MOLECULAR PHARMACOLOGY, 18, 550–552

Nucleolar Cytochrome *P*-450

EDWARD BRESNICK* BRUCE HASSUK,* PAUL LIBERATOR,* WAYNE LEVIN† AND PAUL E. THOMAS†

** Department of Biochemistry and The Vermont Regional Cancer Center, The University of Vermont College of Medicine, Burlington, Vermont 05405, and † Department of Biochemistry and Drug Metabolism, Hoffmann-La Roche Inc., Nutley, New Jersey 07110*

Received January 22, 1980; Accepted May 19, 1980

SUMMARY

BRESNICK, E., B. HASSUK, P. LIBERATOR, W. LEVIN AND P. E. THOMAS. Nucleolar cytochrome *P*-450. *Mol. Pharmacol.* **18:** 550–552 (1980).

Significant amounts of cytochrome *P*-450 have been detected spectrally in purified nucleoli obtained from the livers of 3-methylcholanthrene-treated rats. Using monospecific antibody to microsomal cytochrome *P*-450$_c$ (*P*-448), we have shown that the nucleolar and microsomal enzymes are immunochemically identical. These nucleoli also contained epoxide hydrolase and NADPH–cytochrome *c* reductase activities, both of which were immunochemically identical to their respective microsomal counterparts.

INTRODUCTION

A number of chemically inert polycyclic aromatic hydrocarbons, e.g., benzo[*a*]pyrene, require metabolic activation to proximate and ultimate carcinogenic forms by the concerted actions of a mixed-function oxidase system, i.e., cytochrome *P*-450 and epoxide hydrolase (1). These enzymes which are responsible for the oxidative metabolism of the procarcinogens reside predominantly within the endoplasmic reticulum. Cytochrome *P*-450 has been demonstrated to consist of multiple forms, each of which has a different but overlapping specificity for substrates (2, 3). Benzo[*a*]pyrene is most efficiently metabolized to reactive arene oxides primarily through the activity of cytochrome *P*-450$_c$ (*P*-448),[1] a form of the hemoprotein which is observed after pretreatment of rats with certain polycyclic hydrocarbons. The arene oxides thus produced are further metabolized to *trans* dihydrodiols in a reaction which is catalyzed by epoxide hydrolase which is also present in the endoplasmic reticulum (7). Subsequent metabolism of the *trans*-7,8-dihydrodiol of benzo[*a*]pyrene effected by the mixed-function oxidase system leads to the production of benzo[*a*]pyrene 7,8-diol-9,10-epoxide, an ultimate carcinogenic form of the polycyclic hydrocarbon (8, 9).

Although most of the cellular cytochrome *P*-450 and epoxide hydrolase resides in the endoplasmic reticulum, relatively recent results suggested the presence of the mixed-function oxidase system and epoxide hydrolase in isolated nuclear preparations (10–18). Indeed, we have recently reported the formation of the *trans*-7,8-dihydrodiol derivative and of the *syn* and *anti* diol epoxides of benzo[*a*]pyrene in isolated nuclear preparations (19, 20). Although some question about the contribution of microsomal contamination of the nuclear preparation to the overall mixed-function oxidase activity has been raised, the immunohistochemical demonstration of cytochrome *P*-450$_c$ *within* nuclei has appeared to finally settle this point (21). During the course of the latter studies, it was noted that cytochrome *P*-450$_c$ was present within the nuclei in two forms, referred to as MAD or macrodeposits and MID or microdeposits (21). The former resembled nucleoli in shape and form. The present study was undertaken to establish whether cytochrome *P*-450$_c$ was in fact present in nucleoli obtained from the livers of rats that had been pretreated with a polycyclic hydrocarbon.

MATERIALS AND METHODS

Male Sprague–Dawley rats, 80–100 g, were injected with 3-methylcholanthrene, 20 mg/kg, in corn oil on each of 2 successive days and were killed 24 h later. Livers were removed, washed in cold 0.9% saline, and homogenized in 2.3 M sucrose–10 mM $MgCl_2$. Nuclei were isolated from the heavy sucrose as outlined by Muramatsu and Busch (22) and nucleoli were prepared by the sonication method as described (22). Washed nucleoli were subjected to Ouchterlony immunodiffusion analysis (23) using monospecific antibody to rat liver cytochrome *P*-450$_c$ (23) and epoxide hydrolase (24). NADPH–cytochrome *c* reductase was purified by a modification of the method of Yasukochi and Masters (25) and antibody was prepared as previously described (26).

