

Neurotoxic effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in cerebellar granule cells

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Abbreviations: AhR, aryl hydrocarbon receptor; α -NF, α -naphthoflavone; $[Ca^{2+}]_i$, intracellular free calcium; LTP, long-term potentiation; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein/extracellular signal-regulated kinase; MK-801, dizocilpine maleate; NMDA, *N*-methyl-D-aspartate; PDBu, phorbol 12, 13-dibutyrate; PKC, protein kinase C; ROS, reactive oxygen species; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)buta-diene

Abstract

An environmental pollutant, tetrachloro dibenzo dioxin (TCDD) is known to illicit the cognitive disability and motor dysfunction in the developing brain. TCDD induced effects leading to neurodevelopmental and neurobehavioral deficit may have been defined, however underlying molecular mechanism and possible intracellular targets remain to be elucidated. In this study, we attempted to analyze TCDD-induced neurotoxic effects in the granule cells from cerebellum where certain cognitive abilities and motor function command are known to be executed. [³H]PDBu, (phorbol 12,13-dibutyrate) binding assay indicated that TCDD induced a dose-dependent increase of total PKC activity and its induction was the aryl hydrocarbon receptor (AhR) dependent and *N*-methyl-D-aspartate receptor (NMDAR) independent. TCDD also caused the translocation of both PKC- α and - ϵ in a dose-dependent manner but associated with different receptors; PKC- α via AhR but not PKC- ϵ indicating an isozyme-specific pattern of the induction. Increase of the ROS formation was also observed in the cells treated with TCDD in a dose-dependent and an AhR-dependent manner. The treatment of the cells with the diamino di-

cyano-bis(2-aminophenylthio) butadiene (U0126, MEK-1/2 inhibitor), dizocilpine maleate (MK-801, non-competitive *N*-methyl-D-aspartate glutamate receptor antagonist) and vitamin E attenuated the TCDD-induced ROS production indicating that TCDD-induced ROS formation may be associated with activation of ERK-1/2 in the MAP kinase pathway or the NMDA receptor. TCDD also increased $[Ca^{2+}]_i$, which is associated with ROS formation and PKC activation in the cerebellar granule cells. It is suggested that TCDD activates the NMDA receptor, which may induce a sustained increase of $[Ca^{2+}]_i$ in neurons followed by the ROS formation. Our findings may contribute to understanding the mechanism of TCDD-related neurotoxicity, thereby improving the health risk assessment of neurotoxic compounds in humans.

Keywords: cerebellar granule cells; neurotoxicity; PKC; ROS; TCDD

Introduction

TCDD is an ubiquitous and most notorious environmental pollutant that accumulates in the brain as well as other organs (Kakeyama *et al.*, 2003). Both human and animal studies showed neurotoxic effects of TCDD such as cognitive impairment and motor dysfunctions due to deficit in the neurodevelopmental processes (Legare *et al.*, 2000; Nayyar *et al.*, 2002). Gestational exposure to TCDD in non-human primates showed neurobehavioral and neurodevelopmental deficits in their offspring (Koopman-Esseboom *et al.*, 1994). Recent studies indicated that low-dose exposure to environmental agents, such as dioxin and PCBs during the period of rapid brain growth, known as the "brain growth spurt" in neonatal mice can lead to disruption of the adult brain function, and also to an increased susceptibility to toxic agents at adult ages (Eriksson, 1997; 2000). These effects are manifested as deranged spontaneous behavior, e.g. hyperactivity, and altered behavioral response to the cholinergic agent, nicotine (Viberg *et al.*, 2002). In addition, these behavioral effects are inducible during a restricted period of neonatal life and get worse with age (Eriksson *et al.*, 2001; 2002). Thus, it is important to identify the window of developmental stage sensitive to the exposure and to understand the underlying mechanism during the brain growth spurt period. Since the previous study indicated that PND-14 is the most vulnerable stage for chemical

exposure during the development (Yang *et al.*, 2003), cerebellar granule cells in this study were isolated from 7 days old rats and grown in culture for 7 additional days to mimic PND-14 conditions.

