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INDUCTION BY PHENOBARBITAL IN McA-RH7777 RAT HEPATOMA CELLS OF A POLYCYCLIC HYDROCARBON INDUCIBLE CYTOCHROME P450

Michael E. McManus,[1]* Rodney F. Minchin,[2] Dolores M. Schwartz,[3]
Peter J. Wirth[3] and Brian E. Huber[3]

[1]Department of Clinical Pharmacology, Flinders University,
Bedford Park 5042, South Australia
[2]Laboratory of Experimental Therapeutics and Metabolism and
[3]Laboratory of Experimental Carcinogenesis, National Cancer Institute,
Bethesda, MD 20892

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Summary: The metabolism of 2-acetylaminofluorene (AAF) to its six oxidative metabolites has been used to study cytochrome P450 monooxygenase activity in two rat hepatoma cell lines, McA-RH7777 and Reuber H4-II-E. McA-RH7777 cells exhibited considerably higher basal activities than H4-II-E cells for all metabolic pathways studied. Phenobarbital induced AAF metabolite formation in McA-RH7777 cells to a similar extent as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), but was only a weak inducer of these activities in H4-II-E cells. Northern blot analysis utilizing specific phenobarbital or 3-methylcholanthrene inducible cytochrome P450 cDNA probes indicated that there was at least a 10-fold increase in a 3-methylcholanthrene inducible cytochrome P450 transcript in phenobarbital treated McA-RH7777 cells. These data suggest that in this transformed cell line phenobarbital behaves as a polycyclic hydrocarbon-like inducer. © 1986 Academic Press, Inc.

Hepatoma cell lines have been used extensively to study the cytochrome P450 monooxygenase system (1,2,3). An important property of these monooxygenases is the differential inducibility of different isoenzymes of cytochrome P450. Traditionally, inducers of cytochrome P450 have been classified as either phenobarbital or 3-methylcholanthrene-like. In general, hepatoma cell lines have been shown to respond to 3-methylcholanthrene with induction of forms of cytochrome P450 that are expressed in fetal or extrahepatic tissue but are refractory to induction by phenobarbital (1,2,3). This lack of inducibility by phenobarbital has been taken to reflect the dedifferentiation of these cells towards a fetal phenotype.

* To whom correspondence should be addressed.

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In this study we report that phenobarbital induces 2-acetylaminofluorene metabolism in a rat hepatoma cell line, McA-RH7777, to a similar extent as the potent polycyclic hydrocarbon inducer 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). When cDNA probes specific for phenobarbital (4) and 3-methylcholanthrene (5) inducible cytochromes P450 were hybridized to poly(A) RNA obtained from phenobarbital-treated cells, a ten-fold increase in a 3.1 Kb transcript specific for the 3-methylcholanthrene inducible cytochrome P450 was observed. These results suggest that although phenobarbital induces cytochrome P450 activity in this hepatoma cell line, it nonetheless is acting by inducing a polycyclic hydrocarbon dependent monooxygenase.

METHODS

Chemicals: 2-[9-^{14}C] Acetylaminofluorene (AAF; 47.6 mCi/mmole) was purchased from New England Nuclear (Boston, MA) and was purified to greater than 98% purity by high-pressure liquid chromatography (6). Unlabeled AAF and metabolite standards were obtained as previously described (6). Sodium phenobarbital, β-glucuronidase (Bovine, Type B-3), sulfatase (Limpets, Type V) and dexamethasone (DEX) were from Sigma Chemical Co. (St. Louis, MO). Aroclor 1254 was generously donated by Dr. H. V. Gelboin, National Cancer Institute, and TCDD from Dow Chemical Co. (Midland, MI). All other chemicals were of analytical reagent grade.

