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SYNERGY OF PHENOBARBITAL AND 3-METHYLCHOLANTHRENE IN "SUPERINDUCTION" OF CYTOCHROME P-450c mRNA BUT NOT ENZYME ACTIVITY

PATRICK L. IVERSEN,*† LINDA I. SIEGEL,* KEVIN RAHNER* and EDWARD BRESNICK*†‡§

*The Eppley Institute for Research in Cancer and Allied Diseases, and the Departments of ‡Biochemistry and †Pharmacology, University of Nebraska Medical Center, Omaha, NE 68105-1065, U.S.A.

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Abstract—The combination of phenobarbital and 3-methylcholanthrene in the inductive process of the rat hepatic cytochrome P-450c gene was evaluated. Daily injections of phenobarbital (80 mg/kg, i.p.) had little or no effect on the amount of poly $(A)^+$ RNA encoding cytochrome P-450c, whereas a single injection of 3-methylcholanthrene (25 mg/kg, i.p.) produced a significant accumulation at 15 hr in cytosolic mRNA coding for cytochrome P-450c. Four daily injections of phenobarbital followed by a single dose of 3-methylcholanthrene produced 5–24 times more poly $(A)^+$ RNA coding for P-450c than 3-methylcholanthrene treatment alone. This superinduction of RNA transcripts was also observed for a species coding for cytochrome P-450d, which was increased 3–6 times over 3-methylcholanthrene treatment alone. However, the elevated concentration of transcripts for both the P-450d and P-450c RNA species did not result in an increase in the marker enzyme activity for cytochrome P-450c, 7-ethoxyresorufin *O*-deethylase. These data implicate a regulatory step in the induction of cytochrome P-450c enzyme activity which must occur at a level beyond transcription.

The cytochrome P-450 family of enzymes is responsible, in part, for the metabolic activation of procarcinogens and protoxins, metabolic inactivation of a wide variety of drugs and xenobiotics, and oxidative metabolism of endogenous compounds including steroids and fatty acids [1, 2]. Some forms of enzyme are present constitutively, whereas others are induced to high levels of expression upon exposure of an animal to certain foreign compounds. Polycyclic aromatic hydrocarbons (PAH) are known to induce aryl hydrocarbon hydroxylase activity [3, 4], more specifically, the cytochromes P-450c and P-450d [3]. The PAH induction process appears to involve high affinity binding proteins [5–7] which may function in *trans*-regulation of cytochrome P-450c gene expression [8].

Phenobarbital stimulates the transcription of cytochrome P-450 forms which are distinct from those that respond to PAH administration, i.e. rat cytochrome P-450b and P-450e [9–11]. This induction phenomenon differs from that noted after PAH administration in that to date no binding protein has been identified that could act as a receptor and *trans*-activator. The pleiotropic stimulation of transcription following phenobarbital administration includes the binding protein utilized by 3-methylcholanthrene in the induction of cytochrome P-450c [12, 13].

We have taken advantage of the availability of specific probes for cytochrome P-450c to more fully understand the effects of the phenobarbital and 3-methylcholanthrene combination. These results are reported in this manuscript.

§ Send correspondence to: Edward Bresnick, Ph.D., UNMC-Eppley Institute, 42nd and Dewey Ave., Omaha, NE 68105.

MATERIALS AND METHODS

Treatment of animals. Male Sprague–Dawley rats weighing between 125 and 150 g were purchased from the Sasco Co., Omaha, NE. In all experiments, the animals were allowed access to Purina Rodent Chow and tap water *ad lib*. Controls were given a single injection of 0.9% (w/v) NaCl or corn oil. Phenobarbital (80 mg/kg, i.p.) was given daily for up to 4 days; 3-methylcholanthrene (25 mg/kg, i.p.) was given either 15 or 40 hr prior to killing the animals. In the combined treatment, phenobarbital administration was followed by 3-methylcholanthrene 9 hr later. The animals were killed by decapitation and exsanguination.

