Glutamate Excitotoxicity Is Involved in Cell Death Caused by Tributyltin in Cultured Rat Cortical Neurons

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Tributyltin, an endocrine-disrupting chemical, has been used as a heat stabilizer, agricultural pesticide, and component of anti-fouling paints. In this study, the neurotoxicity of tributyltin was investigated in cultured rat cortical neurons. Tributyltin caused marked time- and dose-dependent increases in the number of trypan blue–stained cells. Measurement of extracellular glutamate concentration showed that glutamate release was induced by tributyltin. Application of the glutamate receptor antagonists MK-801 and CNQX decreased the neurotoxicity. These results suggest that released glutamate and glutamate receptors are involved in tributyltin toxicity. Next, we examined whether various factors, believed to be involved in glutamate excitotoxicity also influence tributyltin toxicity. Cell death induced by tributyltin was found to be reduced by α-tocopherol (a membrane-permeable antioxidant), SB202190 (a p38 mitogen-activated protein kinase inhibitor), and U-0126 (an extracellular signal-regulated protein kinase kinase inhibitor). MK-801 and CNQX decreased the phosphorylation of ERK, but not that of p38. A caspase-3 inhibitor had no effect on tributyltin toxicity, and tributyltin did not change the nuclear morphology. These results suggest that the glutamate excitotoxicity caused by tributyltin is unrelated to apoptosis. In conclusion, we demonstrated that tributyltin induced glutamate release and subsequent activation of glutamate receptors, leading to neuronal death. We propose two independent neuronal death pathways by tributyltin; one is glutamate receptor–dependent cell death via ERK phosphorylation, and the other may be glutamate receptor–independent cell death via p38 activation.

Key Words: tributyltin-glutamate release-NMDA receptors; reactive oxygen species; mitogen-activated protein kinase; caspase.

INTRODUCTION

Organotin compounds have been widely used as heat stabilizers in vinyl chloride polymers, wood preservatives, and agricultural pesticides. Among them, tributyltin chloride (TBT) has been most widely used in marine antifouling paints, even though its residues represent an environmental and health hazard. The use of TBT in antifouling paints has been restricted recently (van Wezel et al., 2004), but TBT and its degradation products dibutyltin and monobutyltin will remain in marine sediments for some time to come, because the half-life of TBT is long, about 2 years (Sarradin et al., 1995). Organotin compounds contaminate human food and water through contributions from industrial effluents and leaching from polyvinyl chloride water pipes (Snoeij et al., 1987). Indeed, 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 nM and 400 nM in human blood.

Tributyltin chloride has been reported to show toxicity to mammals in addition to marine organisms. Ema et al. (1991) reported that TBT caused significant changes in rat behavior. Exposure of mammals to organotin compounds can induce epilepsy, amnesia, and memory defects (Feldman et al., 1993); it is toxic to the developing central nervous system (CNS) (O’Callaghan and Miller, 1988). Little is known about the mechanism of TBT toxicity, but several lines of evidence suggest that TBT causes an increase in intracellular calcium in various cells, including thymocytes (Chow et al., 1992), hepatocytes (Kawanishi et al., 2001), and PC12 cells (Viviani et al., 1995).

In the CNS, glutamate is an important factor for maintaining calcium homeostasis; it is the most abundant excitatory neurotransmitter, and it is widely distributed. Glutamate is associated with various brain functions, such as synaptic plasticity, learning, and long-term potentiation (Collingridge and Laster, 1989). Its physiological and pathological effects in the CNS are mediated mainly via two types of ionotropic glutamate receptors, the NMDA receptor and the non-NMDA receptor. Activation of NMDA receptors allows influx of extracellular calcium $^{2+}$, but excessive release of glutamate causes prolonged stimulation of NMDA receptors, inducing calcium...
overload and neuronal death (so-called excitotoxicity). Excitotoxicity has been speculated to be involved in many neurodegenerative diseases, such as Alzheimer’s disease (Hynd et al., 2004), Parkinson’s disease (Blandini et al., 1996), and amyotrophic lateral sclerosis (ALS) (Rao and Weiss., 2004). Moreover, glutamate neurotoxicity has been reported to play an important role in the neurotoxicity of environmental pollutants, such as methyl mercury (Farina et al., 2003).

