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2,3,7,8-Tetrachlorodibenzo-p-dioxin Activates ERK and p38 Mitogen-activated Protein Kinases in RAW 264.7 Cells

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Abstract. *2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is a widespread environmental contaminant, exposure to it eliciting a broad spectrum of deleterious pathophysiological effects. Since mitogen-activated protein kinase (MAPK) pathways appear to play an important role in both cell survival and the apoptotic process, we assessed the effects of TCDD on the activation of extracellular signal-regulated kinase (ERK), Jun-N-terminal kinase (JNK), p38 MAPKs and caspase-3 in RAW 264.7 cells. TCDD treatment induced a transient upshift in ERK activity, followed by a decline, but a concomitant dramatic activation of p38. However, TCDD did not cause any apparent change in the activity of JNK, though it induced an up-regulation in caspase-3 activity. These results demonstrate that the equilibrium between the ERK and p38 pathways is critical to the fate of the cells, and that the activation of p38, upstream of caspase, plays an important role in the apoptotic process. The data obtained in this study also suggests that TCDD activates the MAPK pathway via an arylhydrocarbon receptor (AhR)-independent mechanism in RAW 264.7 murine macrophages.*

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is the most toxic compound of the halogenated aromatic hydrocarbons, which are a group of widespread environmental contaminants, causing public concern due both to their toxicity and their increasing ubiquity in the environment (1, 2). The adverse biological effects of TCDD observed in animals include immune, reproductive and developmental toxicity, carcinogenesis, wasting syndrome, chloracne and lethality (3-6).

Initially, the majority of studies assessing the immunotoxicity of TCDD focused on the lymphocytes (7), and it was commonly believed that exposure to TCDD had no demonstrable effect on the functions of natural killer cells or macrophages (8, reviewed in 9). However, recent studies have shown that human macrophages contain the arylhydrocarbon receptor (AhR) and arylhydrocarbon receptor nucleus translocator (ARNT) (10), and that TCDD can act directly on the peritoneal macrophages, resulting in increases in TNF production (11). These findings indicate that TCDD can directly influence the signaling pathways operative in the immune functions of macrophages. The classically accepted toxicity model with respect to TCDD treatment was that TCDD first interacts with AhR, and the liganded AhR translocates to the nucleus, where it forms a heterodimer with the nuclear protein, ARNT. The AhR/ARNT complex then binds to the dioxin response elements (DREs), thereby activating transcription (1, 12, reviewed in 13). However, recent studies have shown that the mechanisms of TCDD immunotoxicity involve both AhR-mediated and AhR-independent events (11, 14, 15). A previous report, showing that TCDD activates extracellular signal-regulated kinases (ERKs) and/or Jun-N-terminal kinases (JNKs) (6, 14, 16), suggests that TCDD activates MAPKs (mitogen-activated protein kinases) *via* an AhR-independent mechanism. The ERKs, JNKs and p38s together comprise the family of MAPKs. Since the MAPKs play central roles in the intracellular signal transduction pathways, in response to a variety of cellular stimuli (17, 18), this study investigated the ability of TCDD to activate ERK1/2, p38, JNK and caspase-3 in the TCDD-mediated apoptosis of RAW 264.7 cells.

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Materials and Methods

Culture conditions. RAW 264.7 cells (ATCC TIB71) were cultured at $2x10^6$ cells/ml in Dulbecco's modified Eagles medium (DMEM, Gibco BRL, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco BRL), 10 μg gentamicin (Irvine Scientific, Santa Ana, CA, USA)/ml medium, $5x10^{-5}$ M 2-mercaptoethanol (Sigma, St. Louis, MO, USA), and 1 μg/ml polymyxin-B (Gibco BRL). Duplicate RAW 264.7 cell cultures were either exposed or not exposed to 10 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, Supelco, Bellefonte, PA, USA), and incubated for 30 minutes to 48 hours in a humidified incubator containing 5% CO_2 at 37˚C.

