A RAPID AND SENSITIVE METHOD FOR MEASURING ETHOXYRESORUFIN-O-DEETHYLASE (EROD) ACTIVITY IN CULTURED HEPATOCYTES EXPOSED TO DIOXINS, PCBS, AND RELATED CHEMICALS

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Assessment of the toxicity of complex mixtures of polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and other halogenated aromatic hydrocarbons (HAHs) to humans and wildlife continues to be a difficult scientific problem with important economic and political implications. There is growing interest in using cell culture bioassays which are based upon induction of cytochrome P450IA1-associated monooxygenase activity to obtain estimates of "2,3,7,8-tetrachlorodibenzo-p-dioxin equivalent" (TCDD-EQ) concentrations of complex mixtures of HAHs. Most work in this area has been with the H4IIE rat hepatoma cell line[1-4]. In H4IIE cells, the P450IA1-associated enzymes, ethoxyresorufin-O-deethylase (EROD) and aryl hydrocarbon hydroxylase (AHH), are induced by minute (pg) quantities of TCDD. As well, AHH induction potencies of PCBs, PCDFs, and PCDDs in H4IIE cells correlate well with *in vivo* toxicity of these compounds to rats[5].

Rigorous validation of cell culture bioassays based on EROD and/or AHH induction will require the determination of the dose-response curves of a large number of individual HAHs and mixtures of HAHs in cells derived from different species. Until now, it has been difficult to carry out such studies because methods for measuring the activities of these enzymes are time-consuming and laborious. We have developed a method for measuring EROD activity in primary cultures of chicken embryo hepatocytes which is much faster and more sensitive than other methods[6]. In this "extended abstract", we briefly describe our new method, and show its application to the determination of the EROD dose-response curves of TCDD, 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126) and 3,3′,4,4′-tetrachlorobiphenyl (PCB 77).

METHODS

The following is a brief description of our method[6].

I. Cell Culture

The method described by Fischer and Marks[7] for preparing primary hepatocyte cultures from 19-day old chicken embryos was modified for 48-well cell culture plates. Briefly, cells were plated at a density of approximately 5 X 10^4 cells per well. Each well

contained 0.5 ml of Waymouth's MD 705/1 medium supplemented with insulin (1 μg/ml) and L-thyroxine (1 μg/ml). After incubation for 24 hours at 37°C in an atmosphere of 95% air and 5% CO_2, the medium was removed and replaced with fresh medium. Dimethyl sulphoxide (DMSO) solutions (2.5 μl/well) of TCDD, PCB 126, or PCB 77 were added to the cultures. Control cells received only DMSO. Cells were incubated for another 24 hours, the medium was removed, and plates were frozen on dry ice prior to storage at -80°C.

II. Ethoxyresorufin-O-deethylase (EROD) Assay

Frozen plates were brought to room temperature, and EROD assays were carried out directly in the wells of the cell culture plates using modifications of previously reported methods[8,9]. EROD activity was terminated with methanol, and plates were inserted into a fluorescence multi-well plate reader (Cytofluor 2300, Millipore Ltd.; 530 nm excitation filter, 590 emission filter) without further treatment. Resorufin concentration was determined by fitting data to a resorufin standard curve and enzyme activity was expressed as pmol resorufin/min/mg protein. For dose-response curves, curve-fitting, parameter estimation, and 95% confidence intervals were determined using a quadratic logistic equation. Total protein concentrations were determined on separate plates using the bicinchoninic acid assay[10].

RESULTS AND DISCUSSION

I. Speed and Efficiency

As outlined briefly above, EROD assays were carried out directly in 48-well plates, reactions were stopped with methanol, and plates were inserted into a fluorescence plate reader without further treatment. Automatic scanning of each 48-well plate took approximately 45 seconds. Dose-response curves were generated minutes after the plates were scanned. Assaying EROD directly in the cell culture plates, the use of a fluorescence plate reader, and utilization of computerized curve-fitting programs has resulted in a method which is at least 100 times faster than our previous methods. Our previous methods, which are similar to those currently used for other types of cultured cells, involve several time-consuming steps such as transferring cells to reaction vials prior to carrying out EROD assays, centrifugation, and analysis with a spectrofluorometer. Results obtained with the plate reader are identical to those obtained with a spectrofluorometer[6]. One person can prepare cell cultures, dose the cells, and obtain EROD dose-response curves for at least 50 compounds and/or mixtures of compounds per week.

II. Dose-Response Curves and Sensitivity

Figure 1 shows typical EROD dose-response curves in chicken embryo hepatocytes exposed to TCDD, PCB 126, and PCB 77. Each curve represents the results obtained on a single 48-well plate. Fourteen concentrations of DMSO solutions of each congener were administered in triplicate. Control cells received triplicate doses of DMSO alone. Basal EROD activity was approximately 15 pmol/min/mg protein. DMSO at concentrations of 0.5% in the medium had no effect on EROD activity[6]. For each compound, EROD activity reached a maximum which was followed by a decline at higher TCDD and PCB concentrations. This inhibition/inactivation of EROD activity has been reported by others in cultured chicken embryo hepatocytes, cultured rat hepatocytes and *in vivo*. The mechanism is not known.