The nucleolar preparation was free from membrane contamination as judged by both light and electron microscopy. Furthermore, the contribution of potential mi

These studies were supported in part by a grant from The National Cancer Institute (CA 20711) to Dr. E. Bresnick.

[1] The major form of hepatic microsomal cytochrome *P*-450 after pretreatment of rats with 3-methylcholanthrene has been termed cytochrome P_1-450 (4), cytochrome *P*-448 (5), and more recently, cytochrome *P*-450$_c$ (6).

0026-895X/80/060550-03$02.00/0

crosomal contamination to the nucleoli was determined in the following manner. Male 60-g rats were injected intraperitoneally with L-[4,5-^{3}H]leucine (5.16 Ci/mmol), 67 μCi/50 g body wt 2 h prior to sacrifice. Microsomes were isolated from liver homogenates in 0.05 M Tris (pH 7.5)–1.15% KCl, and the microsomal fraction was washed with 1.15% KCl–10 mM EDTA and suspended in 0.25 M sucrose. The specific activity of the final microsomal preparation was 4.54×10^4 dpm/mg protein. The labeled microsomes, 6.5 mg protein representing 2.96×10^5 dpm, were added to a 20% liver homogenate (16 g wet wt liver) in heavy sucrose prior to the ultracentrifugation step in the preparation of nuclei. The isolation of nucleoli was conducted as described above and the "contamination" with labeled microsomes was ascertained. The final nucleolar preparation (36 mg protein) contained 29 dpm of radioactive protein. This corresponds to 43 μg of total microsomal protein (assuming 1 g wet wt liver contains 25 mg microsomal protein) and indicates that microsomal contamination of nucleoli is <0.2% of the total protein. Consequently, it is concluded that the microsomal content of cytochrome *P*-450$_c$, epoxide hydrolase, and NADPH–cytochrome *c* reductase does *not* contribute to the results obtained with our nucleolar preparations.

The nucleolar preparation was assayed for RNA polymerases I, II, and III to determine maximum contamination of nucleoli with other nuclear protein (RNA polymerases II and III). RNA polymerase was assayed by a modification of the method of Rose *et al.* (27) using endogenous nuclear template in the absence or presence of 10^{-4} M α-amanitin. This concentration of α-amanitin blocks the activity of both RNA polymerases II and III but has no effect on polymerase I activity (28, 29). Results of these studies indicated that 6% of the total nucleolar RNA polymerase activity was present as RNA polymerases II and III. Based on the activity of the RNA polymerases in nuclei, this represents less than 10% contamination of nucleoli with other nuclear protein.

RESULTS AND DISCUSSION

The cytochrome *P*-450 content of liver nucleoli was determined from the CO-binding spectrum (30) (Fig. 1). The maximum CO-binding spectrum occurred at 448.5–449 nm. The average of five cytochrome *P*-450 determinations in liver nucleoli from induced rats is 0.18 ± 0.02 nmol/mg protein.

Previous data from our laboratory (31) have shown that rat liver nuclear cytochrome *P*-450$_c$ and epoxide hydrolase are immunologically identical to the microsomal enzymes. In the present study, we attempted to establish the relationship of nucleolar cytochrome *P*-450 and epoxide hydrolase to their microsomal counterparts by Ouchterlony double diffusion analysis. The results are presented in Fig. 2. These results clearly demonstrate the immunological identity of microsomal cytochrome *P*-450$_c$ and epoxide hydrolase with the nucleolar-derived antigens. During the course of these experiments, we were also able to establish the presence in nucleoli of NADPH–cytochrome *c* reductase activity. Immunochemically, NADPH–cytochrome *c* reductase can also be detected in nucleoli and the enzyme is immunochemically identical to the microsomal-derived antigen as shown in Fig. 3.

