Increased Hepatic Expression and Phosphorylation of the Murine p53 Tumor Suppressor Protein and Expression of p21WAF1 CDK-Inhibitory Protein Following Acute Dosing of TCDD

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1. Introduction

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) exerts a myriad of biologic and toxicologic responses in a wide range of species. These responses include liver necrosis, lymphomyeloid involution, severe weight loss, developmental abnormalities, hyperkeratosis of the epidermis and tumor promoter activity [1-5]. Biochemical changes produced by TCDD *in vivo* and *in vitro* include the induction of cytochrome P-4501A1 [6-8], increases of hepatic uroporphyrin, heptacarboxyporphyrin, and total porphyrins [9], a decrease in EGF binding [10-12], an increase in expression of TGFα [13], and an increase of membrane tyrosine kinase activity in hepatocytes [14,15] and B lymphocytes [16]. More recent studies have reported that TCDD initiates signaling via a protein kinase mediated growth factor signal transduction pathway immediately following transformation of the Ah receptor [17]. These latter biochemical findings implicate alterations in signal transduction pathways and second messengers as a prelude to the tissue specific toxic manifestations of TCDD.

This laboratory previously reported [18] a TCDD-dependent increase in the expression and tyrosylphosphorylation status of p34^{cdc2} kinase (p34). p34 is a member of a family of cell division control enzymes termed cyclin-dependent kinases (CDKs) that, along with the cyclin proteins, serve to control and coordinate the molecular events of cell division in all eukaryotic cells [19-22]. In resting rat hepatocytes p34 is not expressed, but concentrations of p34 increase through G1 and the G1/S transition reaching maximal levels in the S, G2 and M phases [23]. In association with cyclin B, p34 is the serine/threonine kinase subunit of M-phase-promoting factor (MPF); active MPF triggers the G2/M transition in species ranging from yeast to humans [20,24]. Several studies also suggest that p34 functions in the control of the G1/S transition as well as in the initiation of mitosis [25,26].

The kinase activity and substrate specificity of p34 is regulated through (i) post-translational modifications including cycles of phosphorylation and dephosphorylation [27,28], (ii) interactions with cyclin proteins [27,29,30], and (iii) intracellular compartment translocation [30,31]. Another form of p34 regulation involves the interaction of p34 with the tumor suppressor gene product p53 [32,33] and the CDK inhibitory protein p21^{WAF1} [34].

In this study TCDD was evaluated for its ability to affect changes in the expression of p53 and p21^{WAF1}. The data generated support a proposed mechanism of action of TCDD involving the initiation of a cascade of proliferative signaling through the growth factor signal transduction pathway manifest as an increase in the expression of the CDKs and cyclin proteins. This proliferative signaling is followed by a compensatory expression of p21^{WAF1} CDK-inhibitory protein mediated through the p53 tumor suppressor protein.

2. Materials and Methods

Chemicals: TCDD was purchased from AccuStandard, Inc. (New Haven, CT). All antibodies were obtained from commercial suppliers as noted. Bicinchoninic acid was obtained from Pierce (Rockford, IL). Molecular weight standards were supplied through BioRad (Melville, NY). Al other chemicals were purchased from Sigma (St. Louis, MO) and were of the highest purity available.

Animals and Dosing: Adult female Balb/c mice were purchased from Jackson Laboratories (Bar Harbor, ME) and allowed a two-week acclimation period before assignment into treatment groups. Animals were fed ProLab RMH 1000 (Agway, Cortland, NY) and received tap water *ad libitum*. Housing and dosing of the mice was performed as previously described [18]. Five days after dosing S9 liver samples were prepared and stored as outlined [35].

Immunoblotting of p53 and p21 proteins: S9 proteins were solubilized in SDS gel sample buffer [35] and denatured at 100°C for eight min. SDS-PAGE was carried out on 100 µg of each denatured sample as described [36] using 11% polyacrylamide gels. The immunoblotting was carried out as described by Towbin et al. [37]; however, a semi-dry transfer system (BIORAD, Melville, NY), was used to transfer the proteins from the polyacrylamide gels to an Immobilon® membrane (Millipore, Bedford, MA). Complete transfers were accomplished in 25 to 30 min at 300 mA. All additional immunoblotting procedures were performed as detailed elsewhere [18].

Immunoblots were translated into TIFF-formatted files with a MICROTEK 600ZS scanner and quantified using Scan Analysis (BIOSOFT, Cambridge, UK). Summary scans were then printed and peak areas were determined as the product of the peak height and the peak width at half-height. One density unit (Du) was defined as one mm^2 of the resulting peak area.

