM TBT (Fig. 7A). On the other hand, the cytotoxicity due to 500nM TBT was decreased by SP600125 but not by PD98059 or SB202190 (Fig. 7B). Because these results suggested that JNK is involved in 500nM TBT–induced cell death, we examined whether JNK was phosphorylated by 500nM TBT. JNK was found to be activated 1 h after exposure of cells to 500nM TBT, and the phosphorylation level had reverted to the control level by 24 h (Fig. 7D). Interestingly, application of 2µM TBT also activated JNK, although SP600125 did not reduce 2µM TBT–induced cell death (Fig. 7C). Next, we investigated the relationship between JNK phosphorylation and intracellular Ca²⁺ increase. JNK

FIG. 3. Change of nuclear morphology. (A–D) Photographs of nuclear morphology revealed with Hoechst 33258 staining. PC12 cells were exposed to 0.1% DMSO [for 6 h (A) or 24 h (C)] or TBT [2µM for 6 h (B) or 500nM for 24 h (D)].
phosphorylation induced by 500nM TBT was blocked by nifedipine as well as by SP600125 (Fig. 7E).

**Involvement of Caspase in TBT Cytotoxicity**

Because TBT cytotoxicity was inhibited by Ac-DEVD-CMK, we examined the effect of the inhibitors used in this study on TBT-induced caspase activation to determine whether Ca\(^{2+}\), ROS, and JNK are involved in caspase activation. The caspase activation induced by 2\(\mu\)M TBT was reduced by dantrolene, xestospongin C, U-73122, and antioxidants (Fig. 8A).

Nifedipine and SP600125 reduced caspase-3 activation induced by 500nM TBT (Fig. 8B).

**DISCUSSION**

Many reports have demonstrated that TBT increases intracellular Ca\(^{2+}\) concentration and induced apoptosis (Mizuhashi et al., 2000a; Nakatsu et al., 2006b; Oyama et al., 1994; Viviani et al., 1995). Bonfoco et al. (1995) reported that N-methyl-D-aspartate and nitric oxide can induce either apoptotic or necrotic cell death, depending on the strength of the initial insult. Cheung et al. (1998) demonstrated that...
has been suggested that distinct upstream mechanisms exist in apoptosis and necrosis by different concentrations of an organotin compound, trimethyltin (Gunasekar et al., 2001), but whether mechanism of TBT-induced apoptosis is different depending on its concentration has been remained to be elucidated. In this study, we examined the mechanisms of concentration-dependent TBT-induced apoptosis. We selected 500nM TBT as low dose and 2μM TBT as high dose to investigate distinct mechanism of apoptosis caused by TBT because inhibition of caspase-3, apoptosis-related protease, decreased 500nM and 2μM TBT but not 5μM TBT (Figs. 2A–C). Moreover, we confirmed that 500nM TBT– or 2μM TBT–treated cells activated caspase-3 (Figs. 2D and 2E) and showed nuclear fragmentation, one of the apoptotic features (Figs. 3A–D). These results indicate that cell death caused by 500nM and 2μM TBT is apoptosis. Caspase-3 inhibitor reduced 500nM and 2μM TBT cytotoxicity by about 50% (Figs. 2A and 2B), suggesting that caspase-independent pathway may be present. For example, it is known that translocation of apoptosis-inducing factor from mitochondria to nuclear and cathepsin play important roles in caspase-independent apoptosis (Bidere et al., 2003; Foghsgaard et al., 2001; Joza et al., 2001; Susin et al., 1999).

Jurkiewicz et al. (2004) reported that TBT-induced cell death showed apoptotic feature at a range of 2.5–3μM. Reader et al. (1999) have demonstrated that 2μM TBT induced apoptotic cell death in trout hepatocytes. Mizuhashi et al. (2000a) have reported that apoptotic cell death was induced by 3μM TBT in hippocampal slice cultures. Thus, TBT induces apoptotic cell death at a range of 2–3μM in other cell lines as same as PC12 cells.

