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Involvement of Intracellular Ca^{2+}- and PI3K-Dependent ERK Activation in TCDD-Induced Inhibition of Cell Proliferation in SK-N-SH Human Neuronal Cells

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Abstract – 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) has previously shown to induce neurotoxicity through intracellular Ca^{2+} increase in rat neurons. In this study we investigated the role and signaling pathway of intracellular Ca^{2+} in TCDD-induced inhibition of neuronal cell proliferation in SK-N-SH human neuronal cells. We found that TCDD (10 nM) rapidly increased the level of intracellular Ca^{2+}, which was completely blocked by the extracellular Ca^{2+} chelation with EGTA (1 mM) or by pretreatment of the cells with the non-selective cation channel blocker, flufenamic acid (200 μM). However, pretreatment of the cells with dantrolene (25 μM) and TMB-8 (10 μM), intracellular Ca^{2+}-release blockers, or a voltage-sensitive Ca^{2+} channel blocker, verapamil (100 μM), failed to block the TCDD-induced Ca^{2+} increase in the cells. In addition, TCDD induced a rapid and transient activation of phosphatidylinositol 3-kinase (PI3K) and extracellular signal-regulated kinase1/2 (ERK1/2), which was significantly blocked by the pretreatment with BAPTA, an intracellular Ca^{2+} chelator, and LY294002, a PI3K inhibitor. Furthermore, inhibitors of PI3K, ERK, or an intracellular Ca^{2+} chelator further potentiated the anti-proliferative effect of TCDD in the cells. Collectively, the results suggest that intracellular Ca^{2+} and PI3K-dependent activation of ERK1/2 may be involved in the TCDD-induced inhibition of cell proliferation in SK-N-SH human neuronal cells.

Keywords □ TCDD, neurotoxicity, intracellular Ca^{2+}, ERK, PI3K, proliferation, SK-N-SH human neuronal cells

INTRODUCTION

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is one of the most toxic and environmentally stable tricyclic aromatic compounds. It is known to act via initial binding to a cytosolic aryl hydrocarbon receptor (AhR) protein and show a broad range of toxic effects in various tissues in animals and humans. TCDD has shown the developmental neurotoxicity such as brain asymmetry (Henshel *et al*, 1997), decrease in neurotransmitters (Tilson and Kodavanti, 1997), neuronal calcium uptake (Hanneman *et al.*, 1996), cognitive deficits (Schantz and R.E. Bowman, 1989) and death of neurons (Carpenter *et al*, 1997; Kim and Yang, 2005). The toxic effect of TCDD appears to be mediated through alteration of redox balance in hepatic and extrahepatic tissues of mice and rats (Stohs, 1990; Hassoun et al., 1998). Even though most of these reports demonstrated induction of oxidative stress by TCDD, recent reports demonstrated a reduced peroxide level and increase in GSH contents in TCDD-treated hepatocytes (Lawrense *et al.*, 1999; Shertzer *et al.*, 1998). We have also showed that inhibitory effect of TCDD on the proliferation of SK-N-SH human neuronal cells is partially mediated through decreased production of reactive oxygen species (Lee *et al.*, 2002). In addition, our previous study has shown that TCDD induces inhibition of neuronal cell proliferation which is mediated through the AhR-dependent increased expression of p27 and hypophosphorylation of pRB in SK-N-SH cells (Jin *et al.*, 2004). Although TCDD induces AhR-dependent cell cycle arrest at G1, we have also shown that

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TCDD did not induce death of the neuronal cells, implying that there is another survival signaling pathway is involved in along with anti-proliferative signaling pathways by TCDD in the cells.