Isolation of RNA. Total RNA was isolated from rat liver by the modified procedure of Chirgwin *et al.* [14] as described by Siegel and Bresnick [15]. Poly $(A)^+$ RNA was isolated by two consecutive passes over oligo(dT)-cellulose (Collaborative Research Inc., Waltham, MA), as described by Maniatis *et al.* [16]. Aliquots of the liver poly $(A)^+$ RNA were lyophilized and stored at −70°.

Northern blot analysis. One or two micrograms of poly $(A)^+$ RNA was denatured prior to loading on agarose gels as described [15]. Samples were placed on gels in loading buffer containing 50% glycerol (v/v), 1 mM EDTA, 0.4% bromphenol blue (w/v), and 0.4% xylene cyanol (w/v). RNA samples were size fractionated by electrophoresis under denaturing conditions on 1% (w/v) agarose gels (Ultrapure, Bethesda Research Laboratories, Gaithersburg, MD). The RNA fragment sizes were determined by comparing their mobility to end-labeled HindIII restriction endonuclease digests of lambda DNA. Gels were washed as previously described [15]; the RNA was then transferred to nitrocellulose paper

(BA-85, 0.45 μm pore diameter, Schleicher & Schuell Inc., Keene, NH), and the filters were baked in a vacuum oven at 80° for 2 hr. Lanes with lambda standards were cut from filters, and the latter were stored at 4° for autoradiography.

Prehybridization, hybridization, and washing conditions. Filters were prehybridized, hybridized, and washed using the diethylpyrocarbonate (DEPC)-treated nonfat milk conditions described by Siegel and Bresnick [15]. Filters were prehybridized for 2 hr in DEPC-treated BLOTTO (Bovine Lacto Transfer Technique Optimizer, Johnson *et al.* [17]) containing 0.25% (w/v) Carnation instant nonfat dry milk (0.05 × BLOTTO), 6 × SSC (SSC = 0.15 M NaCl–0.015 M sodium citrate), 50% (v/v) deionized formamide at 42°. Filters were probed with pA8, a 5.3 kb subclone, which encodes approximately 80% of the cytochrome P-450c gene [18], or pRSA57 which contains the 5′ coding information of the rat serum albumin gene [19] (obtained from Dr. James Bonner, California Institute of Technology, Pasadena, CA). DNA probes were radiolabeled by nick translation [20], the radiolabeled probe was then denatured by incubation at 37° for 10 min in 0.2 M NaOH, and then it was added to the hybridization solution. The latter was identical to the prehybridization solution except that it contained 2.5–10% dextran sulfate (w/v). All filters were prehybridized and hybridized in Seal-A-Meal bags in a shaking water bath at 42° for 16 hr. Filters were removed and washed (a) twice for 30 min at room temperature with a 0.05 × BLOTTO and 2 × SSC mixture that had been treated for 12–24 hr with 1% DEPC prior to boiling, and to which 0.1% (w/v) sodium dodecyl sulfate (SDS) was added after the solution was permitted to cool; and (b) twice for 30 min at 55° with 0.1 × SSC and 0.1% SDS. Filters were air-dried and placed in Seal-A-Meal bags, and autoradiography was carried out at −75° with Kodak X-Omat AR film in the presence of Dupont Cronex II intensifier screens. Intensity of hybridization was quantified with an LKB model 2202 UltraScan laser densitometer and gel scan computer software. All values were normalized to hybridization with pRSA57.

Rehybridization. Probes were removed from nitrocellulose filters by boiling twice for 15 min in DEPC-treated water. The filters were monitored for background radiation with a Geiger-Muller counter and were neutralized. Filters that were initially probed with pA8 were rehybridized with pRSA57.

Preparation of microsomes. Animals were killed by decapitation, and the livers were perfused *in situ* with cold 0.9% saline. Liver microsomes were prepared according to the conditions of Franklin and Estabrook [21], and enzyme activities were determined on microsomes that had been stored at −80° for up to 5 days in a 10 mM Tris, pH 7.4, 5 mM EDTA, and 10% (v/v) glycerol buffer. Microsomal protein concentrations were determined by the method of Lowry *et al.* [22].