Protein determination: Bicinchoninic acid was used for the spectrophotometric determination of protein concentration as reported [38].

3. Results

Within five days of administration of single doses of 0.25, 0.5 1.0 or 2.0 µg TCDD/kg, both p53 and $p21^{WAF1}$ protein expression were increased relative to corn oil controls (Figure 1 [A] and [B], respectively). The dose-response for the p53 tumor suppressor protein was maximal at 1.0 µg TCDD/kg (21-fold relative to controls), while the $p21^{WAF1}$ protein expression with TCDD-dosing was maximal at 2.0 µg/kg (32-fold increase). Control bands on the Western blots were faint for p53 and $p21^{WAF1}$. Darkly staining bands of p53 (53 kDa) and $p21^{WAF1}$ (21 kDa) immunoreactive protein appeared at the lowest dose of TCDD (0.25 µg/kg) and increased in both intensity of staining and thickness of band with increasing doses of TCDD.

Western blots of p53 immunoreactive protein demonstrated a marked spreading in S9 samples from animals treated with 0.5, 1.0 or 2.0 µg TCDD/kg. p53 immunoreactive doublets were seen at 1.0 and 2.0 µg TCDD/kg. The spreading and doublet formation of a single immunoreactive protein are generally interpreted to represent phosphorylation of the protein. The hyperphosphorylation of p53 protein as evidenced by band spreading and doublet formation was maximal at 1.0 µg TCDD/kg.

4. Discussion

The present study has demonstrated that a single dose of 0.25, 0.5, 1.0 or 2.0 µg TCDD was sufficient to induce the hepatic expression of the p53 tumor suppressor protein and the p53-regulated $p21^{WAF1}$ protein. These results support the inference that TCDD is capable of inducing a G1/S cell cycle block as outlined in Figure 2. With continued exposure to TCDD, the resulting simultaneous reception of proliferative and inhibitory signaling would generate a signaling conflict within the cell. This signaling conflict could create a condition of genomic instability [39] characterized by an increased frequency of gene mutations, gene amplification, allelic loss, and apoptosis [40,41].

The effect of TCDD on signaling pathways and cell cycle control enzymes provides an explanation for the variety of effects observed with TCDD exposure. Escape from the TCDD-mediated cellular arrest may be affected by cell either transformation or apoptosis. Therefore cell proliferation, tissue hypertrophy and cancer [42] are logical outcomes of TCDD exposure. Tissue atrophy, necrosis, developmental toxicity and

immunotoxicity could result from TCDD-induced apoptosis or cell cycle arrest from continued expression of $p21^{WAF1}$ protein. Such a signaling conflict has been proposed as the mechanism of action of HIV-mediated cell killing [43].

Finally, the proposed mechanism of action also serves to explain the apparently contradictory inhibitory effect of the carcinogenic TCDD on tumor cells [44,45]. Accordingly, tumor cells containing a functional p53 gene would undergo growth arrest through the expression of $p21^{WAF1}$ CDK-inhibitory protein following exposure to TCDD during a phase of active growth.

5. References

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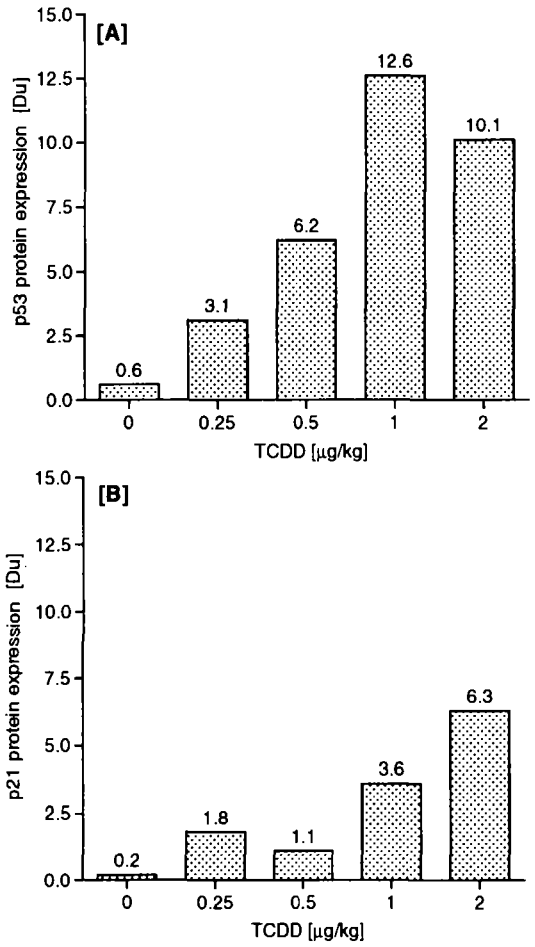