Intracellular calcium is an important regulator of neuronal proliferation and survival during development of the nervous system, triggering important survival signals, including phosphorylation of the antiapoptotic factor Akt (Cheng *et al.*, 2003), the mitogen-activated protein kinase (MAP) kinase ERK (p42/44) (Fahlman *et al.*, 2002) and the transcription of brain-derived neurotrophic factor (Chen *et al.*, 2003). In addition, it has been also reported that ERK1/2 activity was significantly increased in primitive neuroectodermal tumors compared to normal brain (Damodar *et al.*, 2001). Activation of PI3K and its downstream signal molecule, Akt, plays a central role in the signaling of cell survival triggered by neurotrophins, in which Ca^{2+} and calmodulin are also involved in the activation of the Akt (Egea *et al.*, 2001). In fact, TCDD has shown to mimic the action of growth factor in MCF-10A human mammary epithelial cells. Treatment with TCDD increases total tyrosine phosphorylation including insulin like growth factor receptor and PI3K activity in the absence of insulin, and thus, promoting cell growth (Tannheimer *et al.*, 1998).

Thus, in the present study, we investigated whether anti-proliferative action of TCDD is mediated through intracellular Ca^{2+} and PI3K/Akt signaling pathway in SK-N-SH human neuronal cell that is widely used as a good in vitro model for developing human neuronal system (Martin *et al.*, 1992).

MATERIALS AND METHODS

Materials

The SK-N-SH human neuroblastoma cell line was purchased from American Type Culture Collection (Rockville, MA). The powders for Eagle's minimum essential medium, trypsin solution, sodium pyruvate, MTT and all salt powders were obtained from Sigma Chemical CO. (St. Louis, MO). Bis-(o-aminophenoxy)-ethane-N,N,N,N-tetraacetic acid/acetoxymethyl ester (BAPTA/AM), and 1-(2,5-carboxyoxazol-2-yl-6-aminobenzfuran-5-oxyl)-2-(2'-amino-methylphenoxy)-ethane-N,N,N,N-tetraacetoxylmethyl ester (Fura-2/AM) were from Molecular Probes, Inc. (Eugene, OR). TCDD was from Supelco (Bellefonte, PA, USA). Fetal bovine serum and antibiotics (penicillin and streptomycin mixture) were purchased from GIBCO (Grand Island, NY). The stock solutions of drugs were sterilized by filtration through 0.2 μm disc filters (Gelman Sciences: Ann Arbor, MI).

Cell culture

SK-N-SH cells were grown at 37°C in a humidified incubator under 5% CO_2/95% air in an Eagle's minimum essential medium supplemented with 10% fetal bovine serum, 200 IU/ml penicillin, 200 μg/ml of streptomycin and 1 mM sodium pyruvate. Culture medium was replaced every other day. After attaining confluence the cells were subcultured following trypsinization.

Intracellular Ca^{2+} measurement

Aliquots of the HepG2 cells were washed in PBS. Then, 5 mM Fura-2/AM was added, and the cells were incubated for 30 min at 37°C. Unloaded Fura-2/AM was removed by centrifugation at 150*g* for 3 min. Cells were resuspended at a density of 2×10^6 cells/ml in Krebs-Ringer buffer containing 125 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 5 mM $NaHCO_3$, 25 mM HEPES, 6 mM glucose and 2.5 mM probenecid (pH 7.4). Fura-2/AM-loaded cells were maintained at 25°C for 90 min before fluorescence measurement. For each experiment, 0.5 ml aliquot of Fura-2/AM-loaded cells was equilibrated to 37°C in a stirred quartz cuvette. Fluorescence emission (510 nm) was monitored with the excitation wavelength cycling between 340 and 380 nm using a Hitachi F4500 fluorescence spectrophotometer. At the end of an experiment, fluorescence maximum and minimum values at each excitation wavelength were obtained by first lysis of cells with 20 g/ml digitonin (maximum) and then adding 10 mM EGTA (minimum). With the maximum and minimum values, the 340:380 nm fluorescence ratios were converted into free Ca^{2+} concentrations using a software, F-4500 Intracellular Cation Measurement System, provided by Hitachi.