Enzyme analysis. Cytochrome P-450 concentrations were determined from the absorbance difference between 450 and 490 nm upon addition of carbon monoxide gas (1-min exposure at approximately one bubble per sec) to sodium dithionite-reduced microsomes and quantified using an extinction coefficient of 91 $mM^{-1}\,cm^{-1}$, according to Omura and Sato [23] and Estabrook *et al.* [24].

The concentration of cytochrome b_5 was determined according to the method of Raw and Mahler [25] in which the difference between 427 and 500 nm was measured upon the addition of NADH; the concentration was quantified by using the extinction coefficient of 112 $mM^{-1}\,cm^{-1}$.

Ethoxyresorufin *O*-deethylase activity was measured by the method of Klotz *et al.* [26]. 7-Ethoxyresorufin (Pierce Chemical Co., Rockford, IL) was dissolved in methanol to make a stock solution of 400 mM which was stored at −80° in a light tight container for up to 4 weeks. The reaction mixture which contained 0.5 to 1.5 mg microsomal protein, 0.1 mM Tris–Cl, pH 8.0, 2 mM 7-ethoxyresorufin, 0.1 M NaCl was initiated by the addition of 0.5 mM NADPH. The reaction was carried out at 25°, observing the formation of resorufin at 572 nm, and quantified using the extinction coefficient of 73 $mM^{-1}\,cm^{-1}$. The data are expressed as nmol resorufin formed per mg microsomal protein per min.

RESULTS

Effect of phenobarbital pretreatment on induction by 3-methylcholanthrene of cytochrome P-450c mRNA. The steady-state level of transcription of the cytochrome P-450c gene was determined by hybridization of the plasmid pA8 to rat hepatic poly $(A)^+$ RNA. The intensity of the hybridization signal to radiolabeled pA8 was determined from autoradiograms by laser densitometry. With this probe under Northern analysis of poly $(A)^+$ RNA, both 2.7 and 2.0 kb species could be observed corresponding to rat hepatic cytochromes P-450c and P-450d respectively [27]. Rats given phenobarbital intraperitoneally for 1–4 days, followed by an injection of corn oil, produced little or no hybridization with the pA8 probe (Fig. 1). The poly $(A)^+$ RNA obtained from the livers of rats that had been treated with 3-methylcholanthrene intraperitoneally 15 hr prior to sacrifice revealed two hybridization signals, one at 2.7 kb and another less intense band at 2.0 kb. The relative intensity of hybridization of pA8 to hepatic poly $(A)^+$ RNA from rats treated with phenobarbital for 2 or more days prior to 3-methylcholanthrene administration was greater than the hybridization to poly $(A)^+$ RNA from rats treated solely with the polycyclic hydrocarbon (Fig. 1). The amount of rat serum albumin mRNA expressed in these livers is assumed to be constant. The amount of mRNA present in the membrane was determined after rehybridization of the filter membranes with a rat serum albumin gene probe pRSA57. This value was employed as an internal control of the amount of poly $(A)^+$ RNA loaded for gel electrophoresis and subsequently transferred.

The kinetics of phenobarbital pretreatment on 3-methylcholanthrene induction demonstrate that more than 1 day of phenobarbital treatment was required for a synergistic effect to be observed (Fig. 2). The intensity of the hybridization signal for both the 2.7 and 2.0 kb bands increased as a function of time beginning with 2 days of phenobarbital pretreatment. Cytochrome P-450c mRNA, i.e. 2.7 kb

