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INDUCTION OF CYTOCHROME P450IA1 IN RAT HEPATOMA CELL BY POLYCYCLIC HYDROCARBONS AND A DIOXIN

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Abstract—In this study, the effects of benzo[*a*]pyrene (BP), 3-methylcholanthrene (3MC) and tetrachlorodibenzofuran (TCDBF) on the expression of the cytochrome P450IA1 gene in the rat hepatoma cell line H4IIE were examined. The initial rate of increase in the steady-state concentration of the mRNA for this gene was similar with each of these inducers; however, the elevated level of this mRNA was more sustained after TCDBF treatment. Nuclear run-off assays suggested that the elevated level of the mRNA was caused principally by an effect upon transcription.

The cytochrome P450-dependent monooxygenases are a family of isozymes that catalyze the biotransformation of many endogenous and exogenous substances [1, 2]. The cytochromes P450 are either constitutive or inducible, are ontogenically regulated, and in some cases hormonally controlled. Polycyclic hydrocarbons, such as 3-methylcholanthrene (3MC) and benzo[*a*]pyrene (BP), and dioxins, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), are potent inducers of a select subset of these hemoproteins, cytochromes P450IA1 and P450IA2. In addition, cytochrome P450IA1 is responsible for the activation of procarcinogenic forms of the polycyclic hydrocarbons, e.g. BP, to proximal carcinogenic species [3, 4]. These properties have spurred considerable interest in understanding the regulation of the expression of the polycyclic hydrocarbon-inducible cytochromes P450.

The gene structure and the nucleotide sequence of the rat cytochrome P450IA1 gene have been determined [5, 6]. Expression of the cytochrome P450IA1 gene appears to be regulated at both the transcriptional and post-transcriptional levels [7, 8]. There are two cytosolic proteins, a 4S component [9–11] which exhibits saturable, high-affinity interaction with polycyclic hydrocarbons such as BP and 3MC, and an 8S protein or *Ah* receptor that displays saturable, high-affinity interaction with dioxins and like congeners [12–14]. The 4S polycyclic hydrocarbon-binding protein and the *Ah* receptor interact with different 5′-upstream regions of the P450IA1 gene [15–19] and in some manner cause the increase in its transcription. In our laboratory, we have chosen the H4IIE rat hepatoma cell line as the host for transfection and for the study of the regulation of the cytochrome P450IA1 gene expression. We have now studied some of the details of induction of this gene by both polycyclic hydrocarbons, e.g. BP and 3MC, and certain dioxins, e.g. TCDD and tetrachlorodibenzofuran (TCDBF). In this manuscript, we report on the kinetics of induction of cytochrome P450IA1 by these substances.

MATERIALS AND METHODS

The following solutions were utilized: *guanidinium solution:* 4 M guanidinium thiocyanate–5 mM sodium citrate, pH 7.0–0.1 M β-mercaptoethanol–0.5% sarkosyl; *suspension buffer*: 10 mM Tris–HCl, pH 7.4–5 mM EDTA–1% sodium dodecyl sulfate (SDS); *NP-40 lysis buffer*: 10 mM Tris–HCl, pH 7.4–10 mM NaCl–3 mM $MgCl_2$–0.5% Nonidet P-40 (NP-40); *glycerol storage buffer*: 50 mM Tris–HCl, pH 8.3–5 mM $MgCl_2$–0.1 mM EDTA–40% glycerol; *2× reaction buffer with nucleotides*: 10 mM Tris–HCl, pH 8.0–5 mM $MgCl_2$–0.3 M KCl–mixture of 2 mM ATP, CTP and GTP–5 mM dithiothreitol (DTT)–5 mM spermidine. Chemicals used in this study were from the Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated.

Cell culture and induction protocol. The rat hepatoma cells, H4IIEs, were maintained at 37° in Eagle's Minimum Essential Medium (MEM) (GIBCO Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and 1 μg/mL gentamycin. The cells were passaged every third day after dislodgement with 0.25% trypsin. For induction, the cells were plated in T-150 culture flasks at a density of 10^7 cells/flask. When the cells were 60% confluent, they were refed a mixture of MEM–10% FBS–gentamycin containing GIBCO Opti-MEM® I(1:1, v/v) and were incubated for 24 hr at 37°. At this time, 10 mL of GIBCO Opti-MEM® I (obtained from GIBCO) containing a 1 μM concentration of either BP, 3MC, or TCDBF was added. In some cases, a comparable volume of only the vehicle, acetone, was added. Periodically thereafter, the cells were removed and RNA was extracted.