Figure 1. Bar graphs of the densitometry scans of p53 [A] and p21WAF1 [B] Western blots. Murine hepatic 9000 x g supernatant protein was separated on an 11% SDS-PAGE gel (see Materials and Methods). The separated proteins were transferred to a blotting membrane and probed with anti-p53 [A] or anti-p21WAF1 [B] antibody. Immunoblots were scanned and quantified; one densitometry unit (Du) was defined as one mm2 of the resulting peak area. Five animals were treated at each dose of TCDD; ten lanes were run with each gel and two animals were selected randomly from each group for each of the two replicate gels. The representative results depicted for each dose are the means of two animals from a single ten-lane gel.

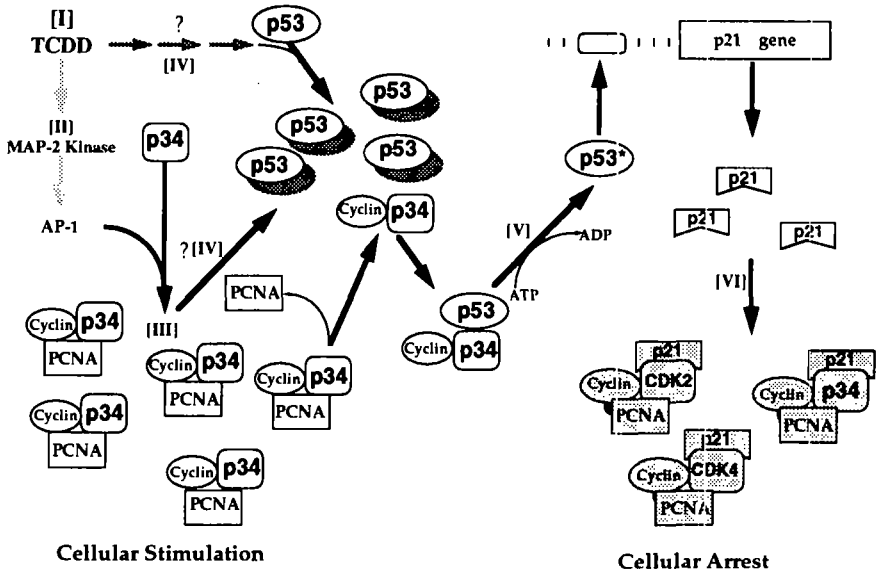

Figure 2. Schematic representation of hypothesized mechanism of the TCDD-induced G1/S arrest underlying the proposed mechanism of dioxin toxicities. **[I]** TCDD enters the cell and interacts with the Ah-receptor; **[II]** transformation of the Ah-receptor complex activates a protein kinase mediated growth factor signal transduction [17] pathway (e.g. MAP-2 kinase); **[III]** expression of immediate early response genes [46] induce a proliferative response in the cell resulting in an increase in cyclin dependent kinase [18], PCNA and cyclin expression; **[IV]** as a result of the persistent signaling for proliferation maintained by TCDD, there is continued expression of cell division control enzymes, DNA synthesis and cell cycling; these activities provide the signal for the expression of the p53 tumor suppressor protein observed in this study; **[V]** p53 protein serves as a transcriptional regulatory factor for the $p21^{WAF1}$ gene [47,48] through phosphorylation by a p34/cyclin complex [32,33]; **[VI]** the $p21^{WAF1}$ protein functions as a universal inhibitor of cyclin dependent kinase activity through the interaction with CDK/cyclin complexes [34,49,50] and consequently induces a G1/S cell cycle arrest.

In this model, p53 serves to mediate the induction of a cell cycle arrest as a mechanism to prevent uncontrolled cell proliferation by xenobiotics capable of inducing growth factor signaling. With continued exposure to TCDD, the resulting simultaneous reception of proliferative and inhibitory signaling would generate a signaling conflict within the cell. This signaling conflict introduces a condition of genomic instability within the cell, predisposing the cell to undergo apoptosis or transformation.