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2,3,7,8-TCDD induces neurotoxicity and neuronal apoptosis in the rat brain cortex and PC12 cell line through the down-regulation of the Wnt/β-catenin signaling pathway

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ABSTRACT

TCDD exposure has various toxic effects on in the human nervous system resulting in various developmental and behavioral deficits. However the underlying molecular mechanism of TCDD-induced adverse effects on the CNS and associated signaling pathways remains largely unknown. Herein we analyzed acute TCDD exposure in the rat brain cortex to investigate whether misregulation of the Wnt/β-catenin signaling pathway plays a role in neurotoxicity. Western blot and immunohistochemical experiments revealed a significant down-regulation of β-catenin and phospho-glycogen synthase kinase-3β (pSer9-GSK-3β) after TCDD exposure. TUNEL assay results showed apoptosis occurs mainly at day 7 after TCDD treatment. Immunofluorescent labeling indicated that β-catenin was localized mainly in the neurons; co-localization of β-catenin and active caspase-3 was found following TCDD exposure. Further, TCDD exposure decreased the level of pSer9-GSK-3β and β-catenin, and increased apoptosis in the PC12 neuronal cell line in a dose-dependent manner. Interestingly the application of lithium chloride, a GSK-3β inhibitor, reversed the suppressive effect of TCDD on β-catenin in PC12 cells and primary cortical neurons restoring cell viability and protecting cells from apoptosis as compared to untreated controls. Taken together, these results indicate that the canonical Wnt/β-catenin signaling pathway may play an important role in TCDD-induced neurotoxicity and neuronal apoptosis.

1. Introduction

The dioxins are a family of ubiquitous environmental contaminants that have attracted significant public concern. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) represents the most widely investigated toxic form of dioxin (Clements et al., 2009). TCDD accumulates readily in the environment and in multiple organ systems due to its high lipophilicity and resistance to metabolism (Van den Berg et al., 1994). Prior studies have demonstrated that acute occupational exposure to TCDD can induce several acute intoxication symptoms such as porphyria, chloracne, transient hepatotoxicity and peripheral and central neurotoxicity (Pelclova et al., 2006). Long-term TCDD exposure can cause teratogenicity, innate and adaptive immune suppression, atherosclerosis, carcinogenesis, hypo- and hyperplasia, diabetes, ocular vascularity changes, and nervous system damage (Fries, 1995; Hogaboam et al., 2008; Knerr and Schrenk, 2006; Marinkovic et al., 2010; Urban et al., 2007). Because TCDD is associated with teratogenic effects in the nervous, immune, and reproductive systems (Birnbaum and Tuomisto, 2000; Pelclova et al., 2006, 2009), exposure to this extremely potent toxin raises important public health concerns.

It is assumed that most TCDD-induced toxicological effects are mediated via binding to the aromatic hydrocarbon receptor (AhR), a cytosolic ligand-activated transcription factor which translocates into the nucleus and dimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT) to regulate transcriptional activation or repression of a diverse array of specific target genes (Puga et al.,

Abbreviations: AhR, aromatic hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; CCK-8, Cell Counter Kit-8; CNS, central nervous system; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; GSK-3β, glycogen synthase kinase-3β; p-GSK-3β, phospho-glycogen synthase kinase-3β; LiCl, lithium chloride; PC12, rat pheochromocytoma; RT, room temperature; TUNEL, terminal deoxynucleotidyl transferase-mediated biotinylated-dUTP nick-end labeling.

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2000). Previous studies have shown that AhR and ARNT are widely distributed throughout the brain and brainstem in the rat brain (Kainu et al., 1995; Petersen et al., 2000).

TCDD is a potent toxin that alters normal brain development and produces cognitive disability and motor dysfunction. These effects have been less intensively studied in experimental animals than its hepatotoxic and carcinogenic effects (Sanchez-Martin et al., 2010). For the most part, studies regarding the adverse effects of TCDD on the CNS have been limited to neurodevelopmental processes (Nayyar et al., 2002; Wormley et al., 2004). However, whether TCDD has any adverse effects on mature neurons and the signaling pathways involved remain largely unknown.

The canonical Wnt/β-catenin signaling pathway plays an important role in a variety of cellular processes including cell proliferation, differentiation, survival, and motility (Inestrosa and Arenas, 2010; Moon et al., 2004). Recent studies have shown that this pathway also plays a role in neuronal function and development (Marinkovic et al., 2010; Zhou et al., 2006). One crucial mediator of canonical Wnt signaling is the *armadillo* protein β-catenin. The stability of β-catenin is controlled by Wnt through the modulation of a large cytoplasmic protein complex comprised of the protein Axin (axis inhibition protein), APC (adenomatosis polyposis coli), CK1α (casein kinase 1 alpha), GβP/frat and GSK-3β (Mikels and Nusse, 2006). GSK-3β, a decisive player in the Wnt/β-catenin signaling pathway, is a core component of the multiprotein complex that directly controls the level of β-catenin phosphorylation, which leads to β-catenin degradation by the ubiquitin-proteasome pathway (Dale, 1998; Lustig and Behrens, 2003; Willert and Nusse, 1998). GSK-3 is an evolutionarily conserved serine/threonine kinase expressed as two isozymes- GSK-3α and GSK-3β (Doble and Woodgett, 2003). Over activation of GSK-3 has been implicated in the etiology of neurodegenerative diseases including Parkinson, amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), and brain aging (Eldar-Finkelman and Martinez, 2011). Thus GSK-3 has been widely considered to be a promising therapeutic target of many neurodegenerative diseases (Eldar-Finkelman and Martinez, 2011). The activity of GSK-3β is under tight phospho-regulation. One critical piece of this regulation is the inhibitory phosphorylation at the S9 of GSK-3β's N-terminus. This provides a phospho-N-terminal domain that acts as a pseudo-substrate for its catalytic domain thereby inhibiting kinase activity (Dajani et al., 2001). Inhibitors of GSK-3β, such as LiCl and SB216763, are commonly used to mimic the action of Wnt molecules and inhibit GSK-3β activity via upregulation of phosphorylation of this site (Jope, 2003). Because of the critical role of Wnt/β-catenin signaling in neuronal survival and function, it makes it an important and possible target for TCDD-induced neurotoxicity.