Isolation of total cellular RNA and Northern blot analysis. Total RNA was extracted from the cells by a slightly modified method of Chirgwin *et al.* [20].

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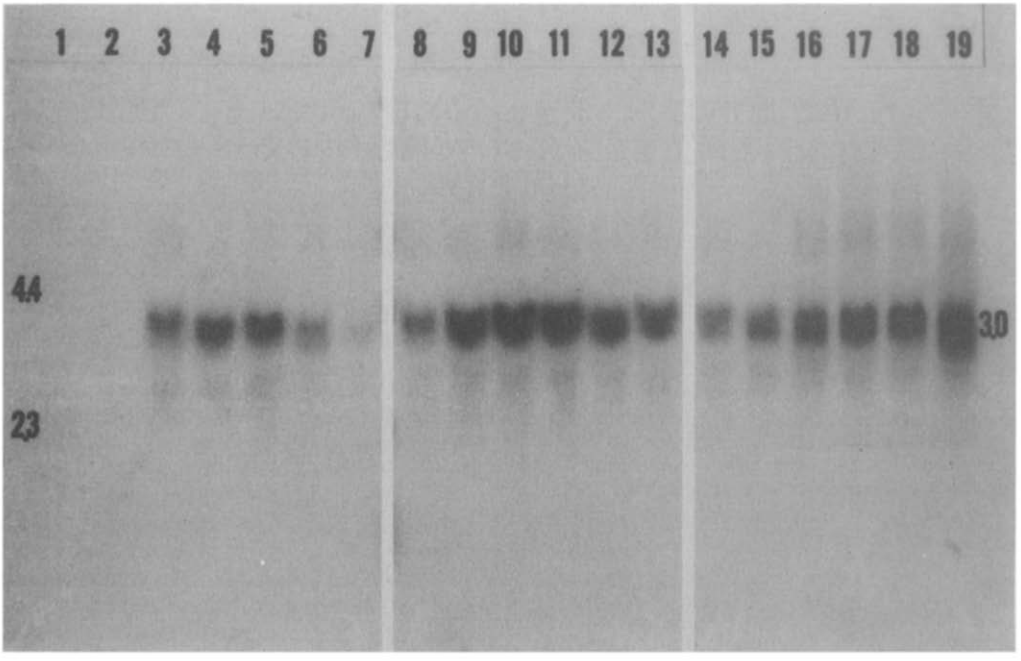

Fig. 1. Induction of cytochrome P450IA1 mRNA in H4IIE cells. H4IIE cells were incubated at 37° for 2–3 days prior to treatment with a 1 μM concentration of BP, 3MC or TCDBF. The total cellular RNA was isolated, electrophoresed through denaturing agarose gels, transferred to Nytran, and probed with pA8 as described in the text. The positions of two standards, 2.3 and 4.4 kb, are indicated in the figure; the position of the 3 kb mRNA for cytochrome P450IA1 is also indicated. Lanes 1 and 2, acetone treatment at 0 and 24 hr; lanes 3–7, BP treatment; lanes 8–13, 3MC; and lanes 14–19, TCDBF. The RNA was extracted after 1.5 hr, lanes 3, 8 and 14; 4 hr, lanes 4, 9 and 15; 8 hr, lanes 5, 10 and 16; 12 hr, lanes 6, 11 and 17; 24 hr, lanes 7, 12 and 18; and 48 hr, lanes 13 and 19. Although the autoradiographs are from one experiment, similar results have been obtained in a repeat.

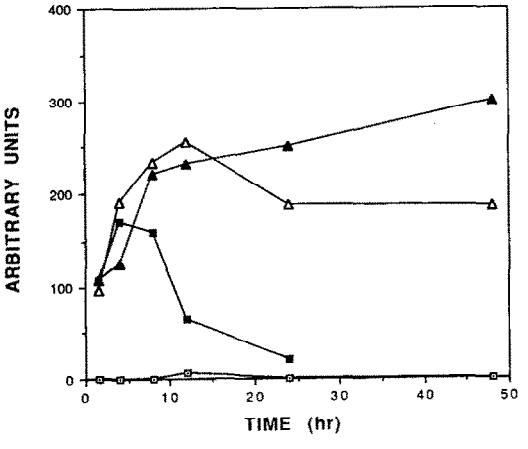

Fig. 2. Kinetics of induction of cytochrome P450IA1 in H4IIE cells. The Northern analysis of Fig. 1 was subjected to densitometry, and the data are presented in arbitrary densitometric units. Key: (□) control; (■) BP; (△) 3MC; and (▲) TCDBF.

