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Apoptosis of cerebellar granule cells induced by organotin compounds found in drinking water: Involvement of MAP kinases

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Abstract

Mono- and dialkyl organotin compounds are used primarily as heat stabilizers in polyvinyl chloride (PVC) plastics. Recently, monomethyltin (MMT), dimethyltin (DMT), monobutyltin (MBT), and dibutyltin (DBT) have been detected in water from homes and businesses served by PVC pipes. While trialkyl organotins such as trimethyltin (TMT) and triethyltin (TET) are well known neurotoxicants, the toxicity of the mono- and dialkyl organotins is not well described. The present study compared the cytotoxicity of organotins found in drinking water with the known neurotoxicant TMT in primary cultures of cerebellar granule cells, and examined the role of MAP kinase signaling in organotin-induced cell death. Twenty-four hour exposure to TMT resulted in a concentration-dependent decrease in cell viability with an EC_{50} of 3 μM. Exposure to MMT, DMT, and MBT at concentrations up to 10 μM had no effect. DBT, however, was very potent, and decreased cell viability with an EC_{50} of 0.3 μM. Staining of organotin-treated cerebellar granule cells with the nuclear dye Syto-13 revealed that TMT and DBT, but not MMT, DMT, or MBT, produced condensation and fragmentation of chromatin characteristic of apoptosis. TMT- and DBT-induced apoptosis was confirmed using TUNEL staining and measurement of PARP cleavage. Activation of MAP kinase pathways was examined after 6 h of exposure to the organotins which induced apoptosis. Both TMT and DBT activated ERK1/2, but only TMT activated the JNK/c-Jun and p38 pathways. Pharmacologic blockade of JNK/c-Jun and p38 activation significantly decreased apoptosis produced by TMT, but not by DBT. These results show that DBT is a potent neurotoxicant in vitro, but unlike TMT, does not induce cell death via activation of MAP kinase signaling.
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Keywords: Organotins; Apoptosis; Cerebellar granule cells; MAP kinase

1. Introduction

The commercial use of organotin compounds has increased dramatically in the past several decades. By 1994, the world output of organotins was approximately 50,000 tonnes, with a majority of their use as heat stabilizers for plastics including polyvinyl chloride (PVC) polymers. While the trialkyl organotins are mainly used as biocides, mono- and dialkyl organotin chlorides are the intermediates used in the production of PVC polymers and are also the major degradation products associated with PVC plastics (Fent, 1996). Monomethyltin (MMT), dimethyltin (DMT), monobutyltin (MBT), and dibutyltin (DBT) have been detected in water from homes and businesses served by PVC pipes. Both mono- and dialkyl organotins were found in water samples, with either the methyl or butyl species predominating depending upon the manufacture of the PVC pipe (Sadiki and Williams, 1999). Mono- and dialkyl organotins have also been found in surface waters (Magos, 1986).

Interest in the potential neurotoxicity of mono- and dialkyl organontins is spurred by the well documented neurotoxicity associated with trialkyl organotins including trimethyltin (TMT) and triethyltin (TET). Accidental human exposure to TMT in the workplace resulted in cases of epilepsy and amnesia associated with hippocampal damage (Rey et al., 1984; Kreyberg et al., 1992), and numerous animal studies have demonstrated that TMT and TET produce pathology including edema, myelin vacuolization, and neuronal death in selective brain regions (reviewed in Verity, 1995). In vitro, TMT appears to have a direct effect on neurons and is often used as a model neurotoxicant for studying neuronal degeneration (Aschner and Aschner, 1992; Philbert et al., 2000). In contrast, much less is known about the neurotoxicity of the mono- and dialkyl

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organotins. Several studies have reported behavioral effects after exposure to mono- or dialkyl organotins. Noland et al. (1982) reported impaired acquisition and extinction learning in 11-day-old offspring of pregnant rats that received MMT in the drinking water during gestation and lactation, and a decrease in locomotor activity and impaired learning was observed in adult rats receiving DBT (Alam et al., 1988). A limited number of in vitro studies also suggest that the dialkyl organotins may be neurotoxic. Both DBT and DMT were cytotoxic after 24 h exposure to N2a neuroblastoma cells, with DBT being the more potent species (Borenfreund and Babich, 1987). DBT was found to be cytotoxic and decrease the expression of both neuronal (choline acetyltransferase) and glial (myelin basic protein, cyclic nucleotide phosphohydrolase) markers in primary cultures of rat telencephalon (Eskes et al., 1999). A recent study using an in vitro model of cell differentiation showed that DBT and DMT, but not MMT, decreased neurite outgrowth and caused cell death in PC12 cells (Jenkins et al., 2004).