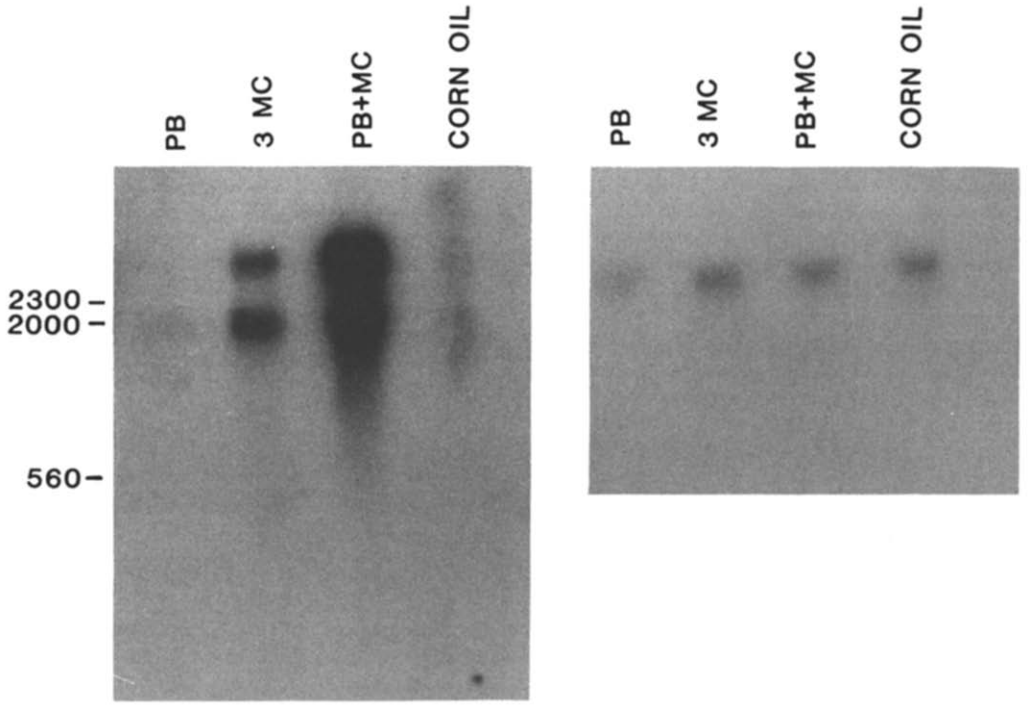

Fig. 1. Effect of phenobarbital pretreatment on the induction of cytochrome P-450c and P450d. Left, rats treated with phenobarbital daily for 4 days, PB; 3-methylcholanthrene, 15 hr, 3MC; combined phenobarbital and 3-methylcholanthrene, PB + MC; and corn oil, right lane. A total of 2 μg poly $(A)^+$ RNA probed with pA8 plasmid produced bands at 2.7 kb (cytochrome P-450c) and 2.0 kb (cytochrome P-450d). Right, the blot on the left was rehybridized with pRSA57, a plasmid containing rat serum albumin coding sequences. Molecular weight markers are indicated on the left.

