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2,3,7,8-Tetrachlorodibenzo-p-dioxin-induced inflammatory activation is mediated by intracellular free calcium in microglial cells

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ABSTRACT

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) has been known to induce inflammatory signaling in a number of cell types and tissues. However, the adverse effects of TCDD on the central nervous system (CNS) have not been entirely elucidated. In this study, using reverse transcriptase PCR (RT-PCR) and ELISA, we showed that TCDD up-regulated the expression and secretion of tumor necrosis factor-alpha (TNF-α) in a time-dependent manner in cultured HAPI microglial cells. TCDD also caused a fast (within 30 min as judged by the increase in its mRNA level) activation of cytosolic phospholipase A2 (cPLA2). This initial action was accompanied by up-regulation of cyclooxygenase-2 (COX-2), an important inflammation marker within 1 h after TCDD treatment. These pro-inflammatory responses were inhibited by two types of Ca^{2+} blockers, bis-(*o*-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid acetoxymethyl ester (BAPTA-AM) and nifedipine, thus, indicating that the effects are triggered by initial increase in the intracellular concentration of free Ca^{2+} ($[Ca^{2+}]i$). Further, TCDD exposure could induce phosphorylation- and ubiquitination-dependent degradation of IκBα, and the translocation of NF-κB p65 from the cytosol to the nucleus in this microglial cell line. Thus, the NF-κB signaling pathway can be activated after TCDD treatment. However, Ca^{2+} blockers also obviously attenuated NF-κB activation and transnuclear transport induced by TCDD. In concert with these results, we highlighted that the secretion of pro-inflammatory cytokine and NF-κB activation induced by TCDD can be mediated by elevation of $[Ca^{2+}]i$ in HAPI microglial cells.

1. Introduction

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is one of the most toxic chemicals that is found ubiquitously in the environment. The spectrum of observed responses attributed to exposure to TCDD and related compounds includes: chloracne, hyperkeratosis, hepatotoxicity, wasting, altered lipid and glucose metabolism, pancytopenia, gastric lesions, urinary tract hyperplasia, edema, carcinogenesis, tumor promotion, neurobehavioral and endocrine effects (Kerkvliet, 2012; Manikkam et al., 2012). Furthermore, recent findings indicate that TCDD also induce rapid cellular pro-inflammatory responses (Matsumura, 2009; Vogel and Matsumura, 2009). TCDD toxicity in the nervous system has been studied during recent years, but the mechanism has not yet been fully elucidated. Most of the studies on TCDD-induced neurotoxicity have been focused on the nerve cells themselves. Thus, it has rarely been studied whether other types of cells in the central nervous system (CNS) were influenced after TCDD exposure, and thereby suffered from its adverse toxic effect.

Brain cells are primarily composed of nerve cells and three types of gliocytes including microglia, astrocytes and oligodendrocytes. Within these three types of gliocytes, microglia are unique, for their ability in being involved in inflammatory and immune responses when the CNS is injured. Microglia comprise 12% of the cell population of the CNS (Xue et al., 2012) and are macrophage-like cell residents within the CNS. Abundant experimental evidences

Abbreviations: TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; AA, arachidonic acid; AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; BAPTA-AM, bis-(*o*-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid acetoxymethyl ester; CNS, central nervous system; COX-2, cyclooxygenase-2; cPLA2, cytosolic phospholipase A2; $[Ca^{2+}]i$, concentration of intracellular free Ca^{2+}; Fura-2/AM, fura-2 acetoxymethyl ester; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-1β, interleukin-1beta; NF-κB, Nuclear factor-kappa B; PGs, prostaglandins; PGE_2, prostaglandin E_2; PGI_2, prostaglandin I_2; PKA, protein kinase A; PKC, protein kinase C; RT-PCR, reverse transcription-polymerase chain reaction; Src, proto-oncogene tyrosine-protein kinase; TNF-α, tumor necrosis factor-alpha; TXA_2, thromboxane A_2.

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have demonstrated that inflammations mediated by microglia contribute to many neurodegenerative diseases, including Parkinson's disease and Alzheimer's disease (Iannaccone et al., 2012; Ryu et al., 2002). Upon activation, microglia are capable of releasing various pro-inflammatory molecules, such as interleukin-1beta (IL-1β) and tumor necrosis factor-alpha (TNF-α), and then induce neuronal cells injury and apoptosis. Thus, in terms of brain toxicology, it is important to investigate whether microglia could respond to TCDD following exposure to this chemical and bring its toxic effect in the CNS.