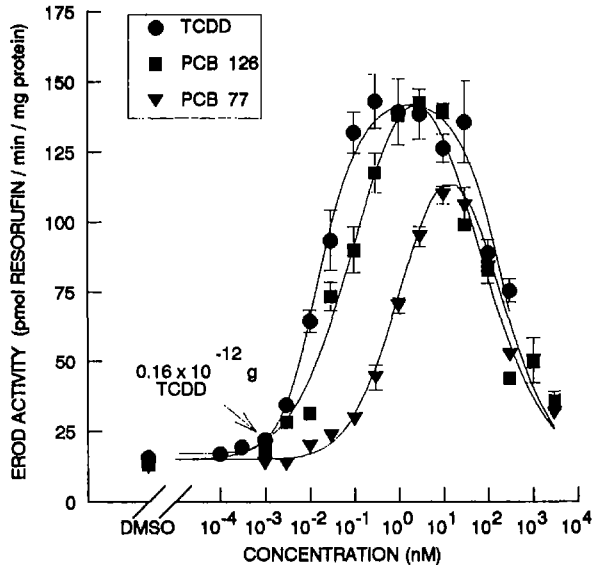

Figure 1. EROD dose-response curves for TCDD, PCB 126, and PCB 77. Each point represents the mean of triplicate doses; bars represent standard errors.

The EC_{50s} for TCDD, PCB 126, and PCB 77 were 0.015 nM, 0.066 nM, and 0.65 nM, respectively. Relative potencies were 1.0, 0.2, and 0.02. These relative values are similar to recently suggested[5] toxic equivalency factors (TEFs) for these compounds (1.0, 0.1, and 0.01, respectively), but are different from relative potencies observed in H4IIE cells (summarized in reference 3).

TCDD and PCB 126 induced EROD activity at concentrations of 0.001 nM or lower (Fig. 1). The volume of the medium in each well was 0.5 ml. Therefore, the detection limit for TCDD and PCB 126-induced EROD was approximately 0.5 X 10^{-15} moles, or 0.16 X 10^{-12} g (0.16 pg). For comparison, the reported detection limit for TCDD-induced EROD activity and AHH activity in H4IIE cells is 10 to 25 pg[1,3].

III. Potential Applications

These methods will be of use for both applied and basic research. Recent studies have shown that our techniques can be applied to H4IIE rat hepatoma cells[11] and to PLHC-1 teleost liver cells[12]. Therefore, these methods should be useful for studies on interspecies differences in responses to the EROD-inducing effects of PCBs, dioxins, and related chemicals.

REFERENCES

1. Bradlaw, JA, Casterline, JL. Induction of enzyme activity in cell culture: A rapid screen for detection of planar polychlorinated organic compounds. *J Assoc Off Anal Chem* 1979; 62:904-916.
2. Zacharewski, T, Safe, L, Safe, S, Chittim, B, DeVault, D, Wilberg, K, Bergqvist, P-A, Rappe, C. Comparative analysis of polychlorinated dibenzo-p-dioxin and dibenzofuran congeners in Great Lakes fish extracts by gas chromatography-mass spectrometry and in vitro enzyme induction activities. *Environ Sci Technol* 1989; 23:730-735.
3. Tillitt, DE, Giesy, JP, Ankley, GT. Characterization of the H4IIE rat hepatoma cell bioassay as a tool for assessing toxic potency of planar halogenated hydrocarbons in environmental samples. *Environ Sci Technol* 1991;25:87-92.
4. Hanberg, A, Ståhlberg, M, Georgellis, A, de Wit, C, Ahlborg, UG. Swedish Dioxin Survey: Evaluation of the H-4-II E bioassay for screening environmental samples for dioxin-like enzyme induction. *Pharm Toxicol* 1991;69:442-449.
5. Safe, S. Polychlorinated biphenyls (PCBs), dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs), and related compounds: Environmental and mechanistic considerations which support the development of toxic equivalency factors (TEFs). *Crit Rev Toxicol* 1990;21:51-88.
6. Kennedy, SW, Lorenzen, A, James, CA, Collins, BT. *Submitted for publication.*
7. Fischer, PWF, Marks, GS. Chick embryo liver cells maintained in serum-free Waymouth MD 705/1 medium. *Tissue Culture Association Manual* 1976; 2:449-452.
8. Poul, RJ, Fouts, JR. A rapid method for assaying the metabolism of 7-ethoxyresorufin by microsomal subcellular fractions. *Anal Biochem* 1980; 107:150-155.
9. Burke, MD, Prough, RA, Mayer, RT. Characteristics of a microsomal cytochrome P-448-mediated reaction. Ethoxyresorufin O-de-ethylation. *Drug Metab Disp* 1977; 5:1-8.
10. Smith, PK, Krohn, RI, Hermanson, GT, Mallia, AK, Gartner, FH, Provenzano, MD, Fujimoto, EK, Goeke, NM, Olson, BJ, Klenk, DC. Measurement of protein using bicinchoninic acid assay. *Anal Biochem* 1985; 150:76-85.
11. Tillitt, DE. *Personal Communication.*
12. Hahn, ME. *Personal Communication.*