TBT (2μM) induced a Ca\(^{2+}\) increase via both IP\(_3\) receptor and ryanodine receptor, while the Ca\(^{2+}\) increase caused by 500nM TBT was mediated through VDCC (Figs. 4A–E). Kawanishi et al. (2001) have reported that 4μM TBT released calcium from intracellular stores, whereas 100nM TBT, nontoxic levels, suppressed the IP\(_3\)-induced calcium release (ICR). Their report suggests that TBT shows concentration-dependent distinct effects on intracellular Ca\(^{2+}\) store and that TBT, which did not change intracellular Ca\(^{2+}\) level, may affect Ca\(^{2+}\) increase through VDCC and ryanodine receptor. It has been reported that TBT inhibits Ca\(^{2+}\) ATPase \textit{in vitro} and \textit{in vivo} (Yallapragada et al., 1990, 1991). Therefore, it suggests that TBT potentiates Ca\(^{2+}\) increase caused by thapsigargin. We investigated whether 50nM TBT, nontoxic levels, changed intracellular Ca\(^{2+}\) concentration, and it induced Ca\(^{2+}\) increase slightly (data not shown). Although 50nM TBT may suppress the IICR, it may induce Ca\(^{2+}\) increase through IP\(_3\) influx from VDCC and its increase seems not to be enough for disruption of calcium homeostasis. Our results also showed that 2μM TBT induced Ca\(^{2+}\) efflux from intracellular Ca\(^{2+}\) store although 500nM TBT did not cause Ca\(^{2+}\) increase via IP\(_3\) receptor or ryanodine receptor. Thus, high dose of TBT seems to be able to induce Ca\(^{2+}\) efflux from intracellular Ca\(^{2+}\) store.
Our data indicate that PLC may be involved in the case of 2μM TBT toxicity because the PLC inhibitor U-73122 decreased the cytotoxicity of 2μM TBT (Fig. 4D). PLC activation may increase the concentration of IP3 in cytoplasm, thereby stimulating the IP3 receptor, leading to Ca^{2+} release. Ca^{2+} release from the IP3 receptor is thought to stimulate the ryanodine receptor (so-called calcium-induced calcium release). On the other hand, 500nM TBT did not appear to...
induce Ca\(^{2+}\) release from ER because inhibition of PLC activation did not reduce the cytotoxicity of 500nM TBT (Fig. 4H). VDCC was involved in the Ca\(^{2+}\) increase caused by 500nM TBT. Ca\(^{2+}\) release from ER is surmised to induce depolarization and to open VDCC, but the VDCC opening induced by 500nM TBT appeared to be unrelated to Ca\(^{2+}\) release from ER because xestospongin C and dantrolene did not suppress the increase of Ca\(^{2+}\) caused by 500nM TBT (data not shown). These results suggest that TBT activates VDCC independently of Ca\(^{2+}\) release from ER.

ROS was involved in the cytotoxicity only in the case of 2\(\mu\)M TBT (Figs. 6A and 6B). Gennari et al. (2000) have reported that TBT generated ROS in rat thymocytes. Mitochondria play an important role in the generation of ROS, but the intracellular Ca\(^{2+}\) increase induced by 500nM TBT may not be large enough to induce generation of ROS in mitochondria.

On the other hand, JNK mediated only 500nM TBT–induced cell death (Figs. 7A and 7B). Many reports have demonstrated that JNK is involved in apoptosis (Tournier et al., 2000; Verheij et al., 1996), and our results support this view. We recently reported that extracellular-regulated kinase and p38 were involved in 500nM TBT neurotoxicity in cortical neurons (Nakatsu et al., 2006a), and Mizuhashi et al. (2000b) showed the results that morphological changes of astrocytes caused by TBT was induced through MEK activation. Thus, the kind of MAPKs involved in TBT toxicity may be different in different cell types. A JNK inhibitor did not influence 2\(\mu\)M TBT cytotoxicity (Fig. 7A), although 2\(\mu\)M TBT activated JNK after 1 h (Fig. 7C). TBT (2\(\mu\)M)-induced cell death seems to be mainly mediated by ROS and not by JNK. JNK phosphorylation caused by 500nM TBT was blocked by nifedipine, as well as SP600125 (Fig. 7E), suggesting that calcium influx through VDCC is required for JNK phosphorylation by 500nM TBT. It has been reported that calcium is involved in JNK phosphorylation (Kim and Sharma, 2004; Yu et al., 2000), and our results are consistent with that.

Both 2\(\mu\)M and 500nM TBT caused caspase-3 activation in PC12 cells (Figs. 2D and 2E). TBT (2\(\mu\)M)-induced caspase activation was reduced by antioxidants, PLC inhibitor, ryanodine receptor antagonist, and IP\(_3\) receptor antagonist (Fig. 8A). These results suggest that ROS and Ca\(^{2+}\) efflux from ER are required for the caspase activation caused by 2\(\mu\)M TBT. Although application of 2\(\mu\)M TBT phosphorylated JNK, SP600125 did not alleviate 2\(\mu\)M TBT–induced cell death. In 2\(\mu\)M TBT–treated cells, ROS seems to play more important roles in caspase-3 activation than JNK. Indeed, we confirmed that SP600125 did not affect 2\(\mu\)M TBT–induced caspase-3 activation (data not shown). On the other hand, 500nM TBT–induced caspase activation was reduced by SP600125 and nifedipine (Fig. 8B), suggesting that caspase-3 activation caused by 500nM TBT was downstream of JNK and VDCC. It is already well established that ROS or JNK is involved in caspase activation (Chen et al., 1998; Morishima et al., 2001). These reports support our results. Thus, the upstream pathways of caspase-3 activation by TBT are concentration dependent.

In summary, we have demonstrated that both 500nM and 2\(\mu\)M TBT induce apoptosis via caspase-3 activation, but the upstream pathways are different. Apoptosis by 2\(\mu\)M TBT was mediated by a large increase of intracellular Ca\(^{2+}\) from the IP\(_3\) receptor and ryanodine receptor, followed by generation of ROS. On the other hand, apoptosis by 500nM TBT was caused by a moderate increase of intracellular Ca\(^{2+}\) through VDCC, followed by phosphorylation of JNK (Fig. 9). Our results provide the first evidence that TBT concentration-dependent distinct upstream mechanisms can lead to apoptotic cell death.

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