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INDUCTION OF CYTOCHROME P-450IA1 IN FETAL RAT LIVER BY A SINGLE DOSE OF 3-METHYLCHOLANTHRENE

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SUMMARY: Pregnant Sprague-Dawley rats were treated with a single ip dose of either olive oil or 40 mg/kg of 3-methylcholanthrene on gestation day 20 and sacrificed at various times after injection. Determination of aryl hydrocarbon hydroxylase activity 24 hr after injection revealed that treatment with 3-methylcholanthrene resulted in a 10.5-fold stimulation of enzymatic activity in liver 800 x *g* supernatants. Western blot analysis with monoclonal antibody 1-7-1 confirmed these results and demonstrated the presence of a 3-methylcholanthrene-inducible P-450 isozyme. Using Northern and slot blot techniques, the induction of steady-state levels of CYPIA1 RNA was shown to occur as early as 4 hr following 3-methylcholanthrene injection. CYPIA1 RNA levels were induced 31.6-fold over values obtained from oil-treated tissues at this time. This appears to be the optimal time to study changes in the

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ABBREVIATIONS: AHH, aryl hydrocarbon hydroxylase; βNF, β-naphthoflavone; BP, benzo[a]pyrene; 3-OH-BP, 3-hydroxybenzo[a]-pyrene; MAb, monoclonal antibody; MC, 3-methylcholanthrene; PAGE, polyacrylamide gel electrophoresis; PAH, polycyclic aromatic hydrocarbon; SDS, sodium dodecyl sulfate; SSC, 0.15 M NaCl/0.015 M Na citrate.

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levels of CYPIA1 RNA gene expression in the fetus following transplacental exposure to polycyclic aromatic hydrocarbons. By 12 to 24 hr postinjection, the induction of CYPIA1 RNA levels declined to 3.5- to 8.5-fold above control values. These results demonstrate that the kinetics of induction of the CYPIA1 gene during the fetal period differed from that seen in adults.

Several laboratories have examined the response of the cytochrome P-450 enzyme system during fetal development (1, 2). PAH -inducible AHH activity has been demonstrated during the fetal period in several rodent species (3), including rats and mice. Although several studies have provided enzymatic (3-11) and immunological (10, 11) evidence for the inducibility of CYPIA1 using various treatment protocols, a recent study using 20-mer oligonucleotide probes specific for the CYPIA1[3] and CYPIA2 genes found no evidence for the prenatal expression of these RNA species in the fetal liver by standard Northern blot techniques following treatment of pregnant rats with two consecutive daily 40 mg/kg doses of MC (13). Expression of the CYPIA1 gene was observed only by employing an extremely sensitive polymerase chain reaction technique whereby the CYPIA1 signal was amplified from a cDNA library (14). In contrast, Marie *et al.* (15) demonstrated that three consecutive, daily, transplacental injections of 20 mg/kg of MC are required in order to observe induction of hepatic RNA species for the IA1 and IA2 genes in fetal rat livers.

Recent studies by our laboratory (16-18), using fetal mouse liver and lung preparations, have shown that the induction kinetics of fetal P-450 RNAs exhibit both tissue- and inducer-dependent specificity and differ from the kinetics observed in the adult. In particular, fetal tissues showed a rapid, early induction of CYPIA1 RNA that reached maximal steady-state levels by 4 hr after transplacental injections of either a 30 or 100 mg/kg dose of MC. Since both Omiecinski and his colleagues (13,14) and Marie *et al.* (15) examined steady-state RNA levels 16 hr after MC administration when, according to our mouse studies (16), the levels of hepatic RNAs may be on the falling phase of the fetal induction curve, we have examined the induction of P-450 RNAs in fetal liver prepara-

[3]Cytochromes P-450IA1 and IA2 are the accepted nomenclature for the rat isoforms P-450c and P-450d, respectively (12).

tions at various times after injection of a single 40 mg/kg dose of MC. Our results show that, as in the mouse, CYPIA1 RNA levels are maximally induced by 4 hr after MC injection in fetal rat liver and decline to much lower levels of expression by 12 to 24 hr. The earlier induction kinetics of this gene during late gestation and limited sensitivity of the oligomer probes during the fetal period are thus important characteristics of this gene system during development.