Cell Lines: The McA-RH7777 and H4-II-E (derived from Reuber hepatoma H-35) rat hepatoma cell lines were kindly provided by Dr. Van R. Potter of the University of Wisconsin, Madison. Cells were maintained at 37°C in 75 cm^2 tissue culture flask (Costar, Cambridge, MA) in 10 ml of the appropriate medium under an atmosphere of 5% CO_2 and 95% air. McA-RH7777 cells were grown in Swims S77 medium (Media Production Unit, National Institutes of Health) supplemented with heat inactivated 10% horse serum and 5% fetal bovine serum (Gibco, Grand Island, NY), and H4-II-E cells in Eagles minimal essential medium (Media Production Unit, National Institutes of Health) supplemented with 10% fetal calf serum, 0.3 mg/ml glutamine, 0.1 mg/ml L-cysteine, 0.1 μg/ml D-biotin, 1.36 μg/ml Vitamin B12, 0.1 mM non-essential amino acids (Gibco, Grand Island, NY). Stock cultures of McA-RH7777 and H4-II-E cells were seeded every five days at a cell density of 0.5 x 10^6 and 1.0 x 10^6 cells per flask, respectively. Freshly isolated hepatocytes were prepared from male Sprague-Dawley rats as previously described (7).

Drug Treatment: All induction studies were conducted in tissue culture cluster dishes containing 12 wells (Costar, Cambridge, MA, Catalogue No. 3512) each of 4.5 cm^2 in area. The doubling time of McA-RH7777 and H4-II-E cells in these wells were 33 and 22 hours, respectively. McA-RH7777 cells were seeded at 2 x 10^5 and H4-II-E cells at 2.5 x 10^5 cells per well (1.0 ml volume) and allowed to attach and grow for the first 24 hr. The medium was then removed and fresh medium added and this procedure was repeated every 24 hr throughout the experiment. Inducing agents were added in 5 μl of dimethylsulfoxide to give the following concentrations: TCDD 10 nM; Aroclor 1254, 1 μM; dexamethasone, 1 μM; and phenobarbital, 1.5 mM. Control cells received 5 μl of the vehicle only. The induction period was 48 hr, which is approximately one and a half cell generation times for both cell types and by this time the cells were confluent.

Acetylaminofluorene Metabolism: Following the induction period the medium was removed and cells were washed once with 2 ml of fresh medium (37°) and then incubated with an additional 1 ml of medium for 5 min to remove inducing agents. ^{14}C-AAF was then added in 0.75 ml of serum free medium to give a final concentration of 15 μM and incubated for varying time periods. This concentration of AAF was not toxic to either cell type. The reaction was stopped by removing the medium to a test tube containing 1 ml of ice cold sodium acetate (1 M; pH 6.0) and then trypsinizing the cells at 37° with 1.25 ml of 0.2% Trypsin in phosphate-buffered saline (0.15 M NaCl/0.014 M KH_2PO_4/0.086 N K_2HPO_4) pH 6.0 for 3 min. The cell suspension was then combined with the sodium acetate-medium fraction and extracted with 7.5 ml of cold ether. Authentic standards of AAF and its metabolites were added to the sodium acetate solution as carriers and to visualize absorbance peaks during chromatography. After one ether extraction, the remaining aqueous phase was incubated overnight with β-glucuronidase (5000 units) and sulfatase (12 units). Additional standards of AAF and metabolites were then added to the incubation mixture prior to extracting twice more with 7.5 ml of ether. The three ether extracts were pooled and evaporated to dryness under nitrogen, and residues were dissolved in 0.1 ml of methanol for chromatographic analysis. AAF disappearance and metabolite formation were then determined as previously described (6). The amount of DNA in the aqueous layer was determined by the method of Erickson et al. (8).