One of the most pivotal messenger molecules involved in neuronal function and development is protein kinase C (PKC). PKC signaling pathways have been implicated as an important factor in learning and memory processes (Walaas *et al.*, 1991; Huang and Huang, 1993). Since TCDD is known to alter PKC activities in brain cells (Hanneman *et al.*, 1996), perturbation of PKC signaling pathway may contribute to TCDD-induced neurotoxicities *via* aberrant PKC-mediated phosphorylation. While activation of PKC is one of the key effects of TCDD, it remains unknown which isozymes are target molecules. Since PKC isozymes are located in different subcellular compartment and have their unique activation profile (Nishizuka, 1988), it is important to analyze the individual PKC isoforms to understand their biological significance in the cellular system.

Reactive oxygen species (ROS) are continuously produced in the cells as byproducts of the electron transport chain in the mitochondria and by increase in $[Ca^{2+}]_i$ with subsequent activation of several signaling cascades (Mariussen *et al.*, 2002). The developing brain is known to be especially vulnerable to oxidative damage. Thus, it is crucial to understand the effects of TCDD on ROS formation in the developing neuronal cells to better assess the neurotoxic risk.

Therefore, the present study attempted to look into the effects of TCDD on PKC isozymes and ROS production in neuronal cells and identify possible intracellular target molecules for TCDD.

Materials and Methods

Cerebellar granule cell culture

Cerebellar granule cell cultures were prepared from the cerebella of 7-day old Sprague-Dawley rat pups as described previously (Yang and Kodavanti, 2001). Cells were plated at 3×10^6 cells/well in 6-well plate. After plating, cells were incubated at 37°C in a humidified incubator with 5% CO₂ atmosphere. Cytosine arabinoside (5 μM) was added after 24 h to prevent growth of non-neuronal cells. Cells were used for the experiments after 7 days in culture. Cultures typically contained > 95% neurons.

Exposure

Cerebellar granule cells grown on 6-well culture plates were exposed to 0.01, 0.1, 1, 10 nM TCDD (> 99% purity; KOR, Boston, MA) respectively, for 15 min. In order to get enough proteins for immunoblots, 4 culture plates were used for each concentration. After the exposure, cultures were washed twice with Locke's buffer and the cells were harvested in a final volume of 2 ml buffer A (described below).

[³H]PDBu binding assay

Cerebellar granule cells grown on 12-well plastic trays (Costar) were tested at 7 days in culture. [³H]phorbol ester binding method was adopted from Vaccarino *et al.* Briefly, the monolayers were washed with Locke's buffer (154 mM NaCl; 5.6 mM KCl; 3.6 mM NaHCO₃; 2.3 mM CaCl₂; 5.6 mM D-glucose; 5 mM Hepes, pH 7.4) containing 0.1% fatty acid-free BSA. Following washing, the cells were incubated in Locke's buffer containing 1 nM 4-β-[³H]phorbol 12,13-dibutyrate ([³H]PDBu; 0.1 μCi/ml) for 15 min at room temperature with TCDD. An equal amount of DMSO was added to controls. After incubation, the medium was aspirated, and cells were washed three times with Locke's buffer and suspended with 1 ml of 0.1 M NaOH. An aliquot of this sample (0.7 ml) was added to 9 ml Ultima Gold (Packard Inst. Co., Meriden, CT) and the radioactivity was determined using scintillation spectroscopy (Beckman LS 6000LL). A small aliquot was used for protein determination (Bradford, 1976). Nonspecific binding was determined in the presence of 1.6 μM phorbol myristate acetate, which was always < 20%, and subtracted from all the values. The unit of [³H]PDBu binding was fmol/mg protein/15 min.