MATERIALS AND METHODS

Sprague-Dawley CR:RAR male and female rats were obtained from the Animal Production Area of the Frederick Cancer Research and Development Center. Females were mated with males and the pregnant rats were treated ip on the 20th day of gestation (day 1 was considered the day when the vaginal plug was detected) with either olive oil alone (1.0 ml/300 gm) or 40 mg/kg of MC dissolved in olive oil, using a high ip injection to avoid direct injection into the uterus. Mothers were sacrificed at various times after injection. Fetal livers from the same litter were pooled and 800 x *g* supernatant fractions were prepared as described previously (16). For Western blot analysis, the proteins were separated by SDS-PAGE (19) on an 8.5% gel. The proteins were then transferred from the gel to a nitrocellulose membrane filter by a modification of a previously described method (20), using the Trans-Blot Cell from Bio-Rad Laboratories (Richmond, CA). MC-inducible protein was detected by overnight incubation with MAb 1-7-1, raised in the mouse against rat cytochrome P-450IA1 (21), and were visualized by incubating the membrane with goat anti-mouse IgG alkaline phosphatase conjugate (Bio-Rad Laboratories) for 2 hr and then reacting with *p*-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt according to the manufacturer's instructions. AHH activity was measured by a fluorescence assay using quinine sulfate as the standard (22, 23). Protein content was determined by the Lowry procedure (24).

RNA was purified as described by MacDonald *et al.* (25) by homogenization in a cold 4 M guanidine thiocyanate/0.1 M Tris-HCl (pH 7.5)/1% 2-mercaptoethanol solution and centrifugation over a 6.1 M CsCl/25 mM NaAc (pH 5.2)/10 mM EDTA solution as described previously (16). Purified RNA was dissolved in 10 mM sodium phosphate buffer (pH 7.0), denatured by treatment with glyoxal, and either blotted directly onto Biodyne A nylon membrane filters (Pall Ultrafine Filtration Corp., Glen Cove, NY) using a Bethesda Research Laboratories Hybri-Slot manifold, or first fractionated by electrophoresis in a 1% agarose gel with constant recirculation of the 10 mM sodium phosphate (pH 7.0) buffer prior to transfer (26). The blots were baked, prehybridized, and hybridized as described previously (26). Plasmid pαT14, a genomic clone of the rat α-tubulin gene (27), was obtained from Dr. Ihor Lemischka and plasmid pA8, a genomic fragment from the rat CYPIA1 gene cloned into pBR322 (28), was obtained from Dr. Edward Bresnick. Digestion of pA8 with *EcoRI* yielded a 5.5 kb fragment that cross-hybridizes to both P-450IA1 and IA2. Both probes were labeled by the random primer labeling technique (29, 30). Following hybridization, the blots were washed three times (5 min each) with 2 x SSC/ 0.1% SDS at room temperature followed by three washes (15 min each) with 0.1 x SSC/0.1% SDS at 50°C. In some experiments, the blots were also probed with a 20 bp oligonucleotide to the rat CYPIA1 gene,

5'-d(TCTGGTGAGCATCCAGGACA)-3' (13), which was synthesized by the NCI-FCRDC Nucleic Acid and Protein Synthesis Laboratory. The oligomer was 5'-end labeled with T4 polynucleotide kinase by Lofstrand Labs Limited. The blots were hybridized and washed as described previously (13). Washed blots were wrapped in Saran Wrap and autoradiographed in the presence of an intensifying screen (Cronex Lightning Plus, DuPont, Wilmington, DE) with preflashed Kodak X-Omat XAR-5 film at -80°C. RNA levels were quantitated by densitometric scanning of the autoradiographs using a LKB Ultroscan XL laser densitometer. Multiple autoradiographic exposures of the blots were scanned to assure quantitation over the linear range of the film.

