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Environmental Contamination and Toxicology

O-Dealkylation of Resorufin Ethers as an Indicator of Hepatic Cytochrome P-450 Isoenzyme Induction in the Cotton Rat (*Sigmodon hispidus*): A Method for Monitoring Environmental Contamination

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Cytochrome P-450, the terminal oxygenase of the mixed-function oxidase system is widely distributed in many tissues but is present in the highest concentration in hepatic endoplasmic reticulum. This enzyme system plays an essential role in the metabolism of a broad range of xenobiotics, and endogenous and exogenous substrates (Guengerich and Liebler, 1985). Evaluation of hepatic cytochrome P-450 induction in wild animals has been suggested as a biological monitor to various environmental contaminants. Hepatic microsomes contain multiple cytochrome P-450 isoenzymes, each possessing broad and overlapping substrate selectivity (Ryan et al., 1982). The different isoenzymes function in both the activation and detoxification of foreign compounds and some forms of cytochrome P-450 have been implicated in the activation of a variety of chemical carcinogens (Wilson et al., 1984).

Many of the model substrates which have been widely used for measuring cytochrome P-450 induction do not differentiate adequately among individual isoenzymes, nor between induced and constitutive isoenzymes (Guengerich et al., 1982). In recent years, O-dealkylation of resorufin ethers has gained a considerable importance (Lubert et al., 1985). This reaction, which is highly specific for the major isoenzymes, can be used as an indicator of cytochrome P-450 isoenzyme induction. Novak and Qualls (1990) have demonstrated that O-dealkylation methoxy-, ethoxy-, pentoxy- and benzyloxyresorufins in cotton rat (Sigmodon hispidus) was similar to Sprague-Dawley rats for 3-methylcholanthrene inducible forms of cytochrome P-450. The objective of this study was to evaluate the induction of major hepatic cytochrome P-450 isoenzymes through O-dealkylation of various resorufin ethers in the cotton rat (Sigmodon hispidus) as an indicator of environmental contamination with toxic chemicals.

MATERIALS AND METHODS

Propoxy-, butoxy-, hexoxy-, octoxy - and decoxyresorufins were synthesized from resorufin by the method of Mayer et al. (1977) using appropriate alkyl iodides. Resorufin, I-iodopropane, 2-iodobutane, I-iodohexane, I-iodoctane and I-iododecane, were purchased from Aldrich Chemical Company (Milwaukee, WI 53201, USA). Methyl-, ethyl-, pentyl- and benzyloxyresorufins were obtained from Molecular Probes Inc. (Junction City, Oregon 97448, USA).

Cotton rats collected from uncontaminated areas (Near Stillwater, Oklahoma) with Sherman live traps were used as breeding stock for a captive outbred colony. Animals were housed in our laboratory animal facility in individual polycarbonate cage with wire

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tops and wood shavings as bedding. Animals were maintained under a 12 hour photoperiod and commercial rat chow was provided ad libitum. After 2 weeks of acclimatization a male was placed with a female for two weeks. Offspring were weaned at three weeks of age and housed in pairs (same sex) until they reach a desired body weight (> 60g). Induction studies were performed using 3-methylcholanthrene (3MC), 25 mg/kg in corn oil IP for 3 days. Four males born to wild-caught females in our outbred colony received each agent. Four males receiving corn oil by IP injection served as controls.

Two known contaminated sites near Criner and Pryor, Oklahoma) were selected for field trials. The Royal Hardage (RH) toxic waste disposal site, a declared superfund site, is located near the town of Criner in McClain County, Oklahoma. This site was used as a toxic waste dump during 1972 to 1980. An industrial site near Pryor, located in Mayes county, Oklahoma is an aroclor 1254 (polychlorinated biphenyl mixture) contaminated site. According to the United States Corps of Engineer (1987), the site has soil concentration of Aroclor 1254 greater than 800 ppm. Cotton rats were live-trapped from contaminated and nearby uncontaminated sites (CO for RH and CP for PO) with ecologically similar habitat. The distances between contaminated and uncontaminated sites for Criner and Pryor were approximately 2.5 and 0.5 km respectively.