In the present study, we examined whether glutamate is involved in TBT toxicity in rat cortical cultures. We investigated whether TBT induces glutamate release and whether NMDA receptor antagonists reduce TBT toxicity. Moreover, we examined whether several factors involved in glutamate excitotoxicity also influence TBT toxicity in cortical neurons.

MATERIALS AND METHODS

Chemicals. Eagle’s minimal essential salt medium (Eagle’s MEM) was purchased from Nissui Pharmaceutical (Tokyo, Japan). Fetal calf serum (FCS) and horse serum (HS) were purchased from JRH Biosciences (Lenexa, KS), and Sigma (St. Louis, MO). L-glutamine (2 mM), D-(-)-glucose (11 mM), NaHCO₃ (24 mM), and HEPES were purchased from Wako (Osaka, Japan). Catalase and glutamine were purchased from Sigma (St. Louis, MO). MK-801 was purchased from Funakoshi (Osaka, Japan). CNQX was purchased from Torcis (Bristol, UK), HEPE was purchased from DOJINDO (Kumamoto, Japan), U-0126, SB202190, and caspase-3 inhibitor (Ac-DEVD-CMK) were purchased from Calbiochem (Darmstadt, Germany), SP600125 was purchased from Biomol Research Laboratories Inc. (Plymouth Meeting, PA). Primary antibodies and horseradish peroxidase–conjugated anti-rabbit antibody were purchased from Cell Signaling Technology (Beverly, MA).

Cell culture. The following procedures were performed under sterile conditions. The animals were handled in accordance with the guidelines for the care and use of experimental animals published by the Japanese Association for Laboratory Animal Science in 1987. Primary cultures were obtained from the cerebral cortex of fetal rats (at 18 days of gestation) as described elsewhere (Taguchi et al., 2003). Fetuses were taken from pregnant Slc; Wistar/ST rats about 90% neurons (Taguchi et al., 1996). The cultures were maintained in a humidified 5% CO₂ atmosphere at 37°C for 3 days, after which the supernatant was transferred to an Eppendorf tube. The supernatant was added to sample buffer containing 125 mM Tris·HCl, 4% SDS, 10% sucrose, 0.004% BPB, and 10% mercaptoethanol, then denatured at 95°C for 3 min. Protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with blocking buffer containing 5% skim milk for 1 h, 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.

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 EDTA, 0.2 mM sodium orthovanadate, and 1 mM PMSF. The mixture was centrifuged at 13,500 rpm for 3 min, after which the supernatant was transferred to an Eppendorf tube. The supernatant was added to sample buffer containing 125 mM Tris·HCl, 4% SDS, 10% sucrose, 0.004% BPB, and 10% mercaptoethanol, then denatured at 95°C for 3 min. Protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with blocking buffer containing 5% skim milk for 1 h, 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.

Hoecbst 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 0.1 mg/ml Hoechst 33258 for 30 min. Cells were visualized and photographed under ultraviolet illumination, with a fluorescence microscope.

Statistics. Data are expressed as mean ± SEM. Statistical evaluation of the data was performed by Student’s t-test (Fig. 1 and Fig. 2A) or by analysis of variance (ANOVA) followed by the Newman-Keul’s test (except Fig. 1 and Fig. 2A). A value of p < 0.05 was considered to be indicative of significance.

RESULTS

Neurotoxicity of TBT in Cortical Neurons

Toxicity of TBT in rat cortical neurons was examined by the trypan blue method (dead cells stained with trypan blue). Cortical neurons were exposed to various concentrations of TBT, and exposure of the cells to 500 nM TBT for 24 h resulted in a remarkable degree of neurotoxicity (survival of 49.8 ± 0.4%) (Fig. 1A). Increased numbers of trypan blue–stained cells were observed after 12 h (Fig. 1B).

