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Trimethyltin-induced apoptosis is associated with upregulation of inducible nitric oxide synthase and Bax in a hippocampal cell line

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Abstract

Trimethyltin (TMT) produces selective neuronal degeneration in the central nervous system (CNS), in which the hippocampus is the most sensitive area. Since previous studies have been conducted in either non-neural cells or mixed primary cultures, an immortalized hippocampal neuronal cell line (HT-22 cell) was used to assess the mechanism and mode of death produced by TMT. The compound produced a time- and concentration-dependent apoptotic death that was caspase-mediated. Excessive generation of reactive oxygen species (ROS) and subsequent reduction of mitochondrial membrane potential ($\Delta\Psi_m$) were involved in the cytotoxicity. Scavenging of ROS by a free radical trapping agent or inhibition of the mitochondrial permeability transition (MPT) pore significantly reduced cell death. Additionally, TMT increased expression of inducible nitric oxide synthase (iNOS) by activation of the redox-sensitive transcription factor NFκB. Pharmacologic inhibition studies showed that the iNOS-mediated NO generation increased expression of Bax and then mitochondrial-mediated apoptosis. It was concluded that excessive ROS generation initiated the apoptotic cell death by upregulating iNOS followed by increased Bax expression which then led to loss of $\Delta\Psi_m$ and caspase-executed cell death. This study is the first to report in a neuronal cell model that TMT stimulates induction of iNOS, which then increases cellular levels of reactive nitrogen species (RNS) to initiate apoptotic death.

Keywords: Trimethyltin; Apoptosis; Nitric oxide synthase; Oxidative stress; Hippocampus

Introduction

Trimethyltin (TMT) is a short-chain trialkyltin that has wide application in industry and agriculture (Feldman et al., 1993). Accidental exposure is associated with a number of neurobehavioral symptoms, including disorientation, aggressiveness, complex partial seizures, and ataxia. TMT produces selective neuronal degeneration in several brain areas. The mechanism of the neurotoxicity and underlying selective vulnerability of the CNS have not been fully elucidated. The hippocampus is the most susceptible brain region, and TMT has been extensively studied in neurons from this site (Snoeij et al., 1987). In a number of different cell lines and primary cells, TMT produces excess generation of cellular oxidative species linked with apoptotic death (Gunasekar et al., 2001a, 2001b; Jenkins and Barone, 2004; Mundy and Freudenrich, 2006). The cell death can be reduced by antioxidants and by increased levels of intracellular glutathione (Cookson et al., 1998; Shin et al., 2005). It is widely accepted that oxidative stress is an initiator of TMT-induced apoptotic cell death.

Nitric oxide (NO) is a free radical generated by nitric oxide synthase (NOS). Excessive NO generation is associated with several neurodegenerative conditions, including ischemia and excitotoxicity (Chabrier et al., 1999). Three isoforms of NOS have been identified in mammalian cells. The calcium-dependent neuronal NOS (nNOS) and endothelial NOS (eNOS) are constitutively expressed and generate low levels of NO for homeostatic regulation. In contrast, inducible NOS (iNOS) can generate high, sustained localized concentrations of NO that are associated with apoptosis in a variety of cells (Mungrue et al., 2003). iNOS-derived NO generation is regulated by transcriptional upregulation of enzyme expression, possibly through activation of redox-sensitive transcription factors, including NFκB (Pahan et al., 2001).

To initiate apoptosis, NO can induce mitochondrial dysfunction by stimulating membrane permeability transition (MPT), thus activating a cascade of reactions leading to cell death