Cotton rats (body weight > 60g) returned to the laboratory from field studies and rats from laboratory studies receiving 3MC and control cotton rats, were fasted overnight and killed by cervical dislocation. Liver microsomes were prepared using methods previously described (Omura and Sato, 1964). Microsomal protein was measured by the method of Smith et al. (1985). The dealkylation of alkoxyresorufins (methoxy-, ethoxy-, propoxy-, butoxy-, pentoxy-, hexoxy-, benzyloxy-, octoxy- and decoxyresorufins) was measured at 37°C using a SLM 48000 spectrofluorometer (SLM Instruments Inc. 810 West Anthony Drive, Urbana, IL 61801, USA). The fluorometer was set to an excitation wavelength of 570 nm, and an emission wavelength of 585 nm with slit widths of both monochrometers at 2 nm. The fluorescence increase as a function of time from resorufin formation measured by the spectrofluorometer was concurrently plotted on HP7475A Plotter (Hewlett-Packard Company, 16399 W. Bernardo Drive, San Diego, CA 92127-1899). Reaction mixtures consisted of microsomal protein (0.30 to 1.50 mg/ml), 1978 ul 1mM Na/K buffer (pH 7.6), 10 ul substrate (1mM in dimethyl sulfoxide). Reactions were initiated by addition of 50 mM nicotinamide adenine dinucleotide phosphate (NADPH) in 1mM Na/K buffer. The rate of formation of resorufin was calculated by comparing the rate of increase in relative fluorescence to the fluorescence of a known amount of resorufin.

The decision tree method was used for selecting the appropriate procedure for hypothesis testing (Gad and Weil, 1984). Data were first subjected to normality test using Univariate procedure (SAS Institute, 1985). Since the data were normal (normality test), the unpaired TTest procedure (SAS Institute, 1985) was used for further comparisons.

RESULTS AND DISCUSSION

Of the nine resorufin ethers (Methoxy, Ethoxy, Propoxy, Butoxy, Pentoxy, Hexoxy, Benzyloxy, Octoxy, Decoxy), the rate of metabolism of methoxy-, ethoxy-, propoxy-, pentoxy- and benzyloxyresorufins by 3MC-induced microsomes differed significantly ($P < 0.05$) between control and treated cotton rats (Fig. 1). It is important to note that in the 3MC-induced microsomes, the substrate showing the highest rate of metabolism was not necessarily the substrate showing the greatest degree over controls. Ethoxyresorufin was the fastest substrate for 3MC-induced microsomes, but the induction of the metabolism of propoxyresorufin was greater (24-fold increase) than the induction of ethoxyresorufin. The study of Novak and Qualls's (1990) study on cotton rats unfortunately, did not include O-dealkylation of propoxyresorufin by 3MC-induced microsomes.

Based on the results from the 3MC induction study, microsomal O-dealkylation of methoxy-, ethoxy-, propoxy-, pentoxy- and benzyloxyresorufins was selected for field studies. Twenty two cotton rats (12 RH and 10 CO) were collected from the Criner study area. Significant differences in O-dealkylation of methoxy ($P < 0.0001$), ethoxy ($P < 0.0009$), propoxy ($P < 0.0012$), pentoxy ($P < 0.0005$) and benzyloxyresorufins ($P < 0.0023$) were observed between RH and CO in male cotton rats (Table 1). Females however, had an apparent increase (3 to 8-fold increase) in O-dealkylation rate for all of five ethers in RH rats but which was not statistically significant from CO rats.

Table 1. Criner study : Effect of environmental contaminants on microsomal O-dealkylation of resorufin ethers

Resorufin ethers	P-450[b]	Methoxy	Ethoxy	Propoxy	Pentoxy	Benzyloxy
Males:						
RH (n=8)[a]	1.050 ±0.030	0.364 ±0.035	0.311 ±0.050	0.204 ±0.035	0.027 ±0.003	0.336 ±0.048
CO (n=5)[a]	0.800 ±0.030	0.103 ±0.019	0.045 ±0.015	0.027 ±0.016	0.009 ±0.002	0.117 ±0.025
Fold induction	1-fold	4-fold	8-fold	8-fold	3-fold	3-fold
Significance	0.0003	0.0001	0.0009	0.0012	0.0005	0.0023
Females:						
RH (n=4)[a]	1.090 ±0.040	0.626 ±0.297	0.954 ±0.554	0.540 ±0.253	0.038 ±0.010	0.525 ±0.204
CO (n=5)[a]	0.920 ±0.080	0.152 ±0.020	0.268 ±0.045	0.180 ±0.029	0.014 ±0.004	0.069 ±0.017
Fold induction	1-fold	4-fold	4-fold	3-fold	3-fold	8-fold
Significance	NS	NS	NS	NS	NS	NS

[a] nmoles of resorufin formed / min / mg of microsomal protein (expressed as Mean ± Standard error); [b] nmoles per mg protein.