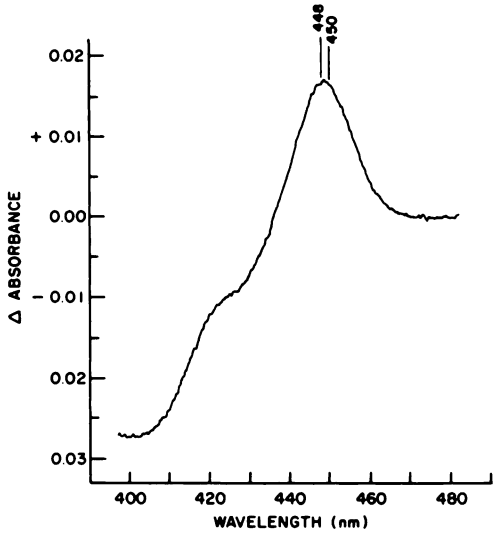

FIG. 1. *CO-reduced difference spectrum of isolated rat liver nucleoli*

Nucleoli were isolated from the livers of rats treated with 3-methylcholanthrene (20 mg/kg) at 24 and 48 h before killing. The spectrum was recorded on an Aminco DW-2 spectrophotometer in the split-beam mode after bubbling CO in the sample cuvette and then adding sodium dithionite to both the sample and the reference cuvettes. The wavelength accuracy was checked against a holmium oxide filter. The nucleoli were homogenized in 0.1 M potassium phosphate buffer (pH 7.4), 20% glycerol, 0.2 mM EDTA, and 1 mM phenylmethylsulfonylfluoride at a final protein concentration of 1.5 mg/ml. The specific content of *P*-450 in this preparation was 0.14 nmol/mg protein.

Thus, all the enzymes necessary for metabolic activation of polycyclic aromatic hydrocarbons such as benzo[*a*]pyrene to potent mutagenic and carcinogenic metabolites are present in hepatic nucleoli.

Although mixed-function oxidases and epoxide hydrolase were once considered to reside exclusively within the endoplasmic reticulum, it is now relatively well estab-

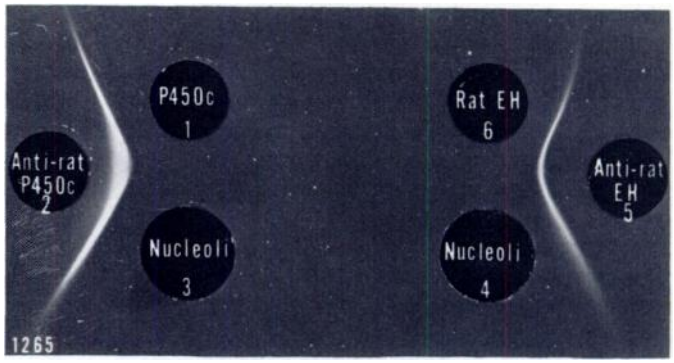

FIG. 2. *Ouchterlony immunodiffusion analysis of isolated rat liver nucleoli using antibody monospecific for rat liver microsomal enzymes, cytochrome P-450$_c$ (anti-rat P450$_c$), and epoxide hydrolase (anti-rat EH)*

Nucleoli were isolated from the livers of rats that had been treated with 3-methylcholanthrene (20 mg/kg) at 24 and 48 h before killing. The solubilized nucleoli (approx 1.4 mg/ml) were in a mixture consisting of 0.3% Emulgen 911, 1.5% sodium cholate, 20% glycerol, and 1 mM phenylmethylsulfonylfluoride. Wells 1 and 6 contained 12 μl of purified hepatic microsomal cytochrome *P*-450$_c$ (1 nmol/ml) and epoxide hydrolase (63 μg/ml), respectively. Wells 3 and 4 were 5 mm in diameter and the rest of the wells were 4 mm in diameter.

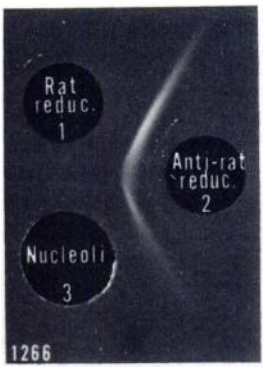

FIG. 3. *Ouchterlony immunodiffusion analysis of isolated rat liver nucleoli using antibody against rat liver microsomal NADPH–cytochrome c reductase (well 2)*

The nucleoli were detergent solubilized as described in Fig. 2 and used at the same concentration. Well 1 was filled with 12 μl purified rat liver microsomal NADPH–cytochrome *c* reductase (67 μg/ml). Well 3 was 5 mm in diameter, whereas the rest of the wells were 4 mm in diameter.

lished that the nucleus can also catalyze both mixed-function oxidase and epoxide hydrolase activities. The present study extends this observation to include the *nucleolus*. This study also serves to identify nucleoli as the intranuclear cytochrome P-450$_c$-containing macrodeposits described in our earlier immunohistochemical study (21). The functional significance of these three enzymes in an organelle known to play an important role in ribosomal RNA processing is presently unknown.