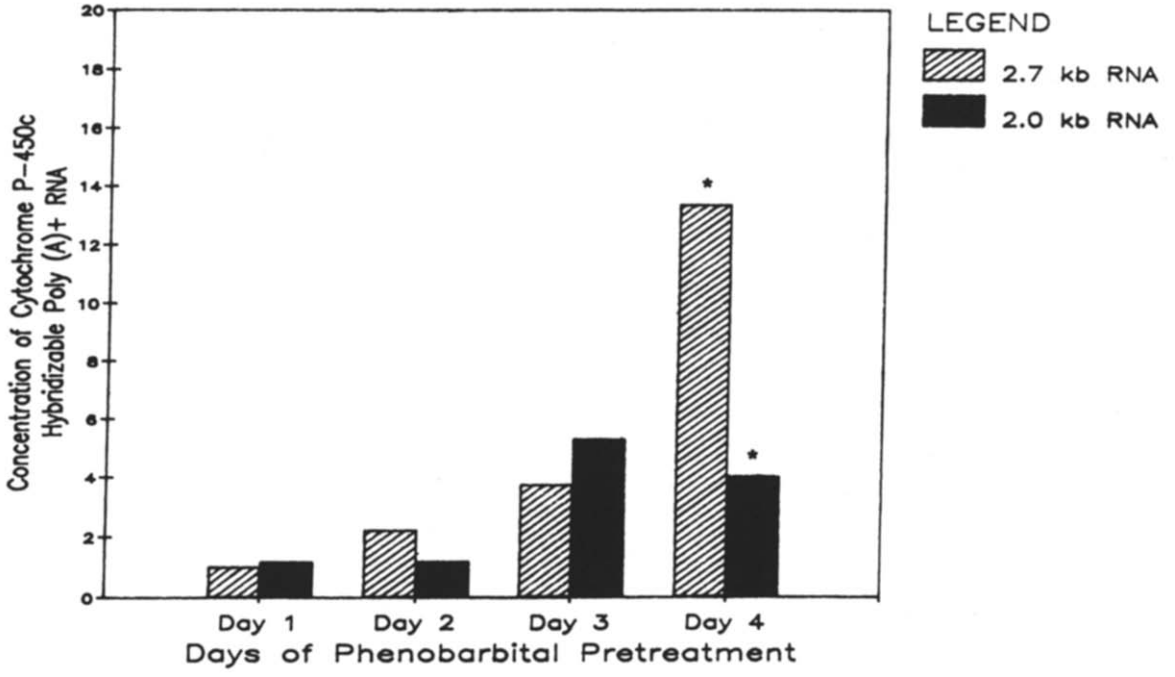

Fig. 2. Time courses of phenobarbital pretreatment on induction of cytochromes P-450c and P450d by 3-methylcholanthrene. The intensity of hybridization bands was determined by laser densitometry and standardized against rehybridization with pRSA57 (see Fig. 1). The values are reported as relative to 3-methylcholanthrene treatment alone. Bars marked with (*) indicate a statistically significant difference from 3-methylcholanthrene alone, $P < 0.01$.

Table 1. Metabolic effects of phenobarbital pretreatment on 3-methylcholanthrene induction in rats

Treatment	Cytochrome P-450 (nmol/mg protein)	Cytochrome b_5 (nmol/mg protein)	7-Ethoxyresorufin *O*-deethylase (nmol product/mg protein/min)
Saline (12)	0.644 ± 0.052	0.368 ± 0.071	0.238 ± 0.086
Phenobarbital (6)	1.41 ± 0.070*	0.209 ± 0.016	0.166 ± 0.046
3-Methylcholanthrene (8)	0.777 ± 0.063	0.311 ± 0.086	1.26 ± 0.333*
Phenobarbital and 3-methylcholanthrene (6)	1.53 ± 0.073*	0.376 ± 0.135	1.98 ± 0.641*

Results are given as means ± SE. The numbers in parentheses indicate the number of animals in that group.

* Statistically significant difference from control, $P < 0.005$.

species, was enhanced 5- to 24-fold with an average of 13-fold, whereas cytochrome P-450d, i.e. the 2.0 kb species, was increased 3- to 6-fold, with an average of 4-fold over 3-methylcholanthrene treatment alone.

Effect of phenobarbital pretreatment on cytochrome P-450. Treatment of rats with phenobarbital for 4 days induced the specific content of cytochrome P-450 from 0.64 in saline-treated animals to 1.41 nmol/mg microsomal protein, nearly 2.5-fold (Table 1), whereas no change in this parameter was observed after 3-methylcholanthrene treatment for 40 hr. In the combination, phenobarbital for 4 days followed by 3-methylcholanthrene for 40 hr, the specific content of cytochrome P-450 was elevated from 0.64 to 1.53 nmol/mg microsomal protein, a level significantly different ($P < 0.005$) from that observed after saline injection but not different from that noted after only phenobarbital. Although the specific content of microsomal cytochrome P-450 was not affected by 3-methylcholanthrene treatment, the total hepatic microsomal cytochrome P-450 (nmol/mg protein times total microsomal protein) did increase from 22.7 in saline-treated animals to 45.7 nmol. The total cytochrome P-450 was also elevated in the livers of animals that received either phenobarbital or the combined phenobarbital/3-MC treatment, to 98.8 and 117 nmol respectively. Neither the specific content nor the total cytochrome P-450 was synergistically induced by the combined treatment. The specific content of cytochrome b_5 did not change significantly following any of the treatment paradigms. This result was expected and is employed as an internal control.

