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PROTEIN TYROSINE PHOSPHORYLATION AS AN INDICATOR OF 2,3,7,8-TETRACHLORO-p-DIOXIN EXPOSURE *IN VIVO* AND *IN VITRO*

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SUMMARY: A dose-dependent increase in tyrosine phosphorylation of five hepatic intracellular proteins with approximate molecular weights of 17, 21, 27, 29, and 34 kDa was seen 24 h after administration of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) to C57BL/6J female mice. The ED_{50} values for tyrosylphosphorylation of these five proteins, respectively, were 0.26, 0.21, 0.26, 0.31, and 0.38μg TCDD/Kg. TCDD induction of 7-ethoxyresorufin O-deethylase activity (EROD) was characterized by an ED_{50} of 2.5μg/Kg. An eighteen h exposure of a human lymphoblastoma cell line (X3) to TCDD increased tyrosylphosphorylation status of ten proteins with approximate molecular weights of 16, 17, 24, 26, 27, 32, 33, 34, 35, and 36 kDa in a dose-dependent manner. The EC_{50} values for these TCDD-dependent tyrosylphosphorylation ranged from 0.01 to 0.07 nM TCDD. EROD induction by TCDD in X3 cells exhibited an EC_{50} of 0.14 nM. These data indicate that TCDD alters intracellular protein tyrosine phosphorylation and these changes are more sensitive biological indicators of TCDD exposure than induction of EROD. © 1992 Academic Press, Inc.

The environmental pollutant TCDD exerts many effects on mammals including severe weight loss, teratogencity, chloracne, and interference in the regulation of both cellular differentiation and proliferation (1-4). TCDD may pose a threat to human health because of its widespread contamination from landfill leachates, industrial accidents and the use of TCDD-containing herbicides (5). Several epidemiological studies have documented the health consequences of human exposure to TCDD (6,7). However, the extent of environmental distribution and human exposure to TCDD are still largely unknown and

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ABBREVIATIONS: B6, C57BL/6J; CI, 95% confidence interval of the estimate; Cytochrome P-450, P450; DMSO. dimethylsulfoxide; EGF, epidermal growth factor; ELISA, enzyme-linked immunosorbant assay; EROD, 7-ethoxyresorufin-O-dealkylase; PBS, phosphate buffered saline; S9, 9000xg supernatant fraction; SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TCDD, 2,3,7,8-tetrachloro-p-dioxin; TGFα, transforming growth factor alpha.

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arguable (6-8). This is due, in large part, to the lack of a sensitive indicator for assessing TCDD exposure to biological systems (9).

For use in risk assessment and molecular epidemiology, TCDD exposure should be defined relative to its mechanism of action. Biochemical changes produced by TCDD in animals and cell cultures include the induction of P4501A1 (10,10-12), an increase of hepatic uroporphyrin, heptacarboxyporphyrin, and total porphyrins (13), a decrease in EGF binding (14-16), an increase in expression of TGFα (17), and an increase of membrane tyrosine kinase activity in hepatocytes (18,19) and B lymphocytes (20). These latter studies imply that increases in membrane protein kinase activity and subsequent alterations in signal transduction function to produce the characteristic toxic manifestations of TCDD.

Previous assessments of molecular changes engendered by TCDD concluded that the induction of P450 was the most sensitive response to TCDD (21,22). In this study TCDD dose-responses of enhanced protein tyrosine phosphorylation and induction of a P4501A1-mediated enzyme activity were compared in two model systems representative of TCDD target tissues.

MATERIALS AND METHODS

Chemicals: All chemicals were purchased from commercial and were of the highest purity available.

Animals: Four to six-week old, female B6 mice were obtained from Harton Sprague Dawley (Indianapolis, IN). Mice were fed Prolab RMH 1000 (Agway, Cortland, NY) and received tap water *ad libitum*. All mice were housed three per cage and maintained on a photoperiod of 12 h. Mice were killed 24 h following an ip injection of TCDD in corn oil at 0, 0.25, 0.5, 1, 2, 4, 10, 50 μg/Kg. Three mice were treated at each dose and the volume of the injections ranged from 0.1 to 0.2 ml per mouse. All procedures were performed on pooled hepatic samples of the three mice per dose. Two preliminary studies were done to determine final doses of TCDD. The experiment as described was repeated twice. Preparation and -80°C storage of hepatic S9 fractions were performed as previously described (23).