RNA Isolation and Northern Blotting: Approximately 6 x 10^8 cells were used for each isolate. RNA was isolated with guanidine thiocyanate by the method of Schweizer and Goerttler (9) and enriched for poly(A) RNA by oligo (dT)-cellulose chromatography (10). Electrophoresis of poly(A) RNA samples on horizontal denaturing formaldehyde agarose gels with subsequent transfer to nitrocellulose membranes was performed as previously described (11). Membranes were hybridized to [^{32}P] nick-translated DNA probes (12) (specific activity > 5 x 10^8 cpm/μg DNA) for 17 hr at 42° in 50% deionized formamide, 0.75 M NaCl, 0.075 M trisodium citrate, 0.05 M Na phosphate buffer (pH 7.4) and 0.1% sodium dodecyl sulfate (SDS) with 0.5 mg/ml yeast tRNA carrier. Blots were washed at moderate stringency (0.3 M NaCl, 0.03 M trisodium citrate, 0.1% SDS at room temperature for 40 min with a total of four changes and 0.075 M NaCl, 0.008 M trisodium citrate, 0.01% SDS at 55° for 30 min with a total of three changes), and exposed to Kodak XAR-5 x-ray film with intensifying screens. After exposure to film, Northern blots were washed (boiled for 5 min) and reprobed with a cDNA actin probe to confirm that all lanes had equal amounts of RNA.

DNA Probes: The DNA probes used in blot analysis were: a 1100 bp phenobarbital-inducible P450 cDNA clone (P450e, R17) kindly supplied by Drs. M. Atchison and M. Adesnik (4); and a 2620 bp 3-methylcholanthrene inducible P450 cDNA clone ($P_1$450) kindly supplied by Dr. F. J. Gonzalez (5).

RESULTS AND DISCUSSION

The cytochrome P450-mediated metabolism of AAF involves oxidation at both nitrogen and carbon atoms. N-Hydroxylation leads to metabolic activation while hydroxylation at positions 1, 3, 5, 7 and 9 on the fluorene ring are considered detoxification pathways. These hydroxylations of AAF appear to be preferentially catalyzed by several forms of cytochrome P450 in rat (6), rabbit (13) and human microsomes (14). Further, these observations have been supported by data obtained using purified forms of cytochrome

Table 1. Induction of 2-acetylaminofluorene metabolism in McA-RH7777 cells

	Formation of AAF Metabolites[a]					
Treatment	7-OH-AAF	9-OH-AAF	5-OH-AAF	3-OH-AAF	1-OH-AAF	N-OH-AAF
None	17.6 ± 0.8	6.0 ± 0.2	19.6 ± 1.9	17.4 ± 1.5	1.5 ± 0.2	1.3 ± 0.1
PB	59.7 ± 3.4	14.5 ± 2.8	68.8 ± 3.4	63.7 ± 2.2	4.7 ± 0.2	4.9 ± 0.7
Aroclor	55.8 ± 3.2	9.6 ± 1.1	61.4 ± 4.2	55.9 ± 2.6	3.8 ± 0.5	4.5 ± 0.1
TCDD	68.2 ± 1.9	13.0 ± 2.0	90.3 ± 1.2	74.8 ± 2.7	5.1 ± 0.4	6.9 ± 0.4
DEX	18.1 ± 3.2	7.8 ± 0.8	20.2 ± 3.9	18.9 ± 2.9	1.6 ± 0.5	1.8 ± 0.4

Values represent mean ± SD, n = 4.

[a]Activity expressed as pmol metabolite formed/μg DNA/20 min.

P450 isolated from rabbit liver (13). Therefore to probe the cytochrome P450 system in two rat hepatoma cell lines, McA-RH7777 and H4-II-E, we have chosen AAF as a substrate.