Involvement of Glutamate in TBT Neurotoxicity

We examined whether glutamate is involved in TBT toxicity, because glutamate is believed to be a factor in neuronal death. First, the amount of glutamate in the medium was measured.
The concentration of extracellular glutamate was increased significantly by exposure of the cells to 500 nM TBT for 30 min, and that increase was exposure time–dependent (Fig. 2A). Next, to investigate the involvement of glutamate release in TBT toxicity, we used NMDA receptor antagonists. Application of 5 μM MK-801, a noncompetitive NMDA receptor antagonist, alleviated TBT neurotoxicity (survival with TBT: 45.7 ± 2.2%; +U-0126: 66.7 ± 1%) and that 10 μM SB202190, a p38 inhibitor, also significantly alleviated the neurotoxicity (survival with +SB202190: 76.7 ± 6.8%). However, 10 μM SP600125, a c-jun-N-terminal kinase (JNK) inhibitor, was ineffective against TBT-induced cell death (Fig. 4A). Further, we investigated whether TBT phosphorylated ERK and p38. As shown in Figure 4B and 4C, at 3 h after application of 500 nM TBT to the culture, p38 was phosphorylated, and at 6 h, ERK was activated. Phosphorylation of ERK and p38 was sustained until 24 h after exposure. We also examined the effects of glutamate receptor antagonists on ERK and p38 phosphorylation and found that MK-801 and CNQX decreased the phosphorylation of ERK, but not that of p38 (Fig. 4D and 4E).

**Effect of Antioxidants on TBT Toxicity**

It is known that oxidative stress contributes to glutamate toxicity (Campisi et al., 2004). Therefore, we examined the effect of antioxidants on TBT toxicity. We used water-soluble antioxidants (ascorbic acid and catalase) and a lipophilic antioxidant (α-tocopherol). Co-administration of 100 μM ascorbic acid or 1000 U/ml catalase during TBT exposure failed to reduce TBT toxicity. In contrast, co-administration of 100 μM α-tocopherol remarkably inhibited TBT-induced neurotoxicity (survival with TBT: 49.3 ± 3.7%; with α-tocopherol 82.7 ± 4.5%) (Fig. 3).

**Effect of MAPK Inhibitors on TBT Toxicity**

Mitogen-activated protein kinase (MAPK) has been implicated in glutamate-induced neuronal death (Cao et al., 2004). Therefore we used inhibitors of the MAP kinase cascade to examine whether MAPKs are involved in TBT neurotoxicity. We found that 10 μM U-0126, an extracellular signal-regulated protein kinase (ERK) kinase inhibitor, reduced TBT neurotoxicity (survival with TBT: 46.4 ± 2.2%; +U-0126: 66.7 ± 1%) and that 10 μM SB202190, a p38 inhibitor, also significantly alleviated the neurotoxicity (survival with +SB202190: 76.7 ± 6.8%). However, 10 μM SP600125, a c-jun-N-terminal kinase (JNK) inhibitor, was ineffective against TBT-induced cell death (Fig. 4A). Further, we investigated whether TBT phosphorylated ERK and p38. As shown in Figure 4B and 4C, at 3 h after application of 500 nM TBT to the culture, p38 was phosphorylated, and at 6 h, ERK was activated. Phosphorylation of ERK and p38 was sustained until 24 h after exposure. We also examined the effects of glutamate receptor antagonists on ERK and p38 phosphorylation and found that MK-801 and CNQX decreased the phosphorylation of ERK, but not that of p38 (Fig. 4D and 4E).

**Effect of Caspase-3 Inhibitor on TBT Toxicity**

Because it has been reported that TBT activates caspase-3 in various cells (Nopp et al., 2002; Jurkiewicz et al., 2004), we used a caspase-3 inhibitor to confirm the involvement of caspase in TBT toxicity in cortical neurons. However, 10 μM of the caspase-3 inhibitor Ac-DEVD-CMK did not reduce TBT toxicity (Fig. 5A). Moreover, we investigated whether 500 nM TBT alters the nuclear morphology (an indication of apoptosis) of cortical neurons, and we found that it did not (Fig. 5C), although 2 μM TBT did induce nuclear fragmentation in PC12 cells, in agreement with a previous report (Fig. 5D) (Viviani et al., 1995).