Immunoblot analysis. The cultured cells were harvested by repeated pipetting, centrifugation at 500 x g for 5 minutes at 4˚C, and washing with phosphate-buffered saline (PBS, 0.1M, pH 7.2). The cell pellet was then mixed with ice-cold lysis buffer, containing 25 mM Hepes (pH 7.5, Sigma), 300 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM ethylenediamine tetraacetic acid (EDTA, Sigma), 0.1 % Triton X-100 (Sigma), 0.5 mM dithiothreitol (DTT, Bio-Rad, San Diego, CA, USA), 20 mM glycerophosphate (Sigma), 0.1 mM Na_3VO_4 (Sigma), 2 μg/ml leupeptin and 1 mM PMSF (Roche Molecular Biochemical Co., Mannheim, Germany). This mixture was then incubated on ice for 30 minutes. The lysates were sonicated on ice for 20 seconds and then centrifuged at 15,000 x g for 20 minutes at 4˚C, after which time the supernatant was collected. The protein concentration was determined by the Bradford method, using a kit from Bio-Rad, with bovine serum albumin (Bio-Rad) as a standard. The total cell lysate was electrophoretically separated on SDS-polyacrylamide gel, and the proteins were transferred to PVDF-plus membranes (Osmonics, Westborough, MA, USA). To detect transferred proteins, the PVDF-plus membranes were incubated with 5% non-fat milk in TTBS (20 mM Tris, 0.5 M NaCl, 0.2% Tween 20, pH 7.4) for 1 hour at room temperature. The membranes were then incubated at 4˚C, overnight, with a 1:500 to 1:1000 dilution of mouse, rabbit, or goat antiserum, raised against ERK, p-ERK, p38, p-p38, p-JNK or caspase-3 (Santa Cruz Biotechnology, Santa Cruz, CA,USA), and then washed in TTBS, and incubated for 1.5 hours with a 1: 1000 to 1:2000 dilution of rabbit anti-mouse, sheep anti-rabbit or anti-goat IgG conjugated with alkaline phosphatase (Santa Cruz Biotechnology). After washing with TTBS, immunoreactive bands on the membranes were developed using NBT/BCIP substrate (Roche Molecular Biochemical Co.). The relative densities of specific immunoreactive bands were evaluated by scanning with a laser Computing Densitometer and using the ImageQuant program version 3.3 (Molecular Dynamics, Sunnyvale, CA, USA).

MTT assay. The colorimetric 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) assay was performed, as described by Hanelt *et al.* (19), in order to assess the proliferation/viability of the RAW 264.7 cell cultures. In brief, MTT (Sigma) was dissolved in sterile PBS at 5 mg/ml, filtered through a 0.22-μm filter (Gelman Science, Ann Arbor, MI, USA) to remove any insoluble residue, and stored in the dark at 4˚C, for no longer than 1 week. The MTT assay was performed in 96-well tissue culture plates (Costar, Cambridge, MA, USA). An aliquot containing 25 μl of stock MTT solution was added to each 200-μl culture well, and the plates were incubated for 3 hours at 37˚C. The plates were centrifuged at 450 x g for 10 minutes, and the supernatants were removed and replaced with 150 μl of DMSO. The plates were shaken to maximize solubilization of the purple formazan crystals, and the absorbance was measured on a *V*max Microplate Reader (Molecular Devices), at a 570 nm test wavelength and a 670 nm reference wavelength.

DNA agarose gel electrophoresis. Isolation of the apoptotic DNA fragment was performed according to the method proposed by Hermann *et al.* (20). In brief, TCDD-treated cells were harvested, washed with PBS and pelleted by centrifugation. The pellets were incubated with lysis buffer (50 mM Tris-HCl, pH 7.5, 0.1% Nonidet P-40, 20 mM EDTA) on ice for 30 minutes, and the supernatants were obtained by centrifugation. The supernatants were then digested with 5 μg/ml RNase A for 1 hour at 37˚C and then incubated for another hour at the same temperature with proteinase K (0.5 mg/ml, Sigma). Next, a 0.5 volume of 10 M ammonium acetate was added. After centrifugation, the DNA pellets were washed with 75% ethanol, briefly dried for 5-10 minutes, dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), and then quantified by taking OD readings at 260 nm. Equal amounts of DNA from different time-treated cells were electrophoretically separated on a 2% agarose gel, containing 1 g/ml ethidium bromide, for 2.5 hours at 100V.

Statistical analysis. Comparisons between the control group and each experimental group were conducted by Student's *t*-tests; $p<0.05$ was considered significant. The data are presented as the means±SD.