The *O*-deethylation of ethoxyresorufin to resorufin is associated with rat hepatic cytochrome P-450c. This assay was employed to determine whether the levels of mRNA for cytochrome P-450c correlated with enzyme activity. Animals subjected to phenobarbital alone did not show any increase in hepatic 7-ethoxyresorufin *O*-deethylase over control in spite of the increase in cytochrome P-450 specific content. Both 3-methylcholanthrene and combined phenobarbital/3-methylcholanthrene treatments demonstrated significant elevation in *O*-deethylase activity, from 0.238 in saline controls to 1.26 in 3-methylcholanthrene-treated rats and 1.98 nmol/mg protein/min in the combined treatment group. While the cytochrome P-450c related enzyme activity in both 3-methylcholanthrene and combined treatment groups was significantly greater than in the saline controls, these two treatment groups were not significantly different from each other.

DISCUSSION

The data presented in this report support two distinct conclusions: first, pretreatment of rats with phenobarbital produces a profound synergy with 3-methylcholanthrene in the induction of cytochrome P-450c mRNA and, second, the elevated levels of mRNA do not correspond to an increase in active cytochrome P-450c enzyme activity. The reason for synergy between phenobarbital pretreatment and 3-methylcholanthrene induction is not currently understood although several possibilities arise. Induction of cytochrome P-450 by phenobarbital alone does not appear to involve gene amplification, increased transport of mRNA into the cytosol, or increase in mRNA translatability [28]; therefore, these mechanisms might not be expected to produce synergy with 3-methylcholanthrene. Phenobarbital has been shown to have no effect on the levels of albumin mRNA [29, 30]. More recently, phenobarbitone administration caused a decrease in levels of albumin mRNA [31]. Therefore, nonspecific stabilization of cytochrome P-450c mRNA by phenobarbital does not appear to be involved in the synergistic induction. Furthermore, we have shown an increase as a result of phenobarbital treatment in the 4S polycyclic hydrocarbon-binding protein which is present in rat hepatic cytosol [12, 13]. The increased binding protein could then result in more *trans*-regulation as a result of subsequent 3-methylcholanthrene treatment. However, neither of these mechanisms has been fully evaluated nor can they account for the lack of enzyme activity.

The amount of steady-state mRNA coding for cytochromes P-450c and P-450d does not correlate with the amount of active enzyme. It has been claimed that cytochromes P_1-450 and P_3-450 mRNA are induced following isosafrole without corresponding increase in enzyme activity [32], but these results may be complicated by the inhibitory action of isosafrole on the cytochrome P-450 enzymes. Translational control of mRNA has been proposed to involve a message-discriminatory initiation factor [33, 34]. This is supported by the non-translatability of some mRNA molecules *in vitro* unless they are first phenol-extracted [35]. However, extension of

this mechanism to the cytochrome P-450c case must account for the lack of any effect upon cytochrome b_5.

Preincubation of cultured rat fetal hepatocytes with dexamethasone acts synergistically with benzanthracene to induce cytochrome P-450c [36]. The combination produced a 1.6-fold increase in translatable cytochrome P-450c mRNA over benzanthracene alone. The synergy of dexamethasone and benzanthracene differs from combined phenobarbital and 3-methylcholanthrene in that no increase in cytochrome P-450c enzyme activity measured by 7-ethoxyresorufin *O*-deethylase could be detected. These results suggest that limitations such as limited capacity of the endoplasmic reticulum are not likely since other synergistic combinations of inducer can produce increased protein.

Finally, the mRNA for cytochrome P-450c and P-450d may be translated, but the latter process may not result in an active enzyme. The presence of apoprotein minus the heme moiety could account for our results. These data could implicate heme synthesis as a rate-limiting step in the synergistic action of phenobarbital on the synthesis of active cytochromes P-450c and P-450d. Details regarding the lack of correlation between mRNA level and active enzyme require further investigation as this complex regulatory scheme may have practical meaning in interpreting human risk and response to environmental contaminants.

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