**DISCUSSION**

Among organotin compounds, trimethyltin is neurotoxic, whereas tributyltin is primarily known as an immunotoxin.
FIG. 2. Involvement of glutamate in TBT neurotoxicity. A. Glutamate release induced by TBT. Cortical neurons were exposed to 500 nM TBT for various times. Then the supernatant was mixed with reaction buffer to measure the amount of glutamate released. (n = 4) ***p < 0.001 versus control (time = 0). Statistical evaluation of the data was performed with Student’s t-test. B, C, and D. Effect of glutamate receptor antagonists and nifedipine on TBT neurotoxicity. MK-801 (5 μM), CNQX (20 μM), or nifedipine (10 μM) was applied for 30 min, then the cells were exposed to 500 nM TBT for 24 h in the presence of the antagonists. ***p < 0.001 versus control, ###p < 0.001 versus TBT (n = 3). Statistical evaluation of the data was performed by ANOVA followed by the Newman-Keul’s test. E. Photographs of cortical neurons stained with trypan blue. Nontreated culture. F. Culture treated with 500 nM TBT for 24 h. G. Culture treated with MK-801+TBT. H. Culture treated with CNQX+TBT. Data are expressed as mean ± SEM. Scale bar = 100 μm.
(Snoeij et al., 1985). However, neurotoxicity of TBT has also been reported (O’Callaghan and Miller, 1988; Tsunoda et al., 2005), although its mechanism has been little investigated. Trimethyltin and tributyltin are thought to have different mechanisms of toxicity. For example, TMT is reported to exert neurotoxicity at least in part by binding specifically to the protein stannin, whereas TBT does not bind to stannin (Thompson et al., 1996, Buck et al., 2003).

Organotin compounds are important environmental pollutants, in part because of their toxicity. Braman et al. (1979) reported that methyltin compounds, including TMT, were detected at concentrations between 0.5 pM and 4 nM in human urine. Further, the butyltin concentration in human blood ranges from 50 nM to 400 nM (Whalen et al., 1999), and that in human liver is about 400 nM (Kannan and Falandysz, 1997; Mizuhashi et al., 2000). The TBT concentration in rock shells caught in polluted sea areas around Japan was about 5 $\mu$M (Horiguchi et al., 1994; Mizuhashi et al., 2000). The concentration of 500 nM TBT used in our experiments thus appears to be similar to levels of human exposure.

O’Callaghan and Miller (1988) reported that TBT decreased brain weight at dose levels that had no influence on body weight. This finding suggests that the brain may be particularly susceptible to injury by TBT. We found that exposure of cortical neurons to 500 nM TBT for 24 h significantly reduced their viability (Fig. 1). Thompson et al. (1996) reported that the 50% toxic concentration of TBT for 48 h of exposure ranges from 5.5 $\mu$M to 9 $\mu$M for non-neuronal cells, such as NIH-3T3, TC7, and B cells, and that primary neuronal cells were more sensitive to TBT than non-neuronal cells. These results suggest that TBT exerts neuronal toxicity via a specific mechanism, at lower concentrations than the levels causing toxicity in other organs.

We examined the involvement of glutamate in TBT-induced neurotoxicity because glutamate is believed to play important roles in various types of neuronal death. We found that TBT increased extracellular glutamate concentration. The mechanism of glutamate release is not clear, but it is unlikely to be due to leakage from dead cells, because significant numbers of dead cells were not detected for up to 6 h after exposure to TBT. In our study glutamate release was not inhibited by NMDA receptor antagonists or a VDCC inhibitor (data not shown).
Thus TBT appears to induce glutamate release via a Ca\textsuperscript{2+}-independent pathway. Application of the glutamate receptor antagonists MK-801 and CNQX reduced the level of TBT toxicity (Fig. 2). These results support the idea that release of glutamate and activation of glutamate receptors are involved in TBT toxicity. It was previously reported that non-NMDA receptors were involved in TBT neurotoxicity in hippocampal slice cultures, but NMDA receptors and VDCC were not involved (Mizuhashi et al., 2000). Activation of both NMDA receptors and non-NMDA receptors may not necessarily be required for excitotoxicity (Kubo et al., 2001; Hakuba et al., 2003). The difference in the involvement of NMDA receptors may reflect differences in experimental conditions, such as brain region or exposure conditions. Also, VDCC plays important roles in other effects of glutamate excitotoxicity, such as ischemia (Hou et al., 2002). In our experimental model, nifedipine blocked neuronal death caused by TBT, and this result indicates that VDCC is involved in TBT toxicity in our system.