Cell fractionation

Cells were scraped off into buffer A (20 mM Tris-HCl, pH 7.5, containing 0.25 M sucrose, 2 mM EGTA, 2 mM EDTA and cocktail of protease inhibitors including 0.5 mM PMSF, 10 μg/ml leupeptin and 10 μg/ml pepstatin). The cells were briefly sonicated and centrifuged at 100,000 g for 1 h. The supernatants were designated as cytosolic fraction. The membrane proteins in the pellets were extracted with buffer B (20 mM Tris-HCl, pH 7.5, containing 1% Nonidet P-40, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA and protease inhibitors) on ice for 30 min followed by centrifugation at 15,000 g and the supernatants were saved as detergent-soluble-membrane fraction.

Immunoblotting for PKC isozymes

Immunoblot analysis was performed as described previously (Yang *et al.*, 1999). Proteins (10 μg) from cytosolic and membrane fractions were separated by 7.5% SDS-PAGE and transferred to nitrocellulose membrane by Semi-Dry Transfer Cell (Bio-Rad, Hercules, CA). The nitrocellulose sheet was blocked with 5% non-fat dry milk in Tris-buffered saline. PKC isozymes were detected with isozyme-specific monoclonal antibodies for PKC-α and -ε isozymes (Transduction Lab, Lexington, KY). The blots were reacted with a peroxidase-conjugated anti-mouse IgG and detected by the Super Signal (Pierce, Rockford, IL). The density of respective bands was analyzed by the Fluor-S (Bio-Rad, Hercules, CA). The data were represented as % controls.

ROS detection

Formation of ROS was elucidated by use of the fluo-

rescent probe DCFH-DA. The detection method was adopted from Mariussen *et al.* Briefly, the cells were preincubated with DCFH-DA (5 μ M), dissolved in methanol, at 37°C in 5% CO₂ atmosphere for 20 min. DCFH readily reacts with ROS such as peroxynitrite (ONOO-) and lipid peroxides to the fluorescent DCF. The medium with the DCFH-DA was then removed, and the cells were added to 1.5 ml prewarmed HEPES-buffered (20 mM) HBSS (pH 7.4) with 5 mM glucose containing TCDD. The dishes were incubated for 15 min at room temperature. Thereafter, the cells were gently removed from the dishes and transferred in triplicate to 250- μ l wells (microtiter plate, 96 wells) for ROS measurement. The formation of the fluorescent oxidized derivative of DCFH, namely DCF (fluorescent at 530 nm), was monitored with a luminescence spectrometer (LB96V, Berthold) at 37°C for 180 min.

Measurement of intracellular free calcium [Ca²⁺]_i in cerebellar granule cells

[Ca²⁺]_i was measured in a plate reader using the fluorescent Ca²⁺-sensitive probe fura-2/AM. The cells were cultured in chambers on coverslips. The cells preincubated with fura-2/AM were treated with TCDD in standard saline solution. Excitations were obtained from filters at 340 nm and 380 nm and emission was at 510 nm. [Ca²⁺]_i was estimated using the equation previously described (Lipton and Nicotera, 1998).

Statistics

The data were analyzed by one way analysis of variance followed by Tukey's multiple comparison test. The significance was set at $P < 0.05$.

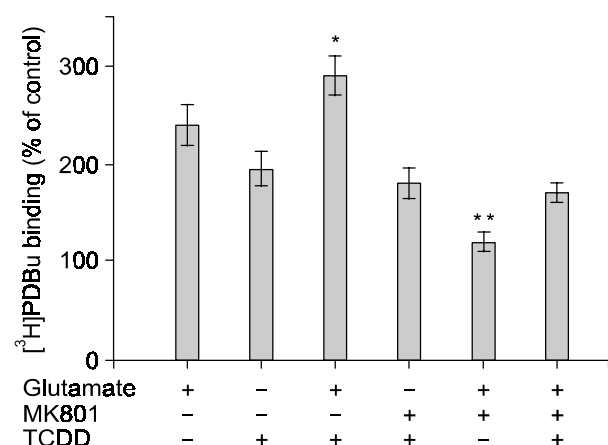


Figure 1. Effects of TCDD and/or MK801 on glutamate-stimulated [³H]phorbol ester binding in cerebellar granule cells. Values are mean \pm SD of four preparations. *significantly different from glutamate or TCDD. **significantly different from TCDD.