Population densities of cotton rats on the Pryor study area were low in both PO and CP. Nine cotton rats (6 PO and 3 CP) were collected from the Pryor study site. Because of the limited number of animals, no definitive conclusions can be drawn regarding the effect of environmental contaminants on O-dealkylation of all ethers tested. Despite this cautionary note, there were significant differences on O-dealkylation of all ethers between PO and CP rats. Cotton rats from PO had significantly higher rate of O-dealkylation reactions ($P < 0.05$) than that of controls (Table 2). Aroclor 1254 (PCB mixture) has been categorized as a "mixed" type of inducer, because it possesses the properties of both phenobarbital-type and 3MC-type inducers (Ryan et al., 1979). Since cotton rats from Pryor were exposed to a known Aroclor 1254 contaminated area, increased rate of O-dealkylation observed in PO rats may be real and could be attributed to Aroclor 1254. However, further study with a large sample size (adequate number of both sexes) is warranted.

Cytochrome P-448 or 3MC-induced microsomes (also designated as P-450c and P-450d

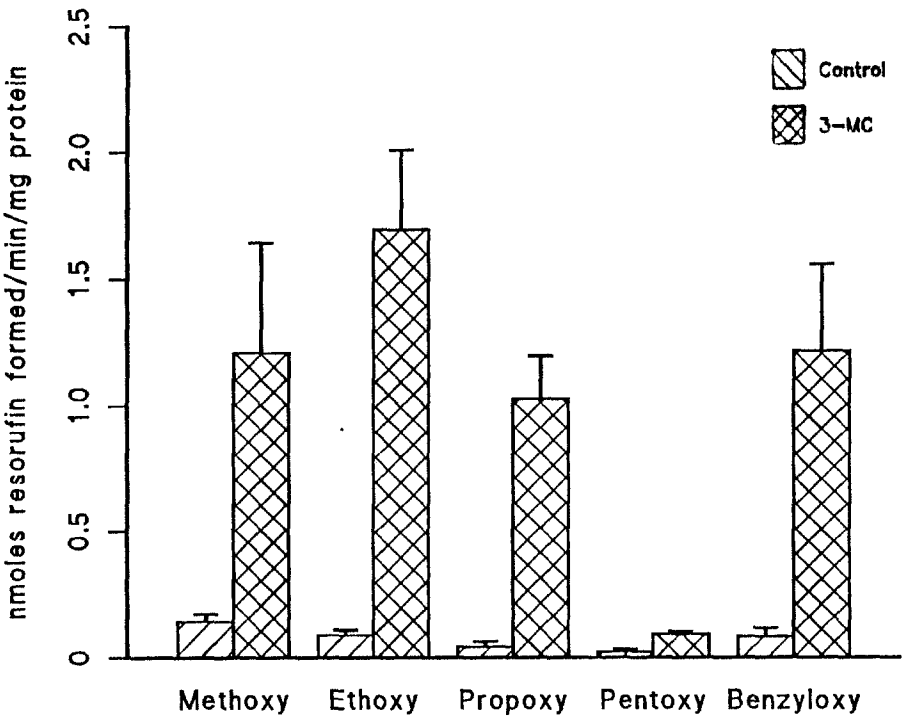

Figure 1. Effect of 3-methylcholanthrene pretreatment on the microsomal O-dealkylation of methoxy, ethoxy, propoxy, pentoxy and benzyloxyresorufins in cotton rats.

agent (Iwasaki et al., 1986). Likewise, at very low doses, induction of cytochrome P-448 may be insignificant.

From the evaluation of alkoxyresorufin O-dealkylation by environmental contaminant-induced microsomes it was apparent that there were individual differences particularly among females. This diminishes the value of using female cotton rats to assess the toxic effects of environmental contaminants. Sex differences occur in the O-dealkylation of alkoxyresorufins in cotton rats, the females exhibiting a higher O-dealkylation rate (Novak and Qualls, 1990). Although the mean rate of alkoxyresorufin O-dealkylation in females was higher than males, this does not conclusively prove that increased metabolic activity was the only factor for individual metabolic differences in females. Variation may also be linked to hormonal (steroid and thyroxine) status (Gustaffson et al., 1983).

Studies have indicated that fetal and newborn animals of most mammalian species including man have limited ability to metabolize xenobiotics because of the low levels of hepatic mixed-function oxidases and other enzymes involved in detoxification that are present at birth. However, postnatal development is rapid, adult levels of activity being reached within a few weeks (Short and Davies, 1970). Finally, marked fall in the maximal activity of phenobarbital-inducible and 3MC-inducible forms of microsomes occurs in senescent animals whether expressed in terms of microsomal protein or unit of cytochrome P-450. However, there are no age-related changes in enzyme (ethoxyresorufin O-deethylase and aldrin epoxidation) activities (Wynne et al., 1987). Age-related effects in alkoxyresorufins O-dealkylation are unlikely in the present study. Since enzyme activities are not affected by increased age, the evaluation of enzyme activity (alkoxyresorufin O-dealkylation) should be more advantageous than total cytochrome P-450 assays which are subjected to age related quantitative change.

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