Bioenergetics (1974), 6, 151-160

Induction of Cytochrome P-450 and P-448 in the Outer Membrane of Mouse Liver Nuclei by Phenobarbital and Benzo[α-]pyrene

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Received 13 May 1974

Abstract

This investigation confirms the presence of the inducible mixed function hydroxylase enzyme system in nuclear membranes. The cytochrome P-450 spectrum and demethylase activity, markers of the enzyme system, were used to define its localization to the outer membrane envelope. Intact BALB/c mouse liver nuclei isolated and purified in Mg^{++} sucrose media of low ionic strength gave CO-dithionite reduced difference spectra of cytochrome P-450 and P-448. Phenobarbital induced P-450 by 40% while the carcinogenic hydrocarbon, benzo[α] pyrene, induced P-448 twofold. A corresponding increase was also observed in the microsomes of the same tissue preparations. No microsomal contamination of nuclear preparations was found. Intact nuclei stripped of their outer membrane by 0.5% Triton X-100 treatment resulted in a striking absence of the P-450 which, however, was found to be present in isolated outer nuclear membranes.

Introduction

The molecular mechanism by which polycyclic hydrocarbons induce cancer is unknown. However, polycyclic hydrocarbons applied to the skin of mice become covalently bound to protein [1, 2, 3] and to DNA

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of the treated tissue [4, 5], and a correlation between the degree of binding to the DNA and carcinogenic activity has been demonstrated by Brookes and Lawley [5]. Mammalian tissues contain aryl hydrocarbon hydroxylase, an inducible, drug-metabolizing system capable of metabolizing carcinogenic polycyclic hydrocarbons. However, the polycyclic hydrocarbon or its hydroxylated products are apparently not carcinogenic, but the intermediate metabolite or reactive species can bind to target molecules, such as DNA [6] as has been demonstrated *in vitro* [7].

The enzyme system is localized outside of the nucleus in the smooth and rough-surfaced endoplasmic reticulum [8, 9], the golgi [9] and the inner membrane of the adrenal cortex mitochondria [10]. Whether a reactive intermediate can survive long enough to be transported from these sites to the nuclear DNA is not known. Yet, Bach's and Johnson's findings of microsome-like activity in the nuclei of HeLa cells [11] led Gelboin to postulate that this compartmentalized enzyme system may be responsible *in vivo* for the chemical reactions leading to the DNA polycyclic hydrocarbon complexes.

Aryl hydrocarbon hydroxylase components, NADPH cytochrome-*c* reductase [12, 13], cytochrome b_5 and P-450 [14, 15] have been found in the nuclear membrane of rat liver. In these studies, 3-methylcholanthrene, but not phenobarbital, induced P-450 formation [16]. In all instances, the activity was less than that observed in the microsomal membrane fraction, from which it was concluded that the enzyme system in the nuclear envelope may be under a different control from that of the microsomal membrane. Using phenobarbital and the carcinogen, benzo[α]pyrene, to induce this enzyme system, the present study sought to determine its localization in the nucleus of another animal, BALB/c mice, and to define specifically which of the two nuclear membranes contains the mixed function hydroxylase enzyme system.

Materials and Methods

Animals

Adult male and female inbred BALB/c mice, average weight of 25 g, were obtained from the Cancer Research Genetics Laboratory, University of California, Berkeley. The animals were housed in plastic cages with sawdust bedding and fed Purina Lab Chow and tap water *ad libitum*. Animals were fasted 24 h prior to sacrifice (by cervical dislocation), then bled and the liver perfused with 0.25 M sucrose, 0°. The liver tissue was removed, freed of the gall bladder, rinsed and held in 0.25 M sucrose, 0°, until used. The livers of 10-12 mice were pooled for one experiment giving 10-14 g of tissue.

Chemicals

Benzo[α]pyrene was obtained from K & K Laboratories, Inc, Plaineview, New York 11803, and phenobarbital from Eastman Kodak Laboratories, New York.