Results

Effects of TCDD on PKC

To determine a total PKC activity following TCDD exposure, [³H]PDBu binding assay was performed. TCDD induced a dose-dependent increase of [³H]PDBu binding. The increase was significant from 1 nM with a maximum of 2 fold increase at 10 nM. However, α -naphthoflavone (α -NF), an AhR blocker, inhibited the increase, indicating that the induction was AhR-dependent (Table 1). Subsequent experiments analyzed the effects of TCDD on [³H]PDBu binding in absence or presence of MK-801, a non-competitive NMDA antagonist. While the effects of TCDD and glutamate appeared to be additive, MK-801 did not interfere with TCDD-induced increase of the binding. It is suggested that TCDD effects on [³H]PDBu binding are not mediated *via* the NMDA receptor (Figure 1).

Subcellular changes in PKC isozymes

To examine effects of TCDD on PKC isozymes, immunoblot analysis was performed. The cells were fractionated and subsequently immunoblotted against the PKC- α and - ϵ monoclonal antibodies. When 1 nM and 10 nM TCDD were exposed, translocations of PKC- α and - ϵ isozymes were observed in a dose-dependent manner (Figures 2A and 2B). When cells were pre-treated with α -NF or/and verapamil, the translocation of PKC- α was blocked by α -NF only (Figure 3A). However, the translocation PKC- ϵ was not affected by either α -NF or verapamil (Figure 3B). The results suggest that the translocation of PKC- α and - ϵ is mediated *via* the AhR-dependent and the AhR-independent pathways, respectively, and that TCDD does not directly activate verapamil-sensitive calcium channels.

Table 1. [³H] PDBu binding in cerebellar granule cells following TCDD exposure for 15 min in presence or absence of α -NF.

	0.01	0.1	1	10 (nM)
TCDD only	98 \pm 12	135 \pm 18	164 \pm 11*	190 \pm 15*
TCDD (nM) + α -NF (10 μ M)	101 \pm 5	115 \pm 14	121 \pm 18	135 \pm 29

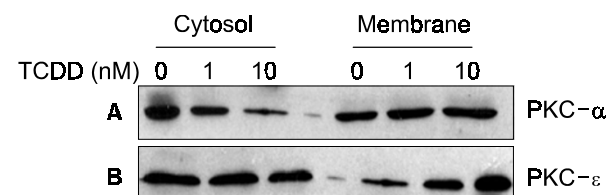


Figure 2. Effects of TCDD on subcellular distribution of PKC- α (A) and PKC- ϵ (B) following 15min exposure. Representative profiles of immunoblots from four separate experiments were given.