Cell culture: Human B-lymphoblastoma cells (X3, Genetest, Woburn, MA) were grown in 175 cm^2 T-flasks containing RPMI 1640 medium with 9% horse serum (Gibco, Grand Island, NY). The cells were cultured at 37°C in a 5% CO_2 balanced air

environment. Cells were subcultured by centrifuging (1000 x g for 3 min) and suspending in 100 mL of fresh media to a cell density of 5.0 x 10^5 cells/mL. TCDD was added at concentrations of 0, 0.02, 0.05, 0.1, 0.5 or 1 nM per flask in DMSO. After the 16 h incubation period, cells were harvested by centrifugation and washed three times in PBS at 4°C and lysed in 20 mM Tris buffer (pH 8.0) with 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1 mM phenylmethyl-sulfonyl fluoride, 0.15 unit/mL of aprotonin, and 1 mM sodium orthovanadate at 4°C for 20 min. Two dose-range finding experiments were performed and the experiment as described was repeated twice.

ELISA: Assays as described (24) were performed with anti-phosphotyrosine monclonal antibody to determine total phosphotyrosine content of hepatic S9 or X3 cell lysate protein.

Gel electrophoresis and immunoblotting: SDS PAGE was carried out as described (25) using 10 or 11% polyacrylamide gels with the modification that hepatic S9 and X3 cell lysates (100 μg protein/well) were subjected to heat treatment (100°C) for 3 minutes. The immunoblotting was carried out as described by Towbin *et al.* (26). Immunoblots were translated into TIFF-formatted files with a Microtech 600GS scanner and quantified using Scan Analysis (BIOSOFT, Cambridge, UK).

P4501A1-associated enzyme activity: EROD was measured as described (27). Rates of metabolism were calculated as pmoles resorufin formed/minute-mg protein or 10^9 cells.

Protein determination: Bicinchoninic acid was used for the spectrophotometric determination of protein concentration as reported (28).

Statistical analysis: Final experiments were repeated twice for B6 mice and X3 cells. Results presented in Figure 1 and Table 1 were representative of all replications of all experiments. Dose responses were defined as 3 or more consecutive doses resulting in an increase in the response variable. The percent of maximal increase over controls was determined for each dose and this variable was transformed into probits for regression analysis; a log-normal distribution was assumed. EC_{50} values and 95% confidence intervals of the estimates were determined using Sigma Plot (Jandel Scientific, San Rafael, CA).

RESULTS AND DISCUSSION

TCDD dosing increased the tyrosine phosphorylation status of 12 intracellular proteins (Fig. 1,Top). Five of these proteins exhibited increases in tyrosylphosphorylation with TCDD in a dose-dependent manner to a maximal response at 1 to 2 μg TCDD/Kg; these proteins had approximate molecular weights of 17, 21, 27, 29 and 34 kDa. Seven proteins, migrating at 15, 16, 18.5, 19, 20, 24, and 33 kDa, appeared to be tyrosylphosphorylated in response to TCDD administration. However, these proteins

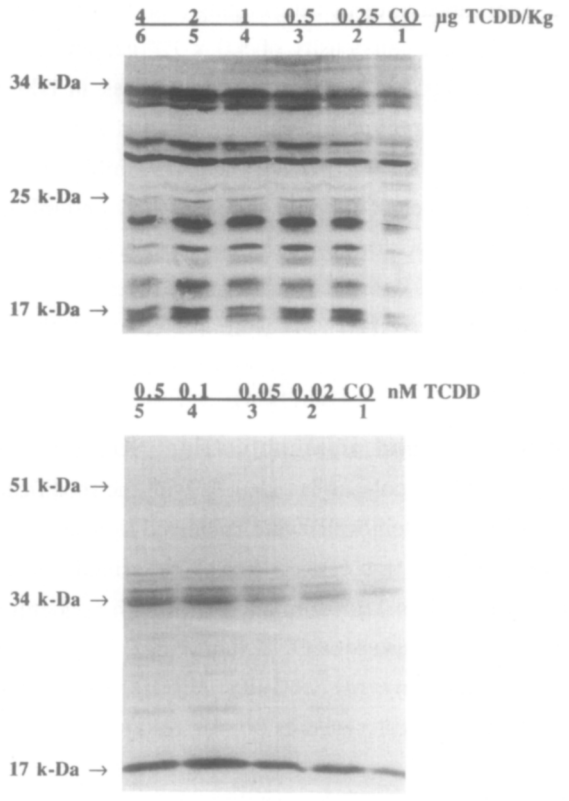

Figure 1. Anti-phosphotyrosine monoclonal antibody immunoblots of SDS PAGE preparations of hepatic S9 from female, C57BL/6J mice (Top) or X3 lymphoblastoma cell lysates (Bottom) treated with TCDD at the doses or concentrations indicated for each lane.

stained maximally at 0.25 or 0.50 µg TCDD/Kg and dose-response curves could not be generated for them. While most phosphotyrosylproteins seen with TCDD pretreatment had readily observable counterparts in the control lane, three proteins in the molecular weight range of 17 to 21 kDa were detected only as faintly staining bands in controls.