Recently, a number of groups reported that TCDD can rapidly increase the concentration of intracellular free Ca^{2+} ($[Ca^{2+}]i$) through a "non-genomic pathway" (Dong and Matsumura, 2008; Li et al., 2010; Sciullo et al., 2008). According to these authors, TCDD can induce pro-inflammatory cytokines, interleukin-6 (IL-6) and TNF-α in U937 and 3T3-L1 cell lines, following early activation of some enzymes and protein kinases, such as cytosolic phospholipase A2 (cPLA2), cyclooxygenase-2 (COX-2), protein kinase C (PKC), and proto-oncogene tyrosine-protein kinase (Src), these activation and pro-inflammatory responses exist even without aryl hydrocarbon receptor nuclear translocator (ARNT) or when ARNT has been knocked down, but aryl hydrocarbon receptor (AhR) still play an important role in this "non-genomic pathway" (Li and Matsumura, 2008; Sciullo et al., 2009). Furthermore, the action of TCDD is clearly blocked by several calcium signaling blockers (Dong and Matsumura, 2008), suggesting TCDD is mediated by calcium-triggered activation of down-stream genes. Activated cPLA2 can cause arachidonic acid (AA) increase, which activates COX-2 leading to an increase in the level of prostaglandin type inflammatory mediators, such as PEG2 (Ueno et al., 2001). COX-2 has been shown to be one of the major mediators of pro-inflammatory responses induced by TCDD (Dong et al., 2010; Sciullo et al., 2010). Additionally, TCDD also causes induction of TNF-α production (Charles and Shiverick, 1997), which appears to contribute greatly to the toxic manifestation of TCDD in mice (Taylor et al., 1992). Through binding to its specific receptor, TNF-α is capable of activating Nuclear factor kappaB (NF-κB), which is known to induce further activation of gene transcription of a number of pro-inflammatory cytokines and chemokines (Lappas et al., 2002).

NF-κB is an inducible transcription factor that is detectable in most cell types and involved in various kinds of cellular processes including cell apoptosis, proliferation, and many inflammatory responses (Camacho et al., 2005; Li and Verma, 2002). Further, it is detectable in most cell types and involved in various kinds of cellular processes, including cell apoptosis, proliferation and many inflammatory responses. It consists of homo- or heterodimers of different subunits and structurally related proteins (Rel/NF-κB proteins), and composes at least five Rel/NF-κB proteins: c-Rel, RelA (p65), RelB, NF-κB1 (p50/p105), and NF-κB2 (p52/p100) (Ghosh and Karin, 2002; Hayden and Ghosh, 2004; Li et al., 2004). The NF-κB pathway is controlled by the inhibitor protein IκBs, among them, IκBα play an important role in anchoring the transcription factor to the cytosol (Baeuerle and Baltimore, 1988). The phosphorylation and ubiquitination-dependent degradation of IκBα can release active NF-κB dimers, which are translocated to the nucleus, inducing the transcription of over 150 target genes (Pahl, 1999). TCDD also induces the activation of NF-κB in a variety of cell lines and tissues (Kobayashi et al., 2008; Puga et al., 2000). In particular, expression of TNF-α is controlled by NF-κB, and in turn, TNF-α is a potent activator of NF-κB, thus resulting in a positive feedback loop. Hence, NF-κB signaling pathway may play a pivotal role in activating microglia function during inflammation.

To sum up, it is important to delineate the secretion of pro-inflammatory cytokine induced by TCDD in microglia for better understanding of the toxic effects of TCDD in the CNS. Since inflammatory responses vary from cell to cell, it is unclear whether microglia respond to TCDD and induce a pro-inflammatory cascade. Recent reports have provided us the initial impetus to study the molecular mechanism of TCDD and to elicit possible inflammatory responses in microglia. Accordingly, we have set our two major objectives: one is to investigate whether TCDD induces the secretion of pro-inflammatory cytokine and activation of NF-κB in microglia, second is to investigate whether the effect is mediated by an increase in $[Ca^{2+}]i$.