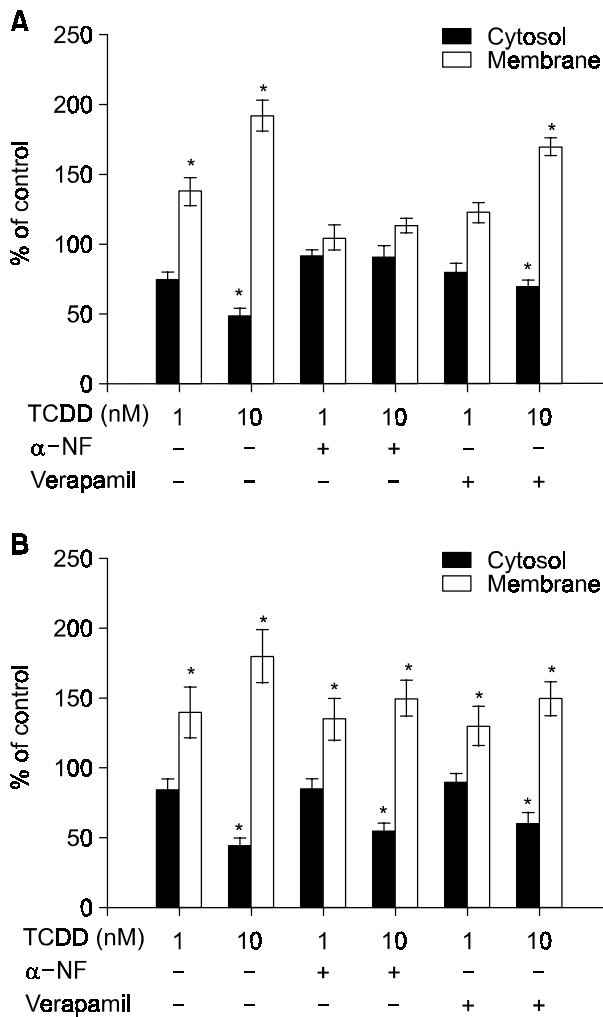


Figure 3. Effects of TCDD on subcellular distribution of PKC- α (A) and PKC- ϵ (B) in SD rat cerebellar granule cells in presence of α -naphthoflavone (α -NF) or Verapamil.

Effects of TCDD on ROS production

TCDD showed a dose-dependent increase in ROS production (Figure 4A). A significant production of ROS was detected from 0.1 nM TCDD with the maximum of 3 fold increase at 10 nM. Increased production was dampened with the treatment of various protective compounds (Figure 4B). Blockage of the TCDD-induced ROS production was most significant by U0126, a MEK-1/2 inhibitor, and followed by MK-801 and vitamin E. The absence of extracellular free calcium seemed to affect the production in a moderate way. However, α -NF did not seem to affect the production, suggesting that the ROS production may be AhR-independent.

Changes in intracellular calcium concentrations

Since increased $[Ca^{2+}]_i$ may be involved in formation of reactive oxygen species (Berridge and Bootman, 2000), intracellular calcium levels following TCDD ex-

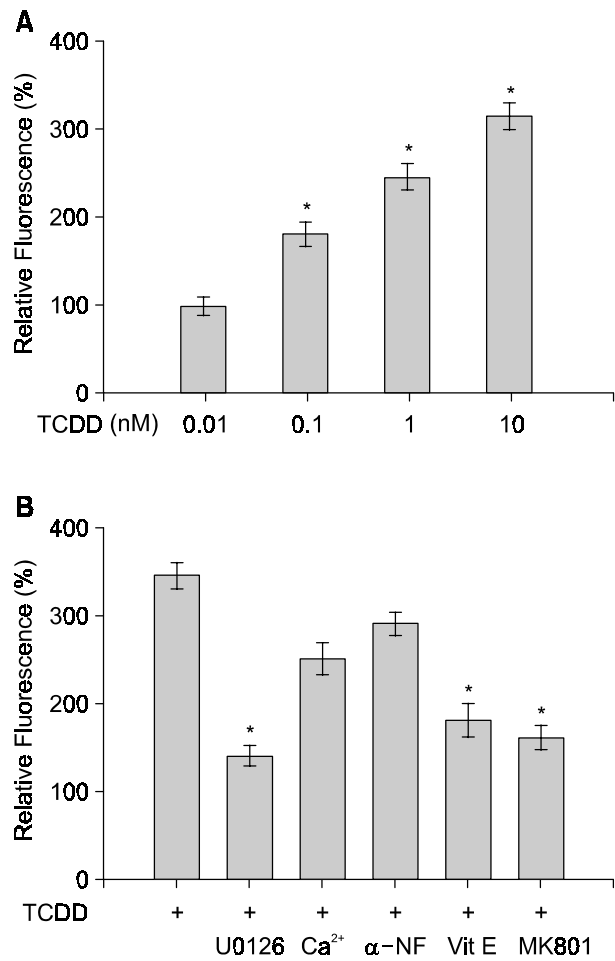


Figure 4. Relative fluorescence as a measure for formation of ROS in cerebellar granule cells following TCDD exposure (A) and in combination with U0126, calcium-free medium, α -NF, vitamin E and MK 801 (B).