Enzyme Induction

Daily intraperitoneal injections of 40 mg/kg body weight of phenobarbital in 0.9% NaCl or 20 mg/kg body weight of benzo[α] pyrene in corn oil (Mazola) were given for five days. Control animals received equal amounts of either 0.9% NaCl or corn oil.

Isolation Procedures

Nuclei were prepared by modifying the procedures of Widnell and Tata [17] and Whittle, *et al.* [18]. Liver tissue was minced with scissors, rinsed twice at 0-4°C with Medium I consisting of 0.33 M sucrose and 3 mM $MgCl_2$, and homogenized with two parts of this medium to one part tissue in a loose-fitting Potter-Elvehjem-type homogenizer with a Teflon pestle of 0.025 inch clearance. Following 25 up-and-down strokes of the pestle turning at 1200 rpm, the homogenate was filtered through nylon cloth (110 mesh) to remove debris. Seven parts Medium I were added to the filtrate and centrifuged at 850 g for 10 min in a Sorvall RC2B centrifuge (SS34 rotor). The crude nuclear pellet was resuspended in Medium I in a Potter-Elvehjem homogenizer and then diluted to 45 ml with Medium II which contained 2.4 M sucrose and 1 mM $MgCl_2$. The final sucrose concentration was 1.6 M.

The nuclei were purified on a discontinuous sucrose gradient consisting of 6 ml of Medium II, 4 ml of 1.8 M sucrose plus 1 mM $MgCl_2$ sucrose, 15 ml of nuclear suspension in 1.6 M sucrose-1 mM $MgCl_2$ and 5 ml of Medium I in a 30 ml tube by centrifuging in the SW 25.1 rotor of a Spinco model L preparative ultracentrifuge at 90,000 g for 3 h. The white nuclear pellet was further purified by resuspension in Medium I, placed over 6 ml of Medium II and centrifuged 2 h at 100,000 g in a number 40 rotor. The pellet was resuspended in 10 ml of Medium I and centrifuged 5 min at 3000 rpm. The final nuclear pellet was then resuspended in 2-3 ml of Medium I.

Removal of Outer Nuclear Membrane

A modified method of Blobel and Potter [19] was used to remove the outer nuclear membrane. Triton X-100, 0.5%, was added to the nuclear suspension (10 mg protein/ml 0.25 M sucrose medium containing 3 mM $MgCl_2$) and gently swirled for 30 sec, which was sufficient to strip off the outer nuclear membrane (see Results).

Microsomes

After removal of the nuclear fraction from the homogenate, the supernatant fraction was centrifuged 15 min at 30,000 g (SS34 rotor). The resulting supernatant was centrifuged in a number 40 rotor for 1 h at 100,000 g. The pellet was suspended in 0.25 M sucrose to a protein concentration of 30 mg/ml.

Cytochrome P-450, P-448 Assay

Difference spectra were recorded at room temperature in a Cary model 14 spectrophotometer. The amounts of cytochrome P-450 and P-448 were estimated from the CO-difference spectrum of dithionite-reduced membranes. Values E-450/490 mM = 91 [20] and E-448/490 mM = 95 [9] were used to calculate the cytochrome concentration.

Oxidative Demethylation of p-nitroanisole

Spectrophotometric assay of p-nitrophenol formed at 410 nm was carried out at 23° in a 3 ml reaction mixture which contained 50 mM Tris-HCL pH 7.4, 150 mM KCL, 3 mM $MgCl_2$, 2 μmoles p-nitroanisole, 1 μmole NADPH and isolated nuclear or microsomal preparations.

Protein Assay

Protein was determined by the method of Gornal, *et al.* [21] using Bovine serum albumin as a standard.

Electron Microscopy

Nuclei, outer nuclear membrane, and microsomal preparations were examined as previously reported by conventional transmission electron microscopy in a Siemens Elmscope I [22].