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(Marriott et al., 2004). In addition to a direct action on mitochondria, excess NO can upregulate pro-apoptotic Bax-like proteins or downregulate expression of anti-apoptotic Bcl-2-like proteins (Tamatani et al., 1998). Both Bcl-2 and Bax belong to the Bcl-2 family that regulates mitochondrial-mediated apoptotic cell death. The balance between activation of anti- and pro-apoptotic Bcl-2 family members ultimately determines whether mitochondria remain functional or become permeabilized to release proteins that promote cell death (Scorrano and Korsmeyer, 2003). The pro-apoptotic activity of NO appears to converge on mitochondria to release cytochrome *c* into the cytosol, which then activates the caspase protease cascade to execute apoptosis. Studies in mixed cell cultures using TMT show that glial cells are a source of NO that can diffuse into the surrounding neuronal cells to initiate cell injury (Reali et al., 2005; Rohl and Sievers, 2005). The role of neuron-derived NO in TMT neurotoxicity is not clear. In this study, HT-22 cells, an immortalized hippocampal neuronal cell line, were used to study involvement of NO in TMT-induced neuronal apoptotic cell death by examining expression of iNOS and Bax.

Materials and methods

Materials. 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide (JC-1) and 2,7-dichlorofluorescin diacetate (DCFH-DA) were purchased from Molecular Probes (Eugene, OR). Pre-stained SDS-PAGE standards and Bio-Rad protein assay system were purchased from Bio-Rad (Hercules, CA). Caspase-3 colorimetric assay kit and caspase inhibitor z-VAD-fmk were purchased from BioVision (Mountain View, CA). SN50 and SN50M were purchased from BioMol (Plymouth Meeting, PA). Phenyl-*n*-*tert*-butylnitrone (PBN), N^G-nitrol-arginine methyl ester (L-NAME), and *n*-[3-(aminomethyl)benzyl]acetamidine (1400W) were purchased from Sigma (St. Louis, MO).

Cell culture. HT-22 cells were obtained from Dr. Val J. Watts, Purdue University. This cell line, an immortalized mouse hippocampal cell line with neuronal properties, such as sensitive to glutamate cytotoxicity via a non-receptor-mediated oxidative pathway (Gursoy et al., 2002), was maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 1 ml penicillin/streptomycin (10,000 U/ml) at 37 °C in an atmosphere of 5% CO_2 and 95% air. The plating density was controlled at the same level in all experiments.

Oligonucleotide (ODN) transfections. Antisense ODN directed against the coding region of Rat Bax gene was commercially synthesized by Integrated DNA technologies (Coralville, IA). Phosphorothionate-modified antisense ODN 5′-AGTCCAGTGTCCAGCCCAT-3′ and scrambled mismatched control ODN 5′-GTCAGTCCACAGTCTAGCC-3′ were used in this study. Twenty-four hours after seeding, ODNs were transfected with lipofectamine2000 (2 μg/ml) into subconfluent HT-22 cells as manufacturer's recommendations. Twenty-four hours later, TMT was added and cells were incubated for additional times as indicated.

Hoechst 33258 staining. Apoptosis was detected by nuclear staining with Hoechst 33258 as described previously (Jones et al., 2000). Briefly, cells were washed twice with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde in PBS. Cells were then exposed to 2 μM Hoechst 33258 dye and examined with an Olympus fluorescence microscope. Cells with condensed and fragmented DNA were considered apoptotic and were quantified by averaging cell counts in four random 40× fields.

In situ terminal deoxynucleotidyl transferase-mediated DNA nick-end labeling (TUNEL) assay. The TUNEL assay was performed on paraformaldehyde (4% in PBS)-fixed cells using the Apoptag (Oncor, Gaithersburg, MD) in situ apoptosis detection kit (Prabhakaran et al., 2002). After experimental treatment, cells were preincubated in equilibration buffer containing 0.1 M potassium cacodylate (pH 7.2), 2 mM $CaCl_2$, and 0.2 mM dithiothreitol for 10 min at room temperature and then incubated in the TUNEL reaction mixture (containing 200 mM potassium cacodylate, 4 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 30 μM biotin-16-2′-deoxyuridine-5′-triphosphate [dUTP], and 300 U/ml TdT) at pH 7.2 in a humidified chamber maintained at 37 °C for 1 h. After incubation in stop/wash buffer for 10 min, the elongated digoxigenin-labeled DNA fragments were visualized using anti-digoxigenin peroxidase antibody solution followed by staining with DAB/H_2O_2 (0.2 mg/ml diaminobenzidine tetrachloride and 0.005% H_2O_2 in PBS, pH 7.4). Cells were then counterstained with hematoxylin. In the microscopic field, the apoptotic cells were determined by both positive TUNEL staining and exhibition of characteristic apoptotic morphology, such as chromatin condensation and margination within the nucleus, and cell shrinkage.