After aspiration of the medium, cells were lysed by adding 2.5 mL of guanidinium solution directly into the flask. The flask was washed twice with 1.5 mL guanidinium solution. The cell lysate was pooled and CsCl was added to each sample to a final concentration of 0.4 g/mL. The lysate was then layered onto 1–2 mL of 5.7 M CsCl–0.1 M EDTA, pH 7.5. The total cellular RNA was obtained from these samples after centrifugation in a SW41 rotor at 32,000 rpm (120,000 *g*) at 20° for 21 hr. The RNA was redissolved and extracted with chloroform: 1-butanol (4:1, v/v). The RNA was precipitated from the aqueous phase by ethanol after the addition of sodium acetate (to 0.3 M). The pelleted RNA was redissolved and reprecipitated with sodium acetate–ethanol. The pelleted RNA was kept at −70° in sodium acetate–ethanol until utilized. At that time, 15 μg of total RNA was denatured by heat and electrophoresed through formaldehyde-1% agarose gels as described by Lehrach *et al.* [21]. The RNA was transferred to Nytran filters in 10×SSC (SSC = 0.15 M NaCl–0.015 M sodium citrate, pH 7.0) overnight as described [22], and the filters were air dried and baked *in vacuo* for 2 hr at 80°.

Prehybridization at 66° for 6 hr and hybridization at this temperature for 16 hr were conducted as described elsewhere [22] using a probe specific for cytochrome P450IA1 in these cells, pA8 [5]. The probe had been labeled to a specific activity of approximately 5×10^8 cpm/mg with [^{32}P]dCTP by the random primer technique [23]. The filter was washed with 2×SSC–0.5% SDS at room temperature for 5 min, 2×SSC–0.1% SDS at room temperature

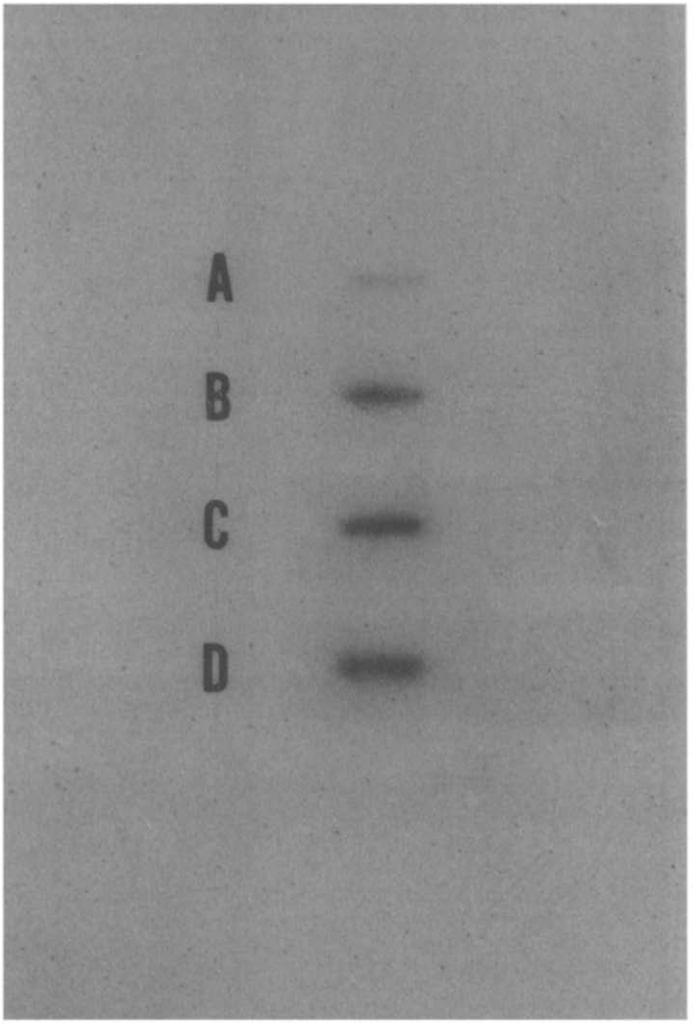

Fig. 3. Nuclear run-off assay with the H4IIE cells. The H4IIE cells were treated with 1 μM BP (B), 3MC (C) or TCDBF (D) and nuclei were isolated. The control group (A) received acetone alone. The nuclei were incubated with [^{32}P]UTP, and the run-off experiments were conducted as described in the text. The nuclear RNA products were prepared and applied to Nytran, and the latter was hybridized with pA8 as the labeled probe.

for 15 min, and finally with 0.1×SSC–0.5% SDS at 60° for 15 min. The filter was then exposed to X-ray film for 72 hr at −70° and developed.