REFERENCES

1. Levin, W., A. W. Wood, A. Y. H. Lu, D. Ryan, S. West, A. H. Conney, D. R. Thakker, H. Yagi and D. M. Jerina. Role of purified cytochrome P448 and epoxide hydrase in the activation and detoxification of benzo[a]pyrene, in *Drug Metabolism Concepts* (D. M. Jerina, ed.). ACS Symposium Series 44, American Chemical Society, Washington, D.C., 99–126 (1977).
2. Lu, A. Y. H. and W. Levin. The resolution and reconstitution of the liver microsomal hydroxylation system. *Biochim. Biophys. Acta* **344:** 205–240 (1974).
3. Coon, M. J., J. L. Vermilion, K. P. Vatsis, J. S. French, W. L. Dean and D. A. Haugen. Biochemical studies on drug metabolism: Isolation of multiple forms of liver microsomal cytochrome P450, in *Drug Metabolism Concepts* (D. M. Jerina, ed.). ACS Symposium Series 44, American Chemical Society, Washington, D.C., 46–72 (1977).
4. Sladek, N. E. and G. J. Mannering. Evidence for a new P-450 hemoprotein in hepatic microsomes from methylcholanthrene treated rats. *Biochem. Biophys. Res. Commun.* **24:** 668–674 (1966).
5. Alvares, A., G. Schilling, W. Levin and R. Kuntzman. Studies on the induction of CO-binding pigments in liver microsomes by phenobarbital and 3-methylcholanthrene. *Biochem. Biophys. Res. Commun.* **29:** 521–526 (1967).
6. Ryan, D. E., P. E. Thomas, D. Korzeniowski and W. Levin. Separation and characterization of highly purified forms of liver microsomal cytochrome P-450 from rats treated with polychlorinated biphenyls, phenobarbital and 3-methylcholanthrene. *J. Biol. Chem.* **254:** 1365–1374 (1979).
7. Jerina, D. M., J. W. Daly, B. Witkop, P. Zaltzman-Nirenberg and S. Udenfriend. Role of the arene oxide-oxepin system in the metabolism of aromatic substrates. I. *In vitro* conversion of benzene oxide to a premercapturic acid and a dihydrodiol. *Arch. Biochem. Biophys.* **128:** 176–183 (1968).
8. Kapitulnik, J., P. G. Wislocki, W. Levin, H. Yagi, D. M. Jerina and A. H. Conney. Tumorigenicity studies with diolepoxides of benzo[a]pyrene which indicate that (±)-trans7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene is an ultimate carcinogen in newborn mice. *Cancer Res.* **38:** 354–358 (1978).
9. Buening, M. K., P. G. Wislocki, W. Levin, H. Yagi, D. R. Thakker, H. Akagi, M. Koreeda, D. M. Jerina and A. H. Conney. Tumorigenicity of the optical enantiomers of the diastereomeric benzo[a]pyrene 7,8-diol-9,10-epoxides in newborn mice: Exceptional activity of (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene. *Proc. Nat. Acad. Sci. USA* **75:** 5358–5361 (1978).
10. Kasper, C. B. Isolation and properties of the nuclear envelope. *Methods Enzymol.* **31:** 279–292 (1974).
11. Khandwala, A. S. and C. P. Kasper. Preferential induction of aryl hydroxylase activity in rat liver nuclear envelope by 3-methylcholanthrene. *Biochem. Biophys. Res. Commun.* **54:** 1241–1246 (1973).
12. Rogan, E. G. and E. Cavalieri. 3-Methylcholanthrene-inducible binding of aromatic hydrocarbons to DNA in purified rat liver nuclei. *Biochem. Biophys. Res. Commun.* **58:** 1119–1126 (1974).
13. Vaught, J. and E. Bresnick. Binding of polycyclic hydrocarbons to nuclear components *in vitro*. *Biochem. Biophys. Res. Commun.* **69:** 578–591 (1976).
14. Pezzuto, J. M., M. A. Lea and C. S. Yang. Binding of metabolically activated benzo[a]pyrene to nuclear macromolecules. *Cancer Res.* **36:** 3647–3653 (1976).
15. Bresnick, E., J. B. Vaught, A. H. L. Chuang, T. A. Stoming, D. Bockman and H. Mukhtar. Nuclear aryl hydrocarbon hydroxylase and interaction of polycyclic hydrocarbons with nuclear components. *Arch. Biochem. Biophys.* **181:** 257–269 (1977).