Phosphatidylinositol 3-kinase assays

The PI3K activity assay was performed using a commercially available kit (PI3Kinase ELISA kit, according to the manufacturer's protocol (Echelon Biosciences Inc., Salt Lake City, UT). Cells were washed twice with ice-cold buffer A (137 mM NaCl, 20 mM Tris-HCl pH 7.4, 1 mM $CaCl_2$, 0.1 mM sodium orthovanadate). After the washing, the cells were lysed immediately with 1 ml lysis buffer (buffer A plus 1% NP40 and 1 mM phenylmethylsulfonyl fluoride) for 30 min at 4°C. The lysates were centrifuged at 12000 rpm for 15 minutes. The supernatants were mixed with 5 ml of anti-PI3K antibody (Upstate Biotechnology, Lake Placid, NY) for 1 h at 4°C, and

then, incubated with 60 ml of 50% protein A-agarose beads in PBS for 1 h at 4°C. The immunoprecipitated enzyme was collected by centrifuging 5 seconds and wash three times with with buffer A containing 1 % NP40, three times with 0.1 M Tris-HCl, pH 7.4, 5 mM LiCl, and 0.1 mM sodium orthovanadate, twice with 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA and 0.1 mM sodium orthovanadate. The collected immunocomplex were used immediately mixed reaction buffer containing 4 mM MgCl2, 20 mM Tris-HCl, pH 7.4, 10 mM NaCl, 25 mM ATP and PI(3,4,5)P3 detector protein for 1h at room temperature. Then, the reaction mixtures were added to the PI(3,4,5)P3-coated microplate for competitive binding. Each well of the plate was washed three times with TBS-T (150 mM NaCl, 10 mM Tris pH 7.5 and 0.05% Tween-20) and added 100 ml of TMB solution. After 30 min incubation, o.5 M H_2SO_4 solution was added to stop the reaction and then the absorbance was read at 450nm. The colorimetric signal was inversely proportional to the amount of PI(3,4,5)P_3 used for standard curve. The activity was normalized by protein amounts.

Western blot analysis

Cells were washed with PBS solution and centrifuged at 1,000*g* for 5 min. Cell pellets was lysed for 15 min at 4oC in whole cell extraction buffer containing 50 mM HEPES (pH 7.4), 0.5% Nonidet P-40, 10% glycerol, 137 mM NaCl, 1 mM EGTA, 10 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl floride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin A, 40 mM α–glycerophosphate, 0.1 mM DTT. Lysates were centrifuged at 20,000*g* for 10 min at 4°C , and supernatant proteins were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to Hybond ECL nitrocellulose membrane (Amersham Life Science, Buckinghamshire, England) at 30 V for overnight. The membrane was blocked with 5% skim milk in Tween-20 containing Tris buffered saline (TTBS) (20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.05% Tween-20) and incubated with primary anti-human PI3K (Upstate Biotechnology, NY, USA), pERK (Santa Cruz Biotechnology, CA, USA), ERK (Cell Signaling, MA, USA), pAkt (Cell Signaling), and Akt (Cell Signaling) antibody in TTBS containing 5% skim milk. After incubation with horseradish peroxidase-conjugated anti-IgG antibody (Santa Cruz Biotechnology, CA, USA), immunodetected proteins were visualized by using enhanced chemiluminescence assay kit (Amersharm Life Science, Buckinghamshire, England).

Cell viability assay (MTT staining)

Cell viability was assessed by the MTT staining method (van de Loosdrecht et al., 1991). Cells from 4- to 5-day-old cultures were seeded in 24-well plates at the density of 5×10^4 cells/well. The volume of the medium in the wells was 1 ml. In control experiments cells were grown in the same media containing drug-free vehicle. After incubation with drug for 48 h, 100 l of MTT (5 g MTT/l in H_2O) were added and cells incubated for a further 4 h. Two hundred microliters of DMSO were added to each culture and mixed by pipetting to dissolve the reduced MTT crystals. Relative cell viability was obtained by scanning with an ELISA reader (Molecular Devices, Menlo Park, CA) with a 540 nm filter.