Measurement of caspase-3 protease activity. Caspase-3 protease activity was assayed with a colorimetric assay kit (BioVision Inc., Mountain View, CA) according to manufacturer's suggestion. The assay is based on spectrophotometric detection of the chromophore *p*-nitroanilide (pNA) after cleavage from the labeled substrate Ac-DEVD-pNA. After different treatments, HT-22 cells were harvested in PBS and centrifuged at 500×*g* for 5 min. Cell pellets were resuspended in 50 μl chilled cell lysis buffer provided by the kit, incubated on ice for 10 min, and then centrifuged at 13,000 rpm for 1 min at 4 °C. The supernatant (diluted to 50 μl cell lysis buffer) was then added to 50 μl of reaction buffer (containing 10 mM dithiothreitol) and 5 μl of DEVD-pNA (final concentration 200 μM) and incubated at 37 °C. Relative caspase-3 activity was measured by determining absorbance at 405 nm in a microtiter plate reader. Each sample contained 50 μg of total proteins.

Western blot analysis. After the various treatments, HT-22 cell lysates were prepared using lysis buffer containing 220 mM mannitol, 68 mM sucrose, 20 mM HEPES, pH 7.4, 50 mM KCl, 5 mM EGTA, 1 mM EDTA, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.1% Triton-X100, and protease inhibitors. Western blot analysis was carried by the protocol provided with the ECF Western blot kit (Amersham Biosciences, Piscataway, NJ). Protein content of the cell extractions was determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA). Samples containing 30 μg protein were subjected to SDS-polyacrylamide gel, electrophoresed, and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) using the Bio-Rad MiniTrans-Blot system (Hercules, CA). After blocking with Tris-buffered saline containing 5% nonfat dry milk and 0.1% Tween 20, the membrane was probed with primary antibody. Reactions were detected with the fluorescein-linked antimouse Ig (second antibody) conjugated to horseradish peroxidase using a Storm 860 fluorescence-Phosphor Imager (Molecular Dynamics, Sunnyvale, CA). Primary antibodies included: anti-mouse β-actin monoclonal antibody (Sigma, St. Louis, MO), anti-mouse monoclonal antibody Bax, Bcl-2, iNOS, and polyclonal antibody caspase-3 (Santa Cruz Biotechnology, Santa Cruz, CA).

Measurement of free radical generation. Free radical generation was determined by the dichlorofluorescin diacetate (DCFH-DA) assay described by Thorburne and Juurlink (1996) with minor modification. Cells were plated 24 h before initiation of the experiment in 48-well plates. In all experiments, cells treated with TMT and untreated, control cells were loaded with DCFH-DA (Molecular Probes, Eugene, OR) at a final concentration of 30 μM for 30 min at 37 °C in the dark. Fluorescence intensity was monitored with a microtiter plate reader at an excitation wavelength of 485 nm and emission wavelength of 535 nm. The data of the treatment groups were expressed as a percentage of DCF fluorescence generated in control cells under identical incubation conditions.

Measurement of mitochondrial membrane potential ($\Delta\Psi_m$). The changes in $\Delta\Psi_m$ were estimated by the use of the cationic fluorescent dye (JC-1) based on the method of Reers et al. (1995). JC-1 exists as a green fluorescent monomer at low membrane potential (120 mV) and as a red fluorescent dimer (J-aggregate) at a membrane potential greater than 180 mV. Following illuminated with 485 nm, the ratio of red (595 nm emission) to green (525 nm emission) fluorescence depends on $\Delta\Psi_m$. There is a reversible shift from red to green upon mitochondrial membrane depolarization. After treatment, HT-22 cells were incubated with JC-1 (3.0 μM) for 30 min at 37 °C in the dark and then washed twice with PBS. Fluorescence was measured with a plate reader (TECAN