16. Alexandrov, K., P. Brookes, M. W. S. King, M. R. Osborne and M. H. Thompson. Comparison of the metabolism of benzo[a]pyrene and binding to DNA caused by rat liver nuclei and microsomes. *Chem. Biol. Interact.* **12:** 269–277 (1976).
17. Bornstein, W. A., H. Chuang, E. Bresnick, H. Mukhtar and J. R. Bend. Epoxide hydrase activity in liver nuclei: Hydration of benzo[a]pyrene 4,5-oxide and styrene oxide. *Chem. Biol. Interact.* **21:** 343–354 (1978).
18. Jernstrom, J., H. Vadi and S. Orrenius. Formation in isolated rat liver microsomes and nuclei of benzo[a]pyrene metabolites that bind to DNA. *Cancer Res.* **36:** 4107–4113 (1976).
19. Bresnick, E., T. A. Stoming, J. Vaught, D. R. Thakker and D. M. Jerina. Nuclear metabolism of benzo[a]pyrene and of (±)-trans-7,8-hydroxy-7,8-dihydrobenzo[a]pyrene. *Arch. Biochem. Biophys.* **183:** 31–37 (1977).
20. Bresnick, E., A. H. L. Chuang and W. A. Bornstein. Benzo[a]pyrene metabolism in neonatal rat liver nuclei. *Chem. Biol. Interact.* **24:** 111–115 (1979).
21. Bresnick, E., D. Boraker, B. Hassuk, W. Levin and P. E. Thomas. Intranuclear localization of hepatic cytochrome P448 by an immunochemical method. *Mol. Pharmacol.* **16:** 324–331 (1979).
22. Muramatsu, M. and H. Busch. Isolation, composition and function of nucleoli of tumors and other tissues, in *Methods in Cancer Research* (H. Busch, ed.). Academic Press, New York, Vol. II, 303–359 (1967).
23. Thomas, P. E., D. Korzeniowski, D. Ryan and W. Levin. Preparation of monospecific antibodies against two forms of rat liver cytochrome P-450 and quantitation of these antigens in microsomes. *Arch. Biochem. Biophys.* **192:** 524–532 (1979).
24. Levin, W., P. E. Thomas, D. Korzeniowski, H. Seifried, D. M. Jerina and A. Y. H. Lu. Liver microsomal epoxide hydrase: Activation, inhibition and immunological properties of the purified enzyme. *Mol. Pharmacol.* **14:** 1107–1120 (1978).
25. Yasukochi, Y. and B. S. S. Masters. Some properties of a detergent-solubilized NADPH-cytochrome c (cytochrome P-450) reductase purified by biospecific affinity chromatography. *J. Biol. Chem.* **251:** 5337–5344 (1976).
26. Thomas, P. E., A. Y. H. Lu, D. Ryan, S. B. West, J. Kawalek and W. Levin. Immunochemical evidence for six forms of rat liver cytochrome P-450 obtained using antibodies against purified rat liver cytochrome P-450 and P-448. *Mol. Pharmacol.* **12:** 746–758 (1976).
27. Rose, K. M., P. A. Ruch and S. T. Jacob. Mechanism of inhibition of RNA polymerase II and poly (adenylic acid) polymerase by the O-n-octyloxime of 3-formylrifamycin SV. *Biochemistry* **14:** 3598–3604 (1975).
28. Lindell, T. J., F. Weinberg, P. W. Morris, R. G. Roeder and W. J. Rutter. Specific inhibition of nuclear RNA polymerase II by α-amanitin. *Science* **170:** 447–449 (1970).
29. Weinman, R. and R. G. Roeder. Role of DNA-dependent RNA polymerase III in the transcription of the tRNA and 5SRNA genes. *Proc. Nat. Acad. Sci. USA* **71:** 1790–1794 (1974).
30. Omura, T. and R. Sato. The carbon monoxide-binding pigments of liver microsomes. *J. Biol. Chem.* **239:** 2370–2378 (1964).
31. Thomas, P. E., D. Korzeniowski, E. Bresnick, W. A. Bornstein, C. B. Kasper, W. E. Fahl, C. R. Jefcoate and W. Levin. Hepatic cytochrome P-448 and epoxide hydrase: enzymes of nuclear origin are immunochemically identical with those of microsomal origin. *Arch. Biochem. Biophys.* **192:** 22–26 (1979).

Send reprint requests to: Edward Bresnick, Department of Biochemistry, The University of Vermont College of Medicine, Burlington, Vt. 05405.