Data analysis

All experiments were performed four times. Data were expressed as mean±standard error of the mean (SEM) and were analyzed using one way analysis of variance (ANOVA) and Student-Newman-Keul's test for individual comparisons. P values less than 0.05 are considered statistically significant.

RESULTS

Induction of Ca^{2+} influx and PI3K activation in TCDD-treated SK-N-SH human neuronal cells

Since TCDD induces AhR-dependent cell cycle arrest at G1 without inducing cell death in SK-N-SH human neuronal cells and intracellular Ca^{2+} is known to trigger important survival signals including phosphorylation of the antiapoptotic factor Akt (Cheng *et al.*, 2003) and the mitogen-activated protein kinase (MAP) kinase ERK (p42/44) (Fahlman *et al.*, 2002), in the present study, we examined whether an intracellular Ca^{2+} signaling pathway is activated in along with anti-proliferative signaling pathways by TCDD in the cells. As shown in Fig. 1, TCDD induced a rapid increase in intracellular Ca^{2+} level. This increase was completely blocked by the extracellular Ca^{2+} chelation with EGTA (1 mM) or by pretreatment of the cells with a non-selective cation channel blocker, flufenamic acid (200 μM). However, pretreatment of the cells with intracellular Ca^{2+}-release blockers, dantrolene (25 μM) and TMB-8 (10 μM), or a voltage-sensitive Ca^{2+} channel blocker, verapamil (100 μM), failed to block the TCDD-induced Ca^{2+} increase in the cells.

Activation of PI3K and ERK1/2 by TCDD in the SK-N-SH cells

Since it has been known that TCDD increases PI3K activity

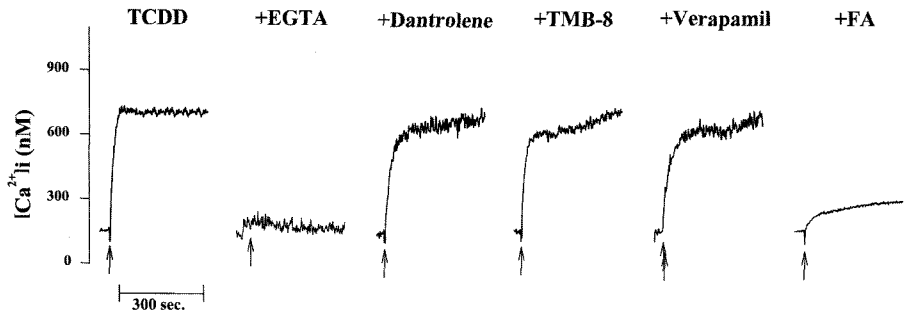

Fig. 1. TCDD induces intracellular Ca^{2+} increase through the activation of nonselective cationic channels. Intracellular Ca^{2+} level was measured by Fura-2 fluorescence method. The arrow shows the time point for addition of TCDD (10 nM). EGTA (1 mM), dantrolene (25 μM), TMB-8 (10 μM), verapamil (100 μM) or flufenamic acid (FA) (200 μM) was added 30 min prior to TCDD treatment.

in the absence of growth promoting signal (Tannheimer *et al.*, 1998) and that the phosphatidylinositide-3-kinase (PI3K)/Akt pathways have central roles in the regulation of cell survival and proliferation, we also examined the activation of those kinases in TCDD-treated SK-N-SH cells. As shown in Fig. 2A, a rapid and time-dependent activation of phosphatidylinositol 3-kinase (PI3K) was sustained more than 30 min after TCDD exposure with no changes in protein expression of PI3K (Fig. 2B). In addition, TCDD rapidly increased the phosphorylation of extracellular signal-regulated kinase1/2 (ERK1/2) up to 10 min, as depicted in Fig. 2C, whereas Akt was not phosphorylated (Fig. 2D).