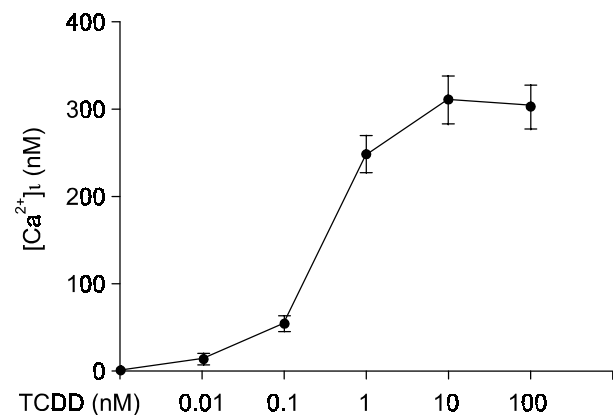


Figure 5. Increase of intracellular calcium concentration after 15 min exposure to the respective TCDD doses. Four separate experiments were carried out for each concentration.

posure were measured. It showed that TCDD induced a dose-dependent increase in $[Ca^{2+}]_i$ in cerebellar granule cells (Figure 5).

Discussion

PKCs are abundant in neuronal tissue and are involved in neuronal survival and functions of neuronal trophic factors, suggesting a crucial role for PKC in the signal transduction between neurons and the etiology of the neuronal diseases (Matsushima *et al.*, 1996). Since functional roles and subcellular distributions of individual PKC isozymes are isozyme-specific (Nishizuka, 1988), identification of specific isozymes targeted for TCDD is required to understand the role of PKC in the TCDD-induced neurotoxicity.

$[^3H]PDBu$ binding assay indicated that TCDD activates PKC in cerebellar granule cells in an AhR-dependent fashion. It provided the evidence that TCDD interferes with the PKC signaling pathways. It is reported that the induction of $[^3H]PDBu$ binding in neuronal cells following the PCB exposures requires the presence of extracellular calcium (Kodavanti *et al.*, 1994). Since extracellular calcium is an important factor on $[^3H]PDBu$ binding in the cerebellar granule cells, we examined a potential route of calcium influx *via* the NMDA receptor, which is known to mediate calcium entry. MK-801 blocked the effects of glutamate on $[^3H]PDBu$ binding, but was ineffective against TCDD-induced increase (Figure 1). The results indicate that TCDD and glutamate may act by the separate mechanism and the NMDA receptor may not be directly involved in the calcium influx required for $[^3H]PDBu$ binding.

Immunoblot analysis revealed that translocations of PKC- α from cytosol to membrane fractions were increased in a dose-dependent manner. PKC- α is selectively associated with lithium-induced memory impairments (Manji *et al.*, 1993). Translocation of PKC- α has been associated with long-term potentiation (LTP) in a hippocampus region (Son *et al.*, 1996). A significant translocation of PKC- α in this study suggests that this particular isozyme may be involved in the TCDD-altered signal transduction pathway. In addition, since PKC- α is Ca^{2+} -dependent isozyme, the translocation may be mediated by the increased free calcium from the exposure of TCDD as shown in Figure 5. However, the treatment of verapamil ruled out the involvement of the voltage-sensitive calcium channel (Figure 3A). Further studies with the additional pharmacological agents are warranted in the future to identify the route of extracellular calcium entry associated with the TCDD-induced PKC activation.

Ca^{2+} -independent forms of PKC are suggested to be involved in the different cellular functions than Ca^{2+} -dependent forms (Konno *et al.*, 1989). Although the physiological roles of Ca^{2+} -independent forms have not been fully clarified, it is known that PKC- ϵ is most abundant in the brain and present mainly in the presynaptic component (Saito *et al.*, 1993). This form has

been suggested to be a candidate isozyme associated with this presynaptic mechanism of LTP (Herreo *et al.*, 1992). Since PKC- ϵ has been associated with a variety of pivotal biological events in neuronal cells, it is feasible that altered subcellular distribution of this particular isozyme may play important roles in the TCDD-induced neurotoxicity. It is of particular interest to note that the translocation of PKC- ϵ was not blocked by the AhR blocker, indicating that certain isozymes of PKC may be activated independent of the AhR. Although TCDD is known to affect a total PKC activity, isozyme-specific PKC profiles following the TCDD exposure has not been determined so far. It is believed that this is the first report demonstrating the subcellular changes of PKC isozyme profiles for TCDD exposure. A significant translocation of PKC- ϵ in this study further suggests that the TCDD-induced neurochemical changes may be, at least in part, mediated *via* calcium-independent pathway. Since neurochemical changes following TCDD or neuroactive PCB exposures have been observed only in the presence of extracellular calcium and most of neurochemical events have been considered Ca^{2+} -dependent (Kodavanti *et al.*, 1994), altered subcellular distribution of the Ca^{2+} -independent isozyme in this study may shed a new light in the mechanistic studies of TCDD-induced neurotoxicity.