The basal activities of AAF metabolism to its six oxidative metabolites is considerably higher in McA-RH7777 cells compared to H4-II-E cells (Tables 1 and 2). Differences in activities vary from 1.8-fold for the 9-hydroxylation, to 11-fold for the 3- and 5-hydroxylations of AAF. For comparison

Table 2. Induction of 2-acetylaminofluorene metabolism in H4-II-E cells

	Formation of AAF Metabolites[a]					
Treatment	7-OH-AAF	9-OH-AAF	5-OH-AAF	3-OH-AAF	1-OH-AAF	N-OH-AAF
None	2.1 ± 0.1	3.4 ± 0.7	1.8 ± 0.2	1.6 ± 0.1	0.4 ± 0.1	0.3 ± 0.1
PB	6.7 ± 0.8	5.1 ± 0.1	6.6 ± 1.0	5.9 ± 0.7	0.9 ± 0.1	0.7 ± 0.1
Aroclor	25.0 ± 2.7	8.5 ± 0.7	26.3 ± 2.7	23.6 ± 2.6	1.6 ± 0.3	1.9 ± 0.3
TCDD	31.3 ± 5.1	9.6 ± 1.3	32.7 ± 5.4	28.8 ± 2.1	2.1 ± 0.4	2.5 ± 0.3
DEX	7.3 ± 0.4	4.8 ± 0.2	6.2 ± 1.0	5.8 ± 0.7	0.8 ± 0.2	0.7 ± 0.1

Values represent mean ± SD, n = 4.

[a]Activity expressed as pmol metabolite formed/μg DNA/20 min.

with values given in Tables 1 and 2 and at the concentration of AAF used in this study, freshly isolated hepatocytes exhibit rates of N-, 1-, 3-, 5-, 7- and 9-hydroxylations of AAF of 3.6 ± 0.4, 12 ± 1, 7 ± 1, 11 ± 3, 56 ± 3, and 112 ± 3 pmol/μg DNA/20 min (X ± SD, n = 4), respectively. For two of these metabolic pathways, 3- and 5-hydroxylations of AAF, McA-RH7777 cells exhibit approximately twice the activity of freshly isolated hepatocytes. The low basal activities reported here for AAF metabolism in H4-II-E cells confirm previous reports of low cytochrome P450 monooxygenase activities in this cell line (15-17).

Phenobarbital pretreatment in both hepatoma cell lines caused a marked induction of AAF metabolism to its six oxidative products (Tables 1 and 2). However, because of the low basal activities of AAF metabolite formation in H4-II-E cells, the fold induction caused by phenobarbital in these cells still results in activities below those of control McA-RH7777 cells. Raphael et al. (16) have reported a similar fold induction by phenobarbital of 7-ethoxycoumarin O-dealkylation in H4-II-E cells to that reported in this study.

For comparison with the inductive effect of phenobarbital pretreatment on AAF metabolism in both hepatoma cell lines, results from three other inducing agents have also been included (Tables 1 and 2). TCDD, a prototype polycyclic hydrocarbon inducer, and Aroclor 1254, a general inducer, caused a marked induction of AAF metabolism to all its oxidative metabolites in both McA-RH7777 and H4-II-E cells. In McA-RH7777 cells phenobarbital is as potent as both TCDD and Aroclor 1254 in inducing AAF metabolism. However, in H4-II-E cells phenobarbital caused only a 2- to 4-fold induction of the 3-, 5- and 7-hydroxylations of AAF compared to at least a 12-fold induction of these pathways by either TCDD or Aroclor 1254. Dexamethasone which has been shown to induce a form of cytochrome P450 similar to that induced by prenenolone 16-α-carbonitrile in vivo and in hepatocyte culture (18) only induced AAF metabolism in H4-II-E cells. The fact that McA-RH7777 cells are refractory to dexamethasone induction but respond to phenobarbital

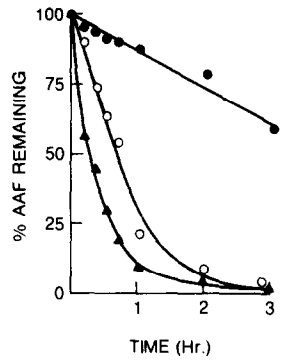

Fig. 1. Disappearance of 2-acetylaminofluorene as a function of time in control (●), phenobarbital (O) and 2,3,7,8-tetrachloro-dibenzo-p-dioxin (▲) pretreated McA-RH7777 cells. Conditions were the same as under methods except that 2-acetylaminofluorene metabolism was determined in separate wells after incubation times of 10, 20, 30, 40, 50, 120 and 180 min.

is in contrast to previous studies where glucocorticoids have been shown to induce in established cell lines those activities more closely associated with phenobarbital induced monooxygenases (15,19).