Involvement of intracellular Ca^{2+} and PI3K signals in the TCDD-induced ERK activation and inhibition of SK-N-SH cell proliferation

Next, we examined whether there is any relationship between intracellular Ca^{2+} increase and the activated PI3K and ERK. The TCDD-induced ERK activation was blocked by the pretreatment with BAPTA, an intracellular Ca^{2+} chelator, and LY294002, a PI3K inhibitor, as shown in Fig. 3A, indicating TCDD-induced ERK activation may be dependent on the intracellular Ca^{2+} and PI3K activity. Moreover, these signals were found to be involved in the inhibitory effect of TCDD on the proliferation of SK-N-SH cells. TCDD-induced inhibition of the cell proliferation was enhanced by the pretreatment with BAPTA, LY294002, or PD98059, an inhibitor of ERK, as demonstrated in the Fig. 3B. Moreover, pretreatment with combination of BAPTA, LY294002 and PD98059 further enhanced the TCDD-induced inhibition of cell proliferation, as illustrated in Fig. 3B.

DISCUSSION

Increased Ca^{2+} has been linked to the TCDD-induced neuro-

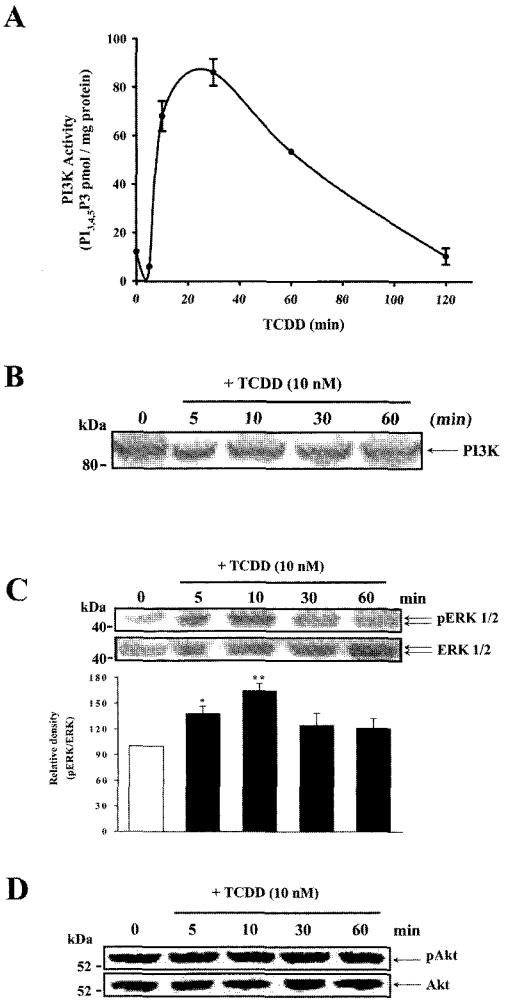

Fig. 2. Effects of TCDD on the activity and expression of PI3K, ERK and Akt. The PI3K activity (A) was measured using a commercially available PI3Kinase ELISA kit, according to the manufacturer's protocol. The expression level of PI3K (B), the phosphorylated ERK/ERK (C), and the phosphorylated Akt/Akt (D) was evaluated by Western blotting analysis. In bar graphs the data represent the mean values of 3 independent experiments with bars indicating S.E.M. $*P<0.05$, $**P<0.01$ compared to untreated control.