While the functional roles of PKC- α and - ϵ in the cerebellar granule cells are not clear, altered subcellular distribution of these isozymes may cause the disruption of normal signal pathways in the developing brain, which ultimately may lead to motor dysfunction and cognitive deficits. In addition, since these isozymes demonstrated a good dose-response relationship upon TCDD exposure, it is suggested that they may be intracellular target molecules sensitive for the exposure of TCDD and related chemicals.

The involvement of ROS formation in TCDD-induced toxicity was assayed using the fluorescence probe DCFH-DA. This is a sensitive and rapid method, which is well suited for detecting overall oxidative stress after stimulation with ROS-forming compounds in low concentrations (Possel *et al.*, 1997; Myhre *et al.*, 2000). TCDD induced production of ROS in a dose-dependent manner. Vitamin E decreased the TCDD-mediated increase in ROS production by 48%. This may be attributed to its properties as an antioxidant, making less ROS available for reaction with DCFH (Packer *et al.*, 2001). ROS reduction by vitamin E further confirms the free radicals generated by TCDD exposure.

The NMDA receptor has been associated with glutamate excitotoxicity and free radical formation (Ankarcrona *et al.*, 1995; Lipton and Nicotera, 1998). NMDA receptor activation may induce a fast and sustained increase in intracellular Ca^{2+} level (Reynolds and Hastings 1995). Since the increase in ROS formation by TCDD was decreased by approximately 54% with MK-801 and TCDD increased the intracellular Ca^{2+} levels, it is suggested that TCDD affects the NMDA receptor, which by activation may cause a sustained elevation of the intracellular Ca^{2+} level in the neuron followed by ROS formation. U0126 also decreased ROS formation by 60%, speculating that activation of

ERK-1/2 in MAPKinase pathway may be involved. The absence of extracellular calcium seemed to dampen the ROS production, indicating that calcium is an important factor for ROS formation. However, α -NF did not seem to affect the TCDD-induced ROS production, suggesting that ROS formation in cerebellar granule cells may be AhR-independent.

While most of TCDD-mediated biological responses are AhR-dependent, AhR-independent pathways have been suggested in recent studies (Hoffer *et al.*, 1996; Yang and Kodavanti, 2001; Butler, *et al.*, 2004). The ROS production and PKC- ϵ translocation by TCDD further support the evidence that the AhR-independent pathways may play pivotal roles in the neuronal cells. Since the health risk assessment of most environmental pollutants is based on the AhR-mediated mechanism of action, whether the responses are AhR-dependent or not is a critical factor in the evaluation of environmental toxic chemicals such as dioxins and other structurally related compounds. The compounds that do not show the association with the AhR on their mechanisms of action have been neglected in the risk assessment processes. Our findings clearly demonstrated that ROS formation and PKC- ϵ translocation by TCDD in neuronal cells were independent of the AhR. Therefore, these neurotoxic effects are not properly assessed under the current risk evaluation concept. The present study provided a scientific basis that a new concept of risk assessment incorporating the AhR-independent mechanisms of action should be developed to assess the neurotoxic effects of the compounds with more accuracy.

This study revealed that TCDD is directly involved in the alteration of the PKC signaling pathway and the ROS production in neuronal cells, which may contribute to understanding of TCDD-related neurotoxicity. Moreover, identification of intracellular target molecules such as PKC- α and - ϵ for TCDD and related compounds may contribute to a better risk assessment of neurotoxicants in humans as well as in our environment.

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