Results

Effect on cell viability and DNA fragmentation. Treatment with 10 nM TCDD resulted in a significant, time-dependent decrease in cell viability in Jurkat T cells (16). Therefore, we attempted to ascertain whether this reference dosage of TCDD also resulted in the apoptosis of macrophages, RAW 264.7 cells. We also investigated any effects TCDD might have on RAW 264.7 cell viability. As shown in Figure 1, this concentration of TCDD resulted in a characteristic DNA ladder formation, which was measured at all times, whereas no apoptosis-associated DNA fragments were observed in the unexposed control group. Although this DNA fragment pattern was less distinct within the first 30 minutes after treatment with TCDD, no distinct differences in DNA cleavage patterns were detected, although they were evaluated at various times following TCDD treatment.

In order to compare the degrees of apoptosis occurring at each time-point, RAW 264.7 cells were exposed to 10 nM TCDD for 0-48 hours. Cell viability decreased over time, in response to TCDD treatment. Moreover, exposure to TCDD for 8-48 hours caused a significant decrease in the percentage of cell viability, relative to the values established before TCDD treatment (83.1%, 79.8%, 77.1% and 64.7%, respectively) (Figure 2). These data indicate that TCDD treatment resulted in immediate apoptosis in the macrophages.

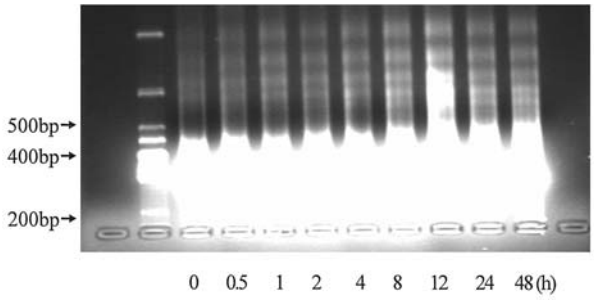

Figure 1. *Effect of TCDD on DNA fragmentation. RAW 264.7 cells were cultured for varying times in DMEM containing 10% FBS, either with or without 10 nM TCDD (time 0). Soluble cytoplasmic DNA obtained at each time-point was analyzed by 2% agarose gel electrophoresis.*

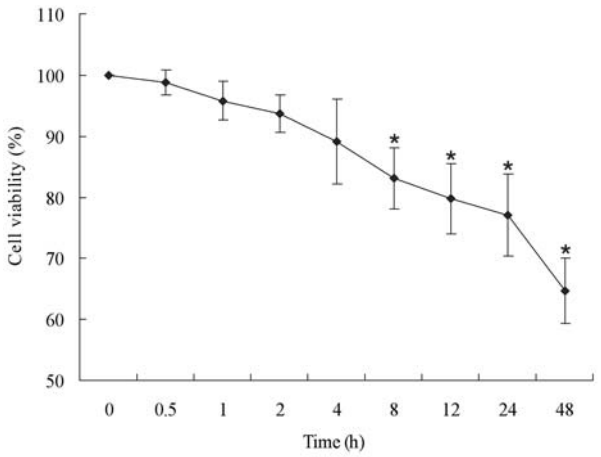

Figure 2. *Effect of TCDD on cell viability. RAW 264.7 cells were treated with TCDD for different times. The proportion of surviving cells was measured by MTT assay. The relative percentages of cell viability are expressed as measured values versus the value before TCDD treatment (time 0 control). The values shown are expressed as the means±SD of three individual experiments. * $p<0.05$ compared with time 0 control.*

Effect on MAPK pathways and caspase-3. In order to ascertain whether or not MAPK pathways are involved in the cellular response to TCDD in the macrophages, the activation of MAPKs was examined, *via* the detection of the phosphorylated state of each kinase. Based on our findings that 10 nM TCDD significantly decreased cell viability 8 hours after treatment, time-points between 0-12 hours were chosen, in order to investigate changes in the kinase cascades which might result from exposure to TCDD.

As shown in Figure 3A, the phosphorylation of ERK became apparent 4 hours after the addition of TCDD to the culture medium, and began to decline 12 hours after exposure. The ERK activation level as a result of TCDD stimulation over time was more apparent with ERK1 than ERK2. The induction of p38 activity was observed 1 hour after exposure, and its activation persisted for 12 hours (Figure 3B). Under the same stimulation conditions, TCDD did not result in the activation of the JNK pathway (Figure 3C). The expressions of total ERK, p38 and JNK were not affected by TCDD.