Nuclear run-off assay. Nuclei were prepared from the treated and untreated H4IIE cells by the NP-40 lysis technique [24] and were frozen in liquid nitrogen. The frozen nuclei (from 10^8 cells) were thawed at room temperature, and 200 μL of 2× reaction buffer with nucleotides was added along with 10 μL of [α-^{32}P]UTP (760 Ci/mmol, 10 mCi/mL). The reaction mixture was incubated for 1 hr at 30°, the nuclei were pelleted at 15,000 *g* for a few seconds, and the supernatant fraction was aspirated to remove most of the unincorporated labeled nucleotide. Nuclear RNA was extracted as described by Chomczynski *et al.* [25] with only a slight modification. The nuclear RNA was precipitated twice with sodium acetate–ethanol, resuspended in water, and used in the hybridization experiments. For the latter, 10 μg of labeled pA8 was blotted onto a Nytran filter that had been prehybridized at 66°. The nuclear RNA was added and hybridization was allowed to continue for 50 hr at 66° in a scintillation vial. The filters were washed sequentially in 2×SSC–0.5% SDS at room temperature for 5 min, 2×SSC–0.1% SDS at room temperature for 10 min, 2×SSC–0.1% SDS at 60° for 30 min, and finally in 0.1×SSC–0.1% SDS at 60° for 15 min. The washed filters were placed in plastic bags and exposed to X-ray film.

RESULTS

Induction of cytochrome P450IA1. H4IIE cells were treated with BP, 3MC, or TCDBF and the steady-state level of cytochrome P450IA1 mRNA was assessed periodically. The Northern analysis is shown in Fig. 1 and the densitometric analysis in Fig. 2. In the absence of BP, 3MC, or TCDBF, no positive

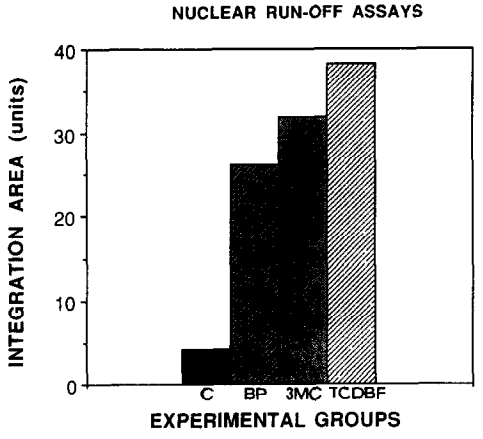

Fig. 4. Densitometric analysis of the nuclear run-off experiments. The treatments were as described in the legend to Fig. 3. The intensity of the hybridization signal is indicated in arbitrary densitometric units. Key: C, control; BP, benzo[*a*]pyrene; 3MC, 3-methylcholanthrene; and TCDBF, tetrachlorodibenzofuran.

signal was apparent in the H4IIE cells. However, as early as 1.5 hr after treatment with any of these substances a significant increase in the pA8-hybridizable mRNA was observed at a mobility of approximately 3 kb, the position for authentic P450IA1 mRNA. No positive signal was noted at 2 kb, the position for P450IA2 mRNA although pA8 does in fact cross-hybridize to the latter [5].

The induction of P450IA1 mRNA demonstrated different kinetics dependent upon the inducer (Fig. 1). This is more easily seen in the densitometric analysis of Fig. 2. Maximum steady-state activity after treatment of the cells with BP, 3MC, and TCDBF was observed at approximately 4, 12, and >48 hr. By 24 hr, the activity in the BP-treated H4IIE cells had returned almost to baseline, whereas that in the 3MC-treated cells was still approximately 75% of the peak level.