While the studies cited above suggest the mono- and dialkyl organotins found in drinking water are potentially neurotoxic, there has not been a systematic study to directly compare their potency in a primary culture. Therefore, the present study compared the cytotoxic effects of MMT, DMT, MBT, and DBT in primary cultures of rat cerebellar granule cells. This culture is comprised of a relatively pure population of neurons (cerebellar granule cells) which make up 95% of the cells present, with the remaining 5% of cells staining positive for the glial marker GFAP (Mundy and Freudenrich, 2000). Cerebellar granule cell cultures have been used extensively for analysis of toxicant-induced cell death and apoptosis. The culture is grown in a medium containing high levels of K^+ (25 mM) which partially depolarizes the cells, mimicking the excitatory glutamatergic input these cells receive in vivo which contributes to their survival (Toescu, 1998). Removal of this trophic support by replacement of the growth medium with a low K^+ medium results in the alteration of a number of signaling cascades, and subsequently, leads to apoptotic cell death (D'Mello et al., 1993; Yan et al., 1994). In the present study, both necrotic cell death and apoptosis were examined 24 h after exposure to the mono- and dialkyl organotins, as well as to the trialkyl organotin TMT which has been previously shown to induce apoptosis in cultured neurons including cerebellar granule cells (Thompson et al., 1996; Gunaskar et al., 2001). Examination of cells switched to a low K^+ medium for 24 h was included as a positive control for apoptosis. Additional studies evaluated the effects of organotins on signaling pathways thought to underlie the induction of apoptosis in cerebellar granule cells. Mitogen-activated protein (MAP) kinases are a family serine/threonine protein kinases that play a major role in signal transduction from the cell surface to the nucleus. Mammalian MAP kinases can be subdivided into the extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and p38 MAP kinases (p38). Each is proving to be integral to the regulation of many important cellular functions including proliferation and differentiation, response to environmental stressors, and apoptosis (Chang and Karin, 2001; Cowan and Storey, 2003). In cerebellar granule cells both JNK and p38 have been implicated in the apoptotic response induced by cellular stress such as removing trophic support (Yamagishi et al., 2001; Coffey et al., 2002). Thus, we examined the role of MAP kinases in apoptosis induced by organotins.

2. Materials and methods

2.1. Materials

Methyltin trichloride (MMT, >97%), dimethyltin dichloride (DMT, >97%), butyltin trichloride (MBT, >95%), and dibutyltin dichloride (DBT, >96%) were purchased from Sigma–Aldrich (Milwaukee, WI). Trimethyltin hydroxide (TMT, >97%) was purchased from Pfaltz and Bauer (Waterbury, CT). Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from GIBCO/Invitrogen (Carlsbad, CA). All other reagents for use in tissue culture were purchased from Sigma Chemical Company (St. Louis, MO). Antibodies to PARP, JNK, phospho-JNK (Thr183 and Tyr 185), p38, phospho-p38 (Thr180 and Tyr182), ERK1/2, phospho-ERK1/2 (Thr202 and Tyr204), c-Jun, and phospho-c-Jun (Ser73) were purchased from Cell Signaling Technology (Beverly, MA). The MEK inhibitor U0126, p38 inhibitor SB203580, and JNK inhibitor SP600125 (JNKII) were purchased from Calbiochem (San Diego, CA). The fluorescent dyes calcein-AM, propidium iodide, and Syto-13 were purchased from Molecular Probes (Eugene, OR). All other chemicals were obtained from Sigma Chemical Company (St. Louis, MO). For MMT, DMT, TMT, and MBT, stock solutions were prepared in distilled water before a final 1/100 dilution into serum-free culture medium. For DBT, a 10 mM stock solution was prepared in ethanol, then stock solutions were prepared in distilled water before a final 1/100 dilution into serum-free culture medium. The concentration of ethanol in the culture medium never exceeded 0.1% and had no effect on any of the measures taken.

2.2. Cell culture

Relatively pure cultures of cerebellar granule cells were prepared from the cerebella of 7 day old Long-Evans rats (Charles River, Raleigh, NC) according to the method of Gallo et al. (1987) with modifications as described in Kodavanti et al. (1993). Cells were grown in DMEM containing 25 mM KCl, 5 mg/l insulin, 5 mg/l transferrin, 50 μM γ-aminobutyric acid, 4.5 mM $NaHCO_3$, 25 mM D-glucose, 5 mM glutamine, and 10% FBS. Cells were plated at a density of 3×10^5 cells/cm^2, which is equivalent to 1×10^6 cells/well in poly-L-lysine precoated 24-well plates (for cytotoxicity measurements), 1.5×10^6 cells/well in 12-well plates (for apoptosis determination), and 3×10^6 cells/well in 6-well plates (for western blot analysis). After plating, the cells were incubated at 37 °C in a humidified incubator under a 95% air/5% CO_2 atmosphere. Cytosine arabinoside (5 μM) was added after 48 h to prevent growth of non-neuronal cells. In our laboratory, cerebellar