Previous reports have shown that caspase activation plays a central role in apoptosis (21), and TCDD has been observed to induce the up-regulation of caspase in both Jurkat and human leukemic T cells (14, 16). In order to more definitively demonstrate that TCDD-induced apoptosis in macrophages occurred *via* AhR-independent mechanisms, we investigated the alternation of the proteolytic activity of caspase in the early post-treatment stages. As shown in Figure 3D, the expression of caspase-3 was induced as early as 30 minutes after exposure, its level tending to increase gradually thereafter.

Discussion

MAPKs are important factors in the transduction of extracellular signals, from the membranes to the nucleus (17). It has been well-established that activation of each MAPK appears to regulate a distinct cellular response. For example, ERK1 and 2 mediate cell proliferation and differentiation and protect cells from apoptotic cell death, whereas SAPK/JNK and p38 MAPK, conversely, inhibit cell proliferation and may promote apoptotic cell death. Accordingly, the balance between the ERK1/2 pathway and the stress-activated SAPK/JNK and p38 MAPK pathways is considered to be a fundamental determinant of cell survival or apoptosis (reviewed in 18). However, reports have predominantly indicated that these signaling pathways exhibit more complex roles than previously thought in the regulation of distinct cellular effects (22). For instance, ERK1/2 kinases have been reported to potentiate apoptosis in leukemic cells (23). On the other hand, SAPK/JNK activation occurs with no effect on rates of cell death (24), and p38 is required for proliferation, not for apoptosis in B lymphocytes (25). Therefore, these previous reports seem to indicate that the MAPK activation pathways are specific to both cell type and stimulus.

Recent data demonstrates that TCDD causes the activation of MAPK pathways in mouse Heap-1 hepatoma cells (6), human leukemic lymphoblastic T cells (14) and Jurkat T cells (16). However, the effect of TCDD on MAPKs in macrophages has remained unstudied. In this study, the relationship between ERK1/2, p38 and JNK activity in the induction of apoptosis in RAW 264.7 murine macrophages treated with TCDD was investigated. Our results show that the activity of ERK underwent a transient upshift after TCDD treatment and then declined, and also that the activation of p38 was immediately and dramatically