In this study, we analyzed the effects of acute exposure to TCDD (25 μg/kg body weight) on neuronal toxicity in the cortex of female Sprague-Dawley rats. We found TCDD exposure resulted in significant neuronal apoptosis. We also found that β-catenin levels were decreased in the cortex and in PC12 cells after TCDD exposure. Inhibition of GSK-3β activity with LiCl blocked TCDD-induced β-catenin degradation and neuronal apoptosis. These findings indicate that TCDD might induce neurotoxicity through modulation of Wnt/β-catenin signaling.

2. Materials and methods

2.1. Animals and treatment

All experiments involving rats were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Chinese National Committee to Use of Experimental Animals for Medical Purposes, Jiangsu Branch; all animal protocols were approved by the Department of Animal Center, Medical College of Nantong University. Adult female Sprague-Dawley rats (weighing 220–250 g) were used in this study. Animals were group-housed under a 12 h light/dark cycle in a pathogen-free area with the room temperature (RT) at 25 ± 2 °C, and had access to standard rodent chow and water ad libitum (Senft et al., 2002). Rats were allowed to acclimate to the facility for 3–5 days before the experiments commenced, during which time they were handled daily.

For the current study, the TCDD (CAS 1746-01-6; MW, 321.9; purity, 99%) was obtained from Cambridge Isotope Laboratories, Inc. Female Sprague-Dawley rats received a single dose of TCDD in corn oil vehicle (25 μg/kg body wt) by intraperitoneal injection. Vehicle-treated control rats were given equivalent volumes of corn oil (Moon et al., 2008). This dosing protocol was chosen because 0.5 LD_{50} dose of TCDD in adult rat is 25 μg/kg and previous studies showed that even a single administration of this dose can up-regulate the expression of Cyp1b1 in *vivo* in rat brain capillaries, and the blood–brain barrier is likely to be a target of AhR environmental pollutant ligands (Jacob et al., 2011). The animals (n = 3 per time point) were sacrificed to extract protein for western blot analysis at 1, 2, 3, 5 or 7 days after TCDD injection. Additional experimental animals (n = 3 per time point) for sections were terminated at the indicated point of time. All efforts were made to minimize the number of animals used and their suffering.

2.2. Cell culture and stimulation

Rat pheochromocytoma (PC12) cells used were terminally differentiated by treatment with nerve growth factor (NGF). PC12 were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% (v/v) fetal bovine serum (Hyclone), 5% (v/v) donor horse serum, and 1% (v/v) antibiotics mixture. Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 until reaching the desired confluence. TCDD was dissolved in DMSO and stored in −20 °C before use. The PC12 cells were plated at a density of 5×10^6 cells/10 cm diameter dish and incubated with different concentrations (1, 10, 100, 300 or 1000 nM) of TCDD for 24 h at 37 °C. As for LiCl treatment, cells were pretreated with 10 mM LiCl for 2 h and then incubated with TCDD for 24 h at 37 °C. For all experiments, PC12 cells were grown to 70–80% confluence and were subjected to no more than 20 cell passages. Protein samples were stored at −80 °C until use.

Primary cultures of cortical neurons were prepared from the brain of E18–E19 Sprague-Dawley rat embryos. In brief, rat embryos (n = 8–10) were sacrificed by cervical dislocation under anesthesia, their brains were quickly removed and the cortexes were harvested on a cold stage, cortical tissues were dissected out and treated with 0.25% trypsin for 15 min at 37 °C in Ca^{2+} and Mg^{2+}-free Hank's balanced salt solution, and washed in Dulbecco's Modified Eagle's medium (DMEM, GIBCO) supplemented with 10% fetal bovine serum (FBS) to stop trypsin activity. All recovered primary cells were mixed and re-suspended in DMEM supplemented with 10% FBS and plated on poly-L-lysine-coated plates for 24 h at 37 °C in a humidified atmosphere of 95% outside air and 5% CO_2. The final cell density was approximately 2×10^5 cells/cm^2 in 2 ml/well of culture medium. After adherence the culture medium was replaced with neuronal culture medium consisting of serum-free Neurobasal medium supplemented with 2% B27, 0.5 mM glutamine, 100 U/ml penicillin and 100 U/ml streptomycin with the media being refreshed every 3 days. Then the cells were characterized by immunohistochemistry for neurofilament (NF) protein and fibrillary acidic protein, revealing that the cell cultures contained about 90% neurons. For LiCl treatment, cells were pretreated with 10 mM LiCl for 2 h and then incubated with TCDD for 24 h at 37 °C.