RESULTS AND DISCUSSION

Determination of enzymatic activity in fetal 800 x *g* supernatant fractions revealed that transplacental MC exposure resulted in a 10.5-fold stimulation of hepatic AHH activity from 0.81 ± 0.17 pmol of 3-OH-BP formed/min/mg protein in oil-treated animals to 8.52 ± 1.53 pmol of 3-OH-BP formed/min/mg protein in MC-treated rats 24 hr after injection. These results were confirmed by Western blot analysis. Duplicate 50 μg aliquots of the 800 x *g* tissue supernatants were fractionated on an 8.5% SDS-polyacrylamide gel and transferred to nitrocellulose. Incubation with MAb 1-7-1, raised against rat CYPIA1 and specific for both the IA1 and IA2 isozymic forms of cytochrome P-450 (21), demonstrated the induction of an immunologically cross-reactive
P-450 protein in MC-treated liver samples (Fig. 1). This band migrated at 56.5 kd, in good agreement with the anticipated size of the CYPIA1 isozyme (31). No protein bands were visible in oil-treated control samples, as expected given the low levels of AHH activity and limited sensitivity of the immunoblot procedure.

We have previously shown in mice that the kinetics of CYPIA1 RNA induction are very different in the fetus than in the adult (16-18). Using this study as a guide, we have examined the induction of CYPIA1 RNA levels 4, 12, and 24 hr following transplacental MC injection. A Northern blot of the RNAs probed with the 5.5 kb *EcoRI* fragment from plasmid pA8, which cross-hybridizes to both the CYPIA1 and CYPIA2 RNA species, is shown in Fig. 2. Densitometric scans of slot blots of the RNA samples are presented in Table 1. All of the values were corrected for the amount of α-tubulin binding to each slot. Northern blot analysis showed that the P-450IA2 RNA species was barely detectable during the fetal period, and was only seen following extended exposure of the film. Therefore, the signals obtained from the slot blots can be used to specifically quantitate the levels of the CYPIA1 isoform, as it is

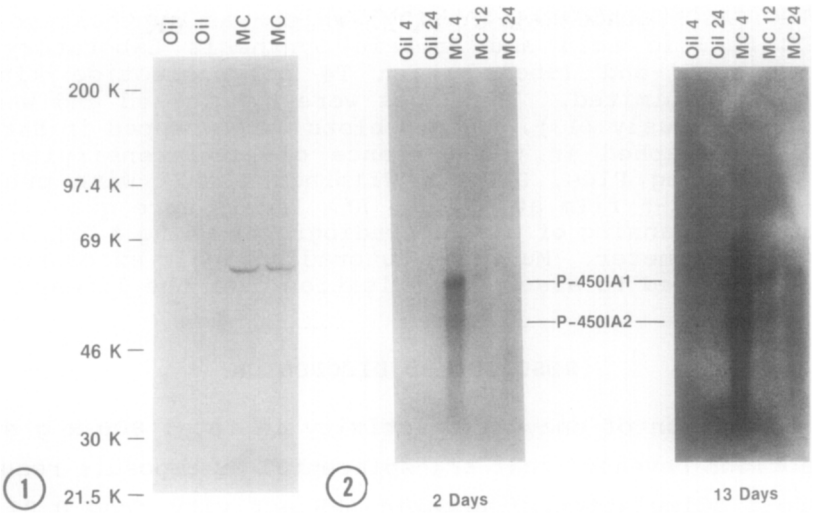

Fig. 1. Induction of CYPIA1 in fetal rat liver tissue by MC. Pregnant Sprague-Dawley rats were treated by high ip injection on day 20 of gestation with either vehicle or 40 mg/kg of MC. The mothers were sacrificed 24 hr later and 800 x *g* supernatant fractions were prepared from fetal livers pooled from the same litter. Duplicate 50 μg aliquots from oil- or MC-treated rats were fractionated on an 8.5% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and the membrane probed with MAb 1-7-1 as described in Materials and Methods. "Rainbow" prestained molecular weight markers (Amersham Corp., Arlington Heights, IL) were used to determine the relative molecular weight of the stained band.