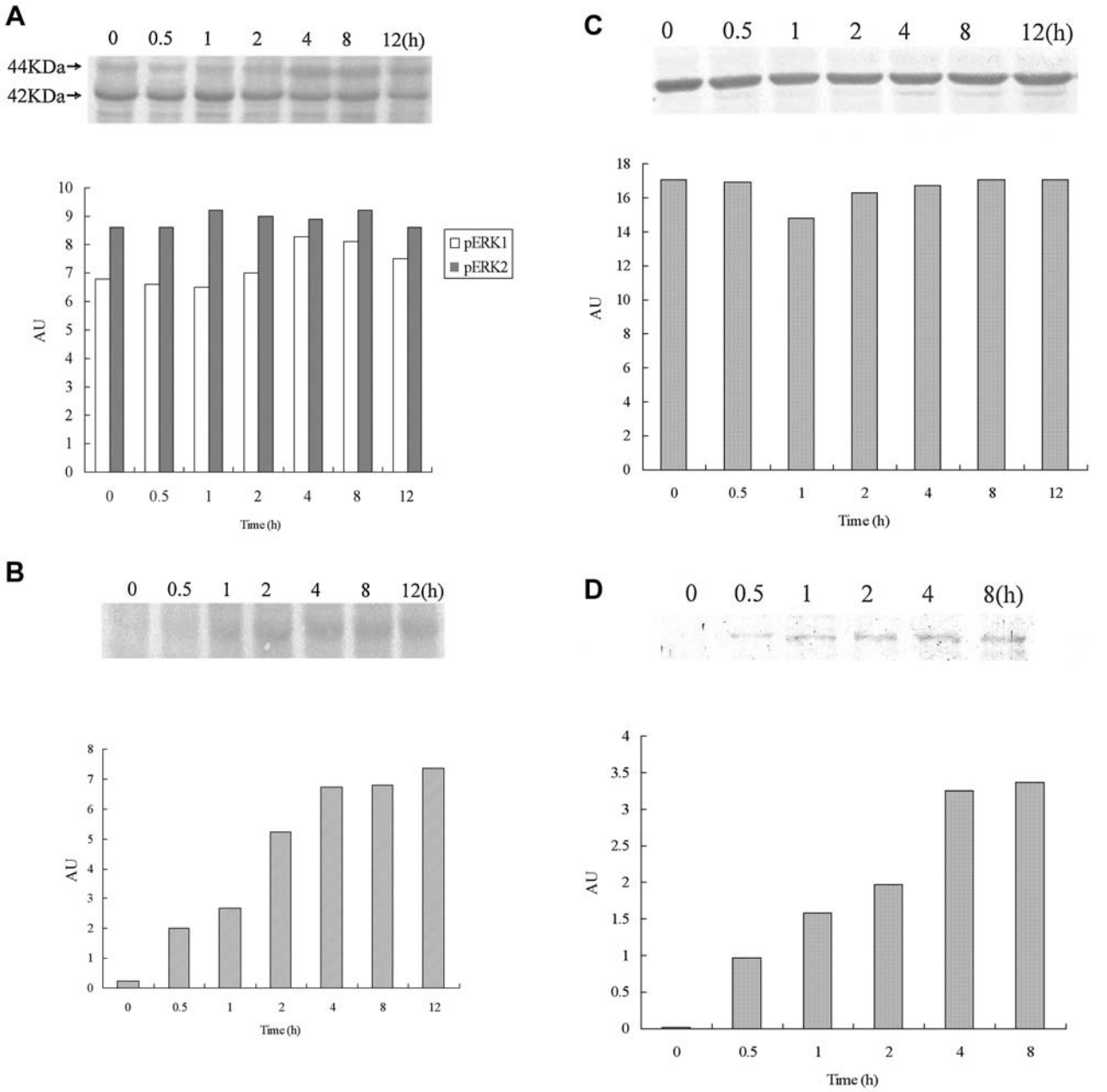

Figure 3. *Effect of TCDD on ERK (A), p38 (B), JNK (C) and caspase (D) activities. Cells were treated with TCDD for the indicated times. Phosphorylation of ERK, p38 and JNK was assessed by SDS-PAGE fractionation of equivalent amounts of protein from each sample, followed by immunoblotting with phospho-specific antibodies. The densities of p-ERK, p-p38, p-JNK and caspase-3 bands were determined using a densitometer and the ImageQuant software. The results are representative of three separate experiments. AU = arbitrary densitometric unit.*

induced. However, TCDD did not result in any apparent changes in the activity of JNK. Since the ERK signaling cascade is required for cell proliferation, while the p38 MAPK pathway is involved in growth arrest and the apoptotic pathway, and the equilibrium existing between the two MAPK pathways may be critical in determining cellular fate (16, reviewed in 18, 22), this maintenance of the activation of the p38 pathway and the late phase decline in the level of active ERK1/2 coincided with decreases in cell viability. This decrease was also associated with apoptosis, as was demonstrated by the presence of DNA fragments, characteristic of apoptosis, in the DNA

fragmentation assay. These results indicate that the activation of two of the three MAPKs tested was affected by exposure to TCDD, and that the p38 pathway, rather than JNK, might be involved in the TCDD-induced apoptosis of RAW 264.7 murine macrophages, since the activation of p38 upstream of caspase plays an important role in the apoptotic process of peritoneal macrophages exposed to UV-B irradiation (26).

The most well-characterized signaling effect of TCDD is its binding to and activation of cytosolic AhR, which subsequently migrates to the nucleus to heterodimerize with ARNT. The AhR/ARNT complex then binds to DREs and induces the expression of target genes (1, 12, reviewed in 13). AhR has been widely considered to be a ligand-dependent transcription factor which controls a variety of developmental and physiological events, including the metabolism of toxins (10). However, MAPK activation is not dependent on ligand-receptor interactions, since it takes place equally efficiently in cells that have AhR as in cells which lack AhR expression (6). Based on these results, we conclude that TCDD activates the MAPK pathway *via* an AhR-independent mechanism in RAW 264.7 murine macrophages. Since the induction of MAPK activities is an essential regulatory component of the ability of AhR to function as a transcription factor (6), TCDD-mediated MAPK activation may also play an important role in the immunotoxicity of this agent, through the activation of AhR.

Since caspases are involved in many kinds of apoptosis in higher eukaryotes (21, 27), the TCDD-induced up-regulation of the proteolytic activity of caspase in macrophages also supports the notion that, besides TCDD-AhR interaction, additional TCDD-activated signal-transduction pathways may also play important roles in the toxicity of this agent (14).

Because macrophages play a crucial role during the chronic phases of immune response, not only by eliminating non-self structures, but also by removing cellular debris (including apoptotic bodies) and remodeling injured tissues (28), these results suggest that TCDD exposure in macrophages may cause impairment of immune function *via* AhR-independent pathways.

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