The effect of phenobarbital pretreatment of McA-RH7777 cells on AAF metabolism is more clearly shown in Figure 1. These data show the disappearance of AAF as a function of time and for comparison data from TCDD pretreated and control cells are included. The approximate half-life of AAF in control, phenobarbital and TCDD pretreated cells were 240, 34 and 23 min, respectively. These data clearly demonstrate that phenobarbital pretreatment of McA-RH7777 cells is capable of inducing AAF metabolism to a similar extent as TCDD. While phenobarbital has previously been shown to be a weak to moderate inducer of cytochrome P450 monooxygenase activity in certain rat hepatoma cell lines (15,16,20-22), this study is to our knowledge the first time an established cell line has been shown to respond in a similar manner to both phenobarbital and TCDD induction.

The similarity in the metabolic profiles of AAF in McA-RH7777 cells following phenobarbital and TCDD induction suggest that these compounds may be inducing the same form(s) of cytochrome P450 in this cell line. Therefore to ascertain the form of cytochrome P450 being induced by phenobarbital in McA-RH7777 cells, Northern blot analysis was performed. RNA was isolated,

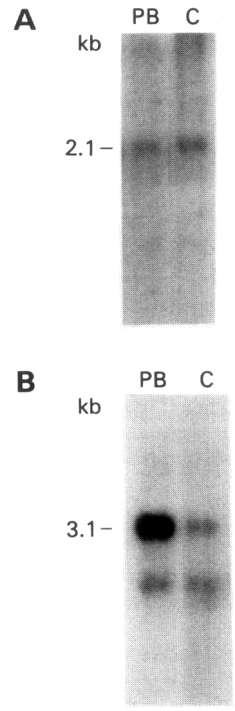

Fig. 2. Northern blot analsis of poly(A) enriched RNA from control (c) or phenobarbital (Pb) pretreated McA-RH7777 cells probed with cDNA probes (A) 450_e (2.1 Kb) and (B) P1450 (3.1 Kb).

electrophoresed, transferred to nitrocellulose membranes and hybridized with a specific phenobarbital (R17, P450e) or 3-methylcholanthrene (P1450) inducible cytochrome P450 cDNA probe. Figure 2A shows that both control and phenobarbital pretreated McA-RH7777 cells display quantitatively identical 2.1 Kb P450e transcripts. In contrast to this result, phenobarbital pretreatment caused an approximate 10-fold increase in a specific P1450 transcript, which suggest that, in this cell line, phenobarbital is functioning as a polycyclic hydrocarbon-like inducer (Figure 2B). These data are clearly consistent with the metabolic profiles of AAF as alluded to above, and confirm previous observations based on metabolic activities that in some hepatoma cell lines phenobarbital induces polycyclic hydrocarbon dependent monooxygenases (3,20,21).

While the mechanism of how polycyclic hydrocarbons induce cytochrome P450 is reasonably well understood, it is currently unclear as to how

phenobarbital and other inducers regulate these monooxygenases (23). The mis-programming of cytochrome P450 gene expression in McA-RH7777 cells compared to fully differentiated hepatocytes following phenobarbital treatment, is a similar phenomenon that has been reported for many other proteins in transformed cell lines (24). The fact that phenobarbital behaves like TCDD in McA-RH7777 cells may suggest a certain degree of similarity in induction mechanisms. Further studies are currently in progress to evaluate the usefulness of this cell line in establishing how phenobarbital regulates cytochrome P450 gene expression.

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