Nuclear run-off assay of P450IA1 transcription. To establish whether the increase in the steady-state level of P450IA1 mRNA after treatment with BP, 3MC, or TCDBF was caused by transcriptional or post-transcriptional events, we conducted nuclear run-off assays with pA8 as the hybridization probe. The nuclei were isolated at 2 hr prior to the time of maximum expression of cytosolic P450IA1 mRNA, i.e. at 2, 8, and 24 hr after BP-, 3MC- and TCDBF-treatment. The results of the nuclear run-off assays with pA8 as probe are shown in Figs. 3 and 4. The slot-blot analysis, i.e. Fig. 3, clearly shows a transcriptional effect which is quantitated in the densitometric analysis of Fig. 4. The rate of transcription was increased by 6 to 7-, 7 to 8-, and 9 to 10-fold after treatment of the H4IIE cells with BP, 3MC, and TCDBF respectively.

DISCUSSION

We used a rat hepatoma cell, the H4IIE, to evaluate the kinetics of induction of cytochrome P450IA1 gene expression. After administration of BP, or 3MC, or a dioxin, the process of induction was rapid with substantial increases in the steady-state level of the P450IA1 mRNA present within 1.5 hr of addition of these agents. That this steady-state level is reflective of the transcription rate may be inferred from the nuclear run-off experiments (at least at a time close to the maximum expression of the mRNA). It is also germane to mention that only P450IA1 expression was noted in these cells; P450IA2 mRNA was not present in either the control or treated cells.

Although the initial slope of increase in steady-state level of P450IA1 mRNA was similar after addition of the polycyclic hydrocarbons and TCDBF, activity fell off rapidly in the case of BP, much less so after 3MC, and not at all after TCDBF. The differences in these kinetics may be due to more rapid metabolism of the benzo[*a*]pyrene to noneffective substances and their extrusion from the cell. TCDBF may be much more resistant to metabolism and may be retained within the H4IIEs at a higher level than BP.

In regard to the regulation of P450IA1 gene expression, early literature [e.g. Refs. 26–28] suggested control exclusively at the transcriptional level since inhibitors of this process eliminated the increase in enzyme activity associated with this hemoprotein. The later literature [7, 29, 30] has indicated that post-transcriptional effects may also play a role. In our studies using nuclear run-off assays with the H4IIE cells, we have observed a 6 to 10-fold increase in transcription after administration of the polycyclic hydrocarbons or TCDBF. This fold increase, however, represents a very conservative estimate since we were only able to detect basal P450IA1 transcription activity at the margin of sensitivity. In the H4IIE cells, then, we would consider transcription as playing a major role in the induction of P450IA1 gene expression as a result of either polycyclic hydrocarbon or TCDBF exposure.

The dioxins with TCDD as the prototype agent induce P450IA1 gene expression in a number of systems, including cells, through the action of the *Ah* receptor, the 8S protein [reviewed in Ref. 17]. In our laboratory, we have found a closer association of the induction by polycyclic hydrocarbons, such as benzo[*a*]pyrene and 3-methylcholanthrene, of P450IA1 gene expression with the 4S polycyclic hydrocarbon-binding protein [e.g. Ref. 31]. We have also reported that with rat liver extracts, BP and to a large extent 3MC do *not* effectively compete with TCDD for binding to the 8S protein [31, 32]. Additionally, TCDBF did *not* effectively block the interaction of labeled BP to the 4S polycyclic hydrocarbon-binding protein; 3MC, on the other hand, is most potent in inhibiting this interaction [31, 32]. We have investigated the presence of both the 4S polycyclic hydrocarbon-binding protein and the 8S *Ah* receptor in our H4IIE cells* using both sucrose density gradient analysis and a hydroxyapatite assay. In neither case did we find any appreciable interaction of either BP or 3MC with an 8S protein;

* Reddy V, Raha A, Xu L-C, Houser WH and Bresnick E, Presence of the 4S PAH-binding protein in rat hepatoma H4IIE cells and its possible role in cytochrome P450IA1 induction. Manuscript submitted for publication.

only 4S binding was noted. As with the rat liver cytosol, TCDBF was not able to block the interaction of BP to this 4S protein. We also observed little specific interaction of labeled TCDD to any 8S protein in our H4IIE cells.

In summary, we have observed a profound induction of the cytochrome P450IA1 gene in H4IIE cells after the addition of BP, 3MC or TCDBF. This effect appeared to be due principally to an increased transcription. The elevated level of the mRNA for P450IA1 was sustained much longer in the TCDBF- and 3MC-treated cells.

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