The ED_{50} for EROD induction was 8.8 times the average ED_{50} for tyrosylphosphorylation of the 17, 21, 27, 29, and 34 kDa proteins (Table 1). However, the ED_{50} of total protein tyrosylphosphorylation of hepatic S9 (1.6 µg TCDD/Kg) was not different from the ED_{50} for EROD induction of 2.5 µg TCDD/Kg. The EROD results are in good agreement with a published ED_{50} value of approximately 3 µg TCDD/Kg for the induction of P4501A1-mediated aryl hydrocarbon hydroxylase activity 3 days post-injection (11).

Table 1. Median and maximally effective doses of 2,3,7,8-tetrachlorodibenzo-p-dioxin for the induction of protein tyrosine phosphorylation and cytochrome P4501A1 in C57BL/6J female mice and X3 lymphoblastoma cells

	Test System			
	C57BL/6J Mouse		X3 Lymphoblastoma Cell	
Response	Median Effective Dose [μg/Kg]	Maximally Effective Dose [μg/Kg]	Median Effective Dose [nM]	Maximally Effective Dose [nM]
Protein tyrosylphosphorylation				
Total protein tyrosylphosphorylation	1.6	2.0	0.09	1.0
Specific protein tyrosylphosphorylations				
pp16 kDa	-	-	0.04	0.10
pp17 kDa	0.26	2.0	0.03	0.10
pp21 kDa	0.21	1.0	-	-
pp24 kDa	-	-	0.07	0.50
pp26 kDa	-	-	0.05	0.50
pp27 kDa	0.26	1.0	0.06	0.50
pp29 kDa	0.31	2.0	-	-
pp32 kDa	-	-	0.06	0.50
pp33 kDa	-	-	0.03	0.10
pp34 kDa	0.38	2.0	0.03	0.10
pp35 kDa	-	-	0.01	0.10
pp36 kDa	-	-	0.03	0.10
Cytochrome P4501A1 induction	2.5 (1.3 - 7.8)	10	0.14 (0.04 - 0.44)	2.0†

Total protein tyrosylphosphorylation was determined by enzyme-linked immunosorbant assay using anti-phophosphotyrosine monoclonal antibody. Individual protein tyrosylphosphorylations were quantified by densitometry of immunoblots developed with anti-phosphosphotyrosine monoclonal antibody. Cytochrome P4501A1 related activity was measured with ethoxyresorufin as substrate; parenthetic value is 95% confidence interval of the estimate.
†Determined from initial dose range finding studies.

TCDD exposure to X3 cells for 18 h resulted in a dose-dependent increase in tyrosylphosphorylation of 10 proteins with apparent molecular weights of 16, 17, 24, 26, 27, 32, 33, 34, 35, and 36 kDa (Fig. 1,Bottom). The ED_{50} values for TCDD-mediated tyrosylphosphorylation of these proteins ranged from 0.01 to 0.07 nM (Table 1). Two other proteins identified at 21 and 23 kDa were tyrosylphosphorylated only at TCDD concentrations of 0.1 and 0.5 nM. Total protein tyrosine phosphorylation displayed an EC_{50} of 0.09 nM TCDD; maximal total protein tyrosine phosphorylation was observed at 1 nM TCDD.

EROD induction by TCDD in X3 cells was described by an EC_{50} of 0.14 nM (CI = 0.04 - 0.44). This result is in general agreement with TCDD induction of AHH activity in Hepa-1 cells that exhibited an ED_{50} of 0.4 to 0.6 nM (29).

EC_{50} values for tyrosylphosphorylation of five proteins at 17, 33, 34, 35 and 36 kDa, however, were approximately 0.1 to 0.2 times the 0.14 nM EC_{50} for EROD. EC_{50}

values for tyrosine phosphorylation of the 16, 24, 26 and 32 kDa proteins of X3 cells were equivalent to the median effective TCDD concentration for EROD induction. Consistent with results from the studies with B6 mice, the EC_{50} values for total protein tyrosylphosphorylation and EROD induction by TCDD in X3 cells were not different.