2. Materials and methods

2.1. Chemicals

The following chemicals were purchased from their suppliers: TCDD (>99.99% purity; Dow Che-micals Co., Midland, MI); nifedipine, (Sigma–Aldrich, St.Louis, MO); bis-(*o*-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid acetoxymethyl ester (BAPTA-AM), (Corvallis, OR, USA). Additional sources and vendors of specific chemicals, such as antibodies, are listed below.

2.2. Cell culture and treatment

HAPI (highly aggressive proliferating cell type) microglial cells (A gift from Professor Qin Shen in Nantong University, Jiangsu Province, China) (Cheepsunthorn et al., 2001; Cui et al., 2010), a MG-like rat cell line, were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO, Shanghai, China) supplemented with 2 mM glutamine (Sigma–Aldrich, Shanghai, China), 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum (Sigma–Aldrich, St. Louis, MO, USA) at 37 °C under humidified atmosphere of 5% CO_2/95% air. In order to study the cytotoxicity induced by TCDD, the cells were treated with 10 nM TCDD (which is diluted by DMSO) for 0.5, 1, 3, 4, 6, 12, 24 or 48 h. Cultures added with 0.1% DMSO was used as a control, For assay of inhibition of $[Ca^{2+}]i$ elevation, cells were pre-treated with Ca^{2+} inhibitors (nifedipine or BAPTA-AM) for 30 min followed by TCDD treatment for 4 h. The cells had been pre-incubated with serum-free medium for 24 h prior to these treatments.

2.3. Animals and treatment

All experiments involving rats were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996, USA) and were approved by the Chinese National Committee for the Use of Experimental Animals for Medical Purposes, Jiangsu Branch. All efforts were made to minimize the number of animals used and their suffering. Adult Sprague-Dawley (SD) rats (230–250 g) were used in this study. Animals were housed in a temperature-controlled (23–25 °C) room at 60 ± 5% humidity under a 12 h light–dark cycle and acclimatized for 7 days with commercial chow and distilled water. For the current study, a single dose of TCDD (10 μg/kg) or an equivalent volume of vehicle (corn oil, 4 ml/kg) were given by gavage to rats. The animals (n = 6 per time point) were killed to extract proteins from either cerebral cortex for Western blot analysis at 0, 1 or 7 day after TCDD treated.

2.4. Measurement of intracellular free calcium concentration ($[Ca^{2+}]i$) in HAPI microglial cells

The membrane-permeant calcium indicator dye fura-2 acetoxymethyl ester (Fura-2/AM; Molecular Probes, Eugene, OR) was dissolved in DMSO to make a 2 mM stock solution. HAPI microglial cells grown on 25 mm glass coverslips were washed twice with 2 ml of Strange buffer (125 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 2 mM $MgCl_2$, 0.5 mM NaH_2PO_4, 5 mM $NaHCO_3$, 10 mM HEPES and 10 mM glucose, pH 7.2) and finally incubated in 2 mL of the same Strange buffer in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C for 1 h to allow complete de-esterification of Fura-2/AM to the calcium sensitive Fura-2. Cells were treated with TCDD or 0.1% DMSO (controls) for 0, 5, 10, 15, 30, 45, 60, 120, 180 or 240 min. Coverslips were placed in a recording chamber and mounted on the stage of an inverted fluorescence microscope (Leica Microsystems GmbH, Germany). Sample specimens were excited by 340 and 380 nm light and images produced by the emission at 510 nm were recorded and quantified. $[Ca^{2+}]i$ was estimated using the equation previously described by (Grynkiewicz et al., 1985).

2.5. Nuclear and cytoplasmic extraction

Cells (1×10^7) in 75 cm^2 plates, at confluency, were washed with ice-cold PBS and suspended in 200 μl of hypotonic buffer (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF and a protease inhibitor cocktail). The cells were allowed to swell on ice for 15 min; then, 2.5 μl of 10% NP-40 was added to the cell suspension. The cell suspension were agitated on a vortex for 10 sec and then centrifuged at 2000 rpm for 5 min. The resulting supernatant is represented as the cytosolic extract. The pellets containing the nucleus were resuspended in 50 μl of ice-cold nuclear extraction buffer (20 mM HEPES [pH 7.9], 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF and a protease inhibitor cocktail) and