Moreover, we investigated the effects of combinations of inhibitors on TBT neurotoxicity, but we found that the combinations did not show potentiated inhibition (data not shown). Non-NMDA receptors are supposed to open after glutamate release, and their activation induces a change in the membrane potential. Glutamate and cancellation of Mg\textsuperscript{2+} blockade (caused by the change of membrane potential) are needed for NMDA receptor activation. Therefore, non-NMDA receptors should be activated before NMDA receptors and VDCC. This chain of activation is presumably why the effects of inhibitor combinations are the same as those of a single inhibitor.

Because reactive oxygen species (ROS) are generated by activation of the glutamate receptor (Campisi et al., 2004), we also examined whether ROS are involved in TBT toxicity. Application of \(\alpha\)-tocopherol blocked cell death induced by TBT, but catalase and ascorbic acid had no effect (Fig. 3). These results suggest that intracellular generation of ROS is important, because \(\alpha\)-tocopherol is more lipophilic and cell-permeable than the other two antioxidants. Ahlemeyer and Krieglstein (2000) showed that \(\alpha\)-tocopherol inhibited mitochondrial reactive ROS production, indicating that \(\alpha\)-tocopherol can decrease intracellular ROS. Ciani et al. (1996) reported that
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\(\alpha\)-tocopherol, but not ascorbic acid, decreased glutamate neurotoxicity. Kim et al. (1998) reported that glutamate exerts toxicity via lipid peroxidation. Several investigators have described that the most effective agents against glutamate toxicity appear to be chain-breaking antioxidants that block lipid peroxidation, such as \(\alpha\)-tocopherol (Ciani et al., 1996; Halliwell, 1992; Siesjo et al., 1989). These findings are consistent with our results.

Next, the effects of MAPK inhibitors were examined, because MAPKs play important roles in glutamate excitotoxicity (Mao et al., 2004). Yu et al. (2000) reported that TBT phosphorylated ERK, JNK, and p38 in human T lymphoblastoid cells CCRF-CEM. In our experimental model, a MEK inhibitor, U-0126, and a p38 MAPK inhibitor, SB202190, decreased TBT toxicity, but a JNK inhibitor did not (Fig. 4). Glutamate receptor antagonists inhibited phosphorylation of ERK, but not that of p38. These results indicate that ERK phosphorylation depends on glutamate receptors, and that there may also be another pathway involving p38 activation. It has been reported that glutamate activates ERK (Jiang et al., 2000). Activation of ERK is supposed to be characteristic of glutamate toxicity, and our results are consistent with this idea.

It is well known that caspase family members are the central executors of apoptosis, and among them, caspase-3 is the most important. Tributyl tin activates caspase-3 in various cells, such as Jurkat T cells, eosinophils, neutrophils, and hepatocytes (Nopp et al., 2002, Jurkiewicz et al., 2004). Caspase-3 inhibitor did not protect neurons from TBT toxicity (Fig. 5A), and TBT did not induce any change in nuclear morphology (Fig. 5C). There have been many reports that glutamate-induced neurotoxicity is apoptotic (Kawasaki et al., 1997; Du et al., 1997) or necrotic (Sohn et al., 1998; Dessi et al., 1993). Our results indicate that glutamate neurotoxicity caused by TBT is not apoptotic, at least in our experimental system.

In summary, we have partially clarified the mechanism of TBT neurotoxicity in cultured rat cortical neurons (Fig. 6).

Tributyl tin at low concentration was toxic to cortical neurons through glutamate release and subsequent activation of glutamate receptors. The mechanism of the glutamate release induced by TBT remains unclear, but it is not simply leakage from dead cells. We demonstrated that intracellular ROS, ERK, and p38 MAPK were also involved in TBT neurotoxicity. It was shown that ERK phosphorylation depended on glutamate receptor activation, but its activation was not involved in p38 phosphorylation. These results suggest that there is a glutamate receptor–dependent pathway and a glutamate receptor–independent pathway. Moreover, the glutamate toxicity induced by TBT was not apoptotic. Thus, glutamate excitotoxicity seems to account for the high sensitivity of cortical neurons to TBT.

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