We have demonstrated that TCDD increases the tyrosylphosphorylation of intracellular proteins in two model systems. These changes in intracellular protein tyrosylphosphorylation occur at TCDD exposure levels lower than those inducing EROD activity. The mechanism by which TCDD induces hyperplasia (30) and can act as a tumor promotor in the mouse skin tumor promoter assay (31,32) is likely through modulation of signal transduction pathways, such as tyrosine phosphorylation, that control the proliferation program of cells (17). Experiments are currently underway to identify several of the prominent proteins that undergo increased tyrosylphosphorylation in response to TCDD administration. Structure-activity studies are also being performed *in vivo* with several congeners of TCDD.

REFERENCES

1. Knutson, J.C. and Poland, A. (1984) J. Cell Physiol.121, 143-151.
2. Knutson, J.C. and Poland, A. (1982) Cell30, 225-234.
3. Safe, S.H. (1986) Annu. Rev. Pharmacol. Toxicol.26, 371-399.
4. Whitlock, J.P.,Jr. (1990) Annu. Rev. Pharmacol. Toxicol.30 p251-77 X.
5. Safe, S. (1990) Crit. Rev. Toxicol.21, 51-88.
6. Fingerhut, M.A., Steenland, K., Sweeney, M.H., Halperin, W.E., Piacitelli, L.A., and Marlow, D.A. (1992) Epidemiology.3, 69-72.
7. Fingerhut, M.A., Sweeney, M.H., Halperin, W.E., and Schnorr, T.M. (1991) IARC. Sci. Publ.31-50.
8. Fingerhut, M.A., Halperin, W.E., Marlow, D.A., Piacitelli, L.A., Honchar, P.A., Sweeney, M.H., Greife, A.L., Dill, P.A., Steenland, K., and Suruda, A.J. (1991) N. Engl. J. Med.324, 212-218.
9. Fingerhut, M.A., Halperin, W.E., and Okun, A.H. (1991) J. Occup. Med.33, 1233-1235.
10. Roberts, E.A., Johnson, K.C., Harper, P.A., and Okey, A.B. (1990) Arch. Biochem. Biophys.276, 442-450.
11. Poland, A. and Glover, E. (1974) Mol. Pharmacol.10, 349-359.

12. Poland, A. and Glover, E. (1976) In: de Serres, F.J., et al. , ed. In vitro metabolic activation in mutagenesis testing. Amsterdam, North-Holland. Publ.
13. Yao, C. and Safe, S. (1989) Toxicol. Appl. Pharmacol100, 208-216.
14. Kawamoto, T., Matsumura, F., Madhukar, B.V., and Bombick, D.W. (1989) J. Biochem. Toxicol.4, 173-182.
15. Moriya, M., Matsumura, F., and Kalimi, G.H. (1986) J. Biochem. Toxicol.1, 45-54.
16. Madhukar, B.V., Brewster, D.W., and Matsumura, F. (1984) Proc. Natl. Acad. Sci. U. S. A.81, 7407-7411.
17. Choi, E.J., Toscano, D.G., Ryan, J.A., Riedel, N., and Toscano, W.A.,Jr. (1991) J. Biol. Chem.266, 9591-9597.
18. Madhukar, B.V., Ebner, K., Matsumura, F., Bombick, D.W., Brewster, D.W., and Kawamoto, T. (1988) J. Biochem. Toxicol.3, 261-277.
19. Bombick, D.W. and Matsumura, F. (1987) Life. Sci.41, 429-436.
20. Clark, G.C., Blank, J.A., Germolec, D.R., and Luster, M.I. (1991) Mol. Pharmacol.39, 495-501.
21. Roberts, L. (1991) Science252, 911.
22. Roberts, L. (1991) Science254, 377.
23. Ma, X.F., Gibbons, J.A., and Babish, J.G. (1991) Toxicol. Lett.59, 51-58.
24. Stefanescu, M., Cremer, L., Cristescu, C., Matache, C., and Szegli, G. (1991) Roum. Arch. Microbiol. Immunol.50, 187-192.
25. Laemmli, U.K. and Favre, M. (1973) J. Mol. Biol.80, 575-599.
26. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A.76, 4350-4354.
27. Burke, M.D., Thompson, S., Elcombe, C.R., Halpert, J., Haaparanta, T., and Mayer, R.T. (1985) Biochem. Pharmacol.34, 3337-3345.
28. Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., and Klenk, D.C. (1985) Anal. Biochem.150, 76-85.
29. Jaiswal, A.K., Nebert, D.W., and Eisen, H.W. (1985) Biochem. Pharmacol.34, 2721-2731.
30. Abbott, B.D. and Birnbaum, L.S. (1990) Teratology41, 71-84.
31. Pitot, H.C., Goldsworthy, T., Campbell, H.A., and Poland, A. (1980) Cancer Res40, 3616-3620.
32. Poland, A., Palen, D., and Glover, E. (1982) Nature300, 271-273.