Fig. 2. Northern blot of fetal rat liver RNA probed with pA8. Pregnant Sprague-Dawley rats were treated by high ip injection on day 20 of gestation with either vehicle or 40 mg/kg of MC. The mothers were sacrificed at various times after injection and total RNA was isolated from fetal livers pooled from the same litter. Total RNA was fractionated on a 1% agarose gel and then transferred to a nylon membrane. The membrane was then hybridized with a 5.5 kb *EcoR1* fragment from the pA8 plasmid. Two different exposures (2 and 13 days) of the same autoradiograph are shown.

Table 1

Densitometric analysis of RNA slot blot data

RNA samples were blotted directly onto nylon membranes using the BRL Hybri-Slot manifold. The blots were first probed with the 5.5 kb EcoRI fragment from plasmid pA8, the fragment removed by washing the membrane according to the manufacturer's instructions, and then reprobed with pαT14. The data obtained with the pA8 fragment was corrected for α-tubulin expression. All values are expressed as relative absorbance units compared to the 4 hr oil-treated samples for each tissue.

	Relative Absorbance Units		
Treatment	4 hr	12 hr	24 hr
Oil	1.00	ND[a]	1.21
MC	31.61	3.53	8.49

[a]ND = not determined.

doubtful that the extremely low levels of P-450IA2 expression contributed significantly to the autoradiographic signals.

As can be seen from the blot in Fig. 2, the levels of CYPIA1 RNA were undetectable in oil-treated liver preparations on either gestation day 20 or 21 (4 or 24 hr after oil injection) in the Northern blots, although a faint signal could be detected on the slot blots (Table 1). Treatment with MC resulted in a marked induction of the CYPIA1 RNA levels 4 hr after injection. From the low levels of basal expression detected on the slot blots, we estimated an induction of 31.6-fold. The CYPIA1 RNA levels decreased dramatically to 3.5-fold at 12 hr and rose to 8.5-fold by 24 hr.

Two independent studies from other laboratories (13, 15) have failed to detect CYPIA1 or CYPIA2 induction in fetal livers 16 hr following a single or two daily injections of MC to pregnant rats by standard blotting procedures. The results obtained in this report differ from these studies in several important parameters. We have examined induction of the CYPIA1 genes at three time points, since our earlier mouse studies had indicated that the 16 hr time point was on the downward portion of the induction curve in the liver when CYPIA1 RNA levels might be difficult to detect. Our results in fetal rat liver confirm this finding, as the CYPIA1 RNA levels declined markedly after the maximal induction observed 4 hr following a single transplacental injection of MC. We have also probed our Northern blots with the same 20 bp oligonucleotide to the CYPIA1 gene used by Giachelli and Omiecinski (13) and were barely able to detect the CYPIA1 RNA at the 4 hr time point (data not shown), when the RNA levels were at their maximal values. Thus, the larger 5.5 kb *EcoRI* fragment from plasmid pA8 proved to be a more sensitive probe than the 20 bp oligomer, and was also probably a more sensitive probe than the 950 bp fragment used by Marie *et. al.* (15). While we used the same concentration of MC as Giachelli and Omiecinski (13), our 40 mg/kg dose of MC was twice the dose used by Marie *et al.* (15).

Our results clearly demonstrate at the biochemical and molecular levels that rat fetuses are able to boost their CYPIA1 enzyme levels in response to exposure to environmental toxicants. The ability to enhance the expression of various P-450 isozymes as a result of exposure to various exogenous compounds may be an important determinant of the susceptibility of rat fetuses to chemically-induced damage. Recent studies have shown in mouse fetuses that genetic differences for inducibility of CYPIA1 by PAHs

in both the fetuses and the mother play a major role in determining susceptibility to PAH-mediated tumor initiation in the lung and liver (reviewed in 2). Similar results have been demonstrated in adult rats (32). More recently, Yang *et al.* (33) have shown that inducibility of the CYPIA1 gene in fetal rats can be detected as early as gestation day 10 in whole embryos and may play an important role in activating potential teratogenic agents to their more reactive species during critical periods of organogenesis. Continued work with these rodent model systems, which exploit the differences in expression of various gene systems in fetal and adult animals, may provide important insights into the complex array of gene regulatory pathways that influence and are influenced by the neoplastic process.

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