Results

Aryl Hydrocarbon Hydroxylase and Cytochrome P-450 and P-448

This study first established that the aryl hydrocarbon hydroxylase enzyme system was present and inducible in the microsomal membranes of the liver, as evidenced by an increase in the terminal oxidase, cytochrome P-450 or P-448 content following five daily intraperitoneal injections of phenobarbital or benzo[α]pyrene, respectively (Table I). Additional evidence of the induction was demonstrated by an increase in the oxidative demethylation of p-nitroanisole (Table I).

TABLE I. Induction of Cytochrome P-450 and Oxidative Demethylation in Microsomal and Nuclear Fractions

	Microsomes		*Nuclei*	
Animal Treatment	Cytochrome P-450 (μmoles/μg protein)	Demethylation (μmoles/min/mg protein)	Cytochrome P-450 (μmoles/μg protein)	Demethylation (μmoles/min/mg protein)
None	0.835 (4)[a]	0.6 (6)	0.012 (4)	0.12 (3)
Phenobarbital	1.340 (3)	1.2 (3)	0.017 (2)	0.20 (2)
Benzo[α] pyrene	1.535 (3)[b]	1.5 (4)	0.025 (3)	0.30 (3)

[a] Number of experiments in brackets
[b] Cytochrome P-448 induction

To show that the nucleus contains this enzyme system, intact mouse liver nuclei were isolated and assayed for P-450. The CO-difference spectrum clearly showed that the nuclear preparation contained P-450 (Fig. 1) and P-420. Phenobarbital-injected animals showed a 40% increase in P-450 during the five-day induction period whereas benzo[α]pyrene-treated mice demonstrated a similar increase in P-448, characteristic of the endoplasmic reticulum and golgi [9]. Furthermore, as in the microsomal preparations, the inducible oxidative demethylase

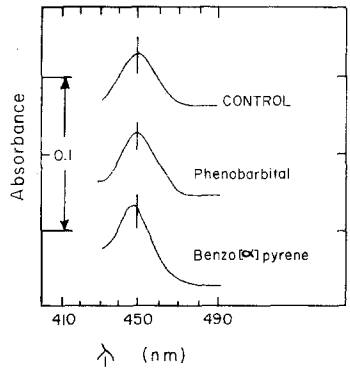

Figure 1: Comparison of dithionite-reduced CO-difference spectra of mouse liver nuclear fractions in control phenobarbital- and benzo[α]pyrene-injected animals.

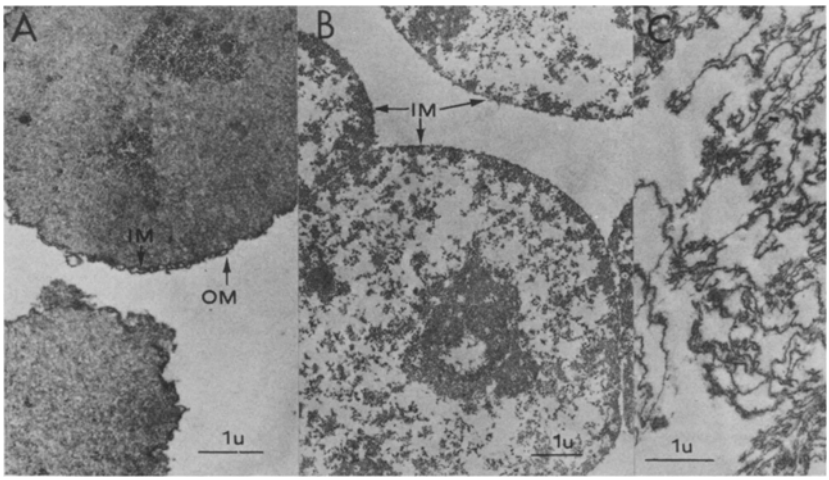

Figure 2: Electron micrographs of isolated liver nuclei. (A) intact (B) treated with 0.5% Triton X-100, to remove (C) outer membrane. Arrows indicate inner (IM