2,3,7,8-tetrachlorodibenzo-p-dioxin activates ERK and p38 mitogen-activated protein kinases in RAW 264.7 cells

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Introduction

The classically accepted toxicity model with respect to 2,3,7,8-tetrachlorodibenzo-p-dioxin (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 activate transcription (1).

A previous report, showing that TCDD activates extracellular signal-regulated kinases (ERKs) and/or Jun-N-terminal kinases (JNKs)(2, 3), 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 responses to a variety of cellular stimuli, 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 treated with TCDD

Materials and Methods

Culture conditions: RAW 264.7 cells (ATCC TIB71) were cultured at 2 X 106 cells/ml in Dulbecco's modified Eagles medium (Gibco BRL), supplemented with 10% FBS (Hyclone), 100 U/ml penicillin, 100 mg/ml streptomycin (Gibco BRL), 10 mg/ml gentamicin (Irvine Scientific), 5 X 10-5 M 2-mercaptoethanol (Sigma). Duplicate cell cultures were either exposed or not exposed to 10 nM TCDD (Supelco), and incubated for 30 min to 48 h in a humidified incubator containing 5% CO2 at 37 oC.

Immunoblot analysis: SDS-PAGE-separated cell lysates were transferred to PVDF-plus membranes (Osmonics). The membranes were incubated at 4 oC, overnight, with mouse, rabbit, or goat antiserum, raised against ERK, p-ERK, p38, p-p38, p-JNK or caspase-3 (Santa Cruz Biotechnology), followed by incubation with rabbit anti-mouse, sheep anti-rabbit or anti-goat IgG conjugated with alkaline phosphatase (Santa Cruz Biotechnology). Stained proteins were visualized by using NBT/BCIP substrate. The relative densities of specific immunoreactive bands were evaluated by using the ImageQuant program version 3.3 (Molecular Dynamics).

MTT Assay: The colorimetric 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) assay was performed.

DNA agarose gel electrophoresis: Isolation of the apoptotic DNA fragment was performed according to the method proposed by Hermann et al. (4). Equal amounts of DNA from different time-treated cells were electrophoretically separated on a 2% agarose gel 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. Data are presented as the means ± SD.

Results

Effect on cell viability and DNA fragmentation: As shown in Fig 1, 10nM 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, we were unable to detect any distinct differences in DNA cleavage patterns, although they were evaluated at various times following TCDD treatment.

In order to compare the degrees of apoptosis occurring at each time point, cells were exposed to TCDD for 0-48 h. Cell viability decreased over time, in response to TCDD treatment. Moreover, exposure to TCDD for 8-48 h caused a significant decrease in the percentage of cell viability, relative to the values established before TCDD treatment (83.1%, 79.8%, 77.1%, 64.7%, respectively)(Fig 2).

Effect on MAPK pathways and caspase-3: Based on our findings that 10 nM TCDD significantly decreased cell viability 8 h after treatment, in this section we were chosen time points between 0-12 h, in order to investigate changes in the kinase cascades which might be resultant from exposure to TCDD. As shown in Fig 3A, the phosphorylation of ERK became apparent 4 h after the addition of TCDD to the culture medium, and began to decline 12 h after exposure. ERK activation level as a result of TCDD stimulation over time was more apparent on ERK1 than on ERK2. The induction of p38 activity was observed 1 h after exposure, and its activation persisted for 12 h (Fig 3B). Under the same stimulation conditions, TCDD did not result in the activation of the JNK pathway (Fig 3C). Expressions of total ERK, p38, and JNK were not affected by TCDD.

In order to more definitively demonstrate that TCDD-induced apoptosis in macrophages occurred via AhR-independent mechanisms, we investigated that the expression of caspase-3 was induced as early as 30 minutes after exposure, and caspase-3 level tended to increase gradually thereafter (Fig 3D).

Discussion

In this study, we have investigated the relationship between ERK1/2, p38, and JNK activity in the induction of apoptosis in RAW 264.7 cells treated with TCDD. Our results indicate that the activation of two of the three MAPKs tested were 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 cells, since the activation of p38 upstream of caspase plays an important role in the apoptotic process of peritoneal macrophages exposed to UV-B irradiation (5). The most well-characterized signaling effect of TCDD is its binding to and activation of cytosolic AhR (1). 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 (6). 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 (3). 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 the AhR to function as a transcription factor (3), 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 (7), 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 (8).

References

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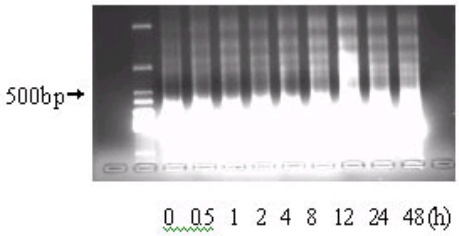
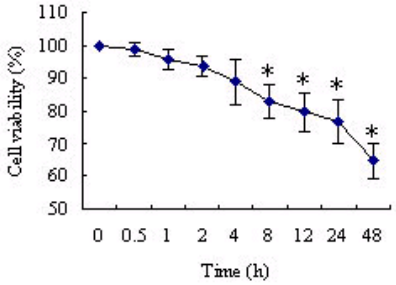
Fig. 1



Effect of TCDD on DNA fragmentation

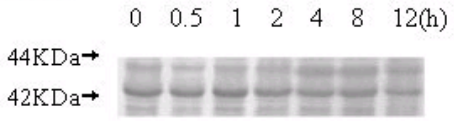
Fig 2.

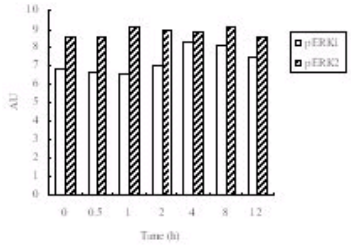


Effect of TCDD on cell viability

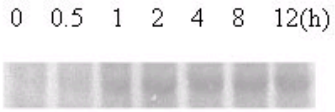
Effect of TCDD on ERK (A), p38 (B), JNK (C) and caspase (D) activities

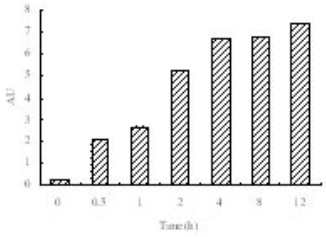
Fig. 3A.





3B.





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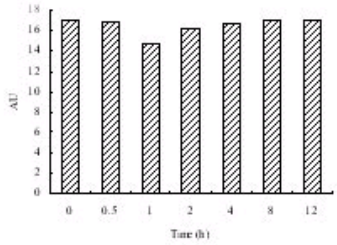

3D.