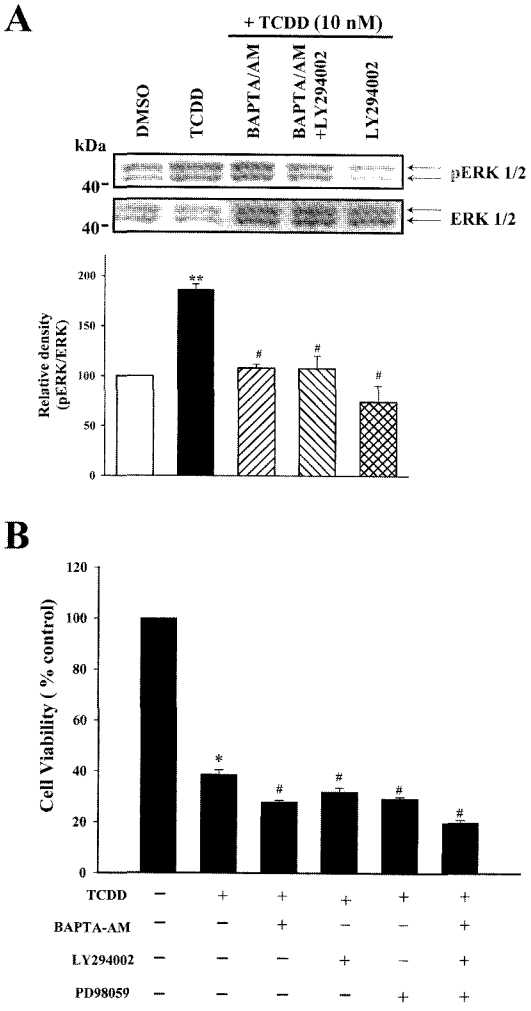

Fig. 3. Effects of various protein kinase inhibitors and a Ca^{2+} chelator on TCDD-induced ERK activation and the viability of SK-N-SH cells. Cells were treated with TCDD for 48 hr in the presence or absence of inhibitors. BAPTA/AM (2 μM) was pretreated for 4 hrs and LY294002 (10 μM) and PD98059 (25 μM) were pretreated 1 hr prior to TCDD treatment. *P< 0.05 compared to control. #P<0.05 compared to the group treated with TCDD alone.

toxicity in primary cultured rat astroglia, hippocampal and cerebellar neurons as well as cardiotoxicity in developing chick embryo (Hanneman WH et al., 1996; Legare *et al.*, 1997; Kim and Yang, 2005). In human cells, the effect of TCDD on the intracellular Ca^{2+} level seems to be contradictory. In human mammary epithelial cells, it is shown that TCDD increases intracellular Ca^{2+} concentration, whereas it does not elevate Ca^{2+} in MCF-10A mammary epithelial cell line (Tannheimer *et al.*, 1997; Tannheimer *et al.*, 1999). In the present study, we also showed that TCDD induced Ca^{2+} influx in another human neuronal cell line, SK-N-SH.

The present study also demonstrated that TCDD-induced intracellular Ca^{2+} increase activated ERK and prevented further decrease in the cell growth induced by TCDD in the cells. Our results showing that TCDD increased PI3K activity and that pretreatement with a PI3K inhibitor not only suppressed ERK activation but enhanced the TCDD-induced decrease in cell proliferation indicate that intracellular Ca^{2+} and PI3K activation may, at least in part, suppress the anti-proliferative signaling induced by TCDD through ERK. This is contrary to the report that TCDD mimics the action of a growth factor through Ca^{2+}-independent PI3K activity in promoting the proliferation of MCF-10A human mammary epithelial cells in the absence of insulin (Tannheimer *et al.*, 1998). The difference between two results seems to be due to the presence of growth factors in the culture medium. Although Akt is well known downstream target of PI3K activation, our result showed that TCDD did not affect the Akt activity in the cells in spite of PI3K activation. Instead, ERK seems to be a downstream target molecule of PI3K activation in TCDD-treated SK-N-SH cells. Indeed, there are reports that TCDD transiently activates the ERK pathway in MCF10A cells (Davis *et al.* 2001), and TCDD-induced differentiation of thymocytes toward CD8 positive T cells requires the activation of ERK (Tsukumo *et al.*, 2002).

Previous results have shown that TCDD-induced inhibition of neuronal cell proliferation is mediated through decreased production of reactive oxygen species(ROS) (Lee et al., 2002) and the AhR-dependent increased expression of p27 and hypophosphorylation of pRB in SK-N-SH cells (Jin *et al.*, 2004). In addition to those signals, our present results suggest that Ca^{2+}- and PI3K-dependent activation of ERK1/2 by TCDD may also be involved in and exert an opposite effect on the ROS-p27-pRB pathways leading to the TCDD-induced growth suppression in SK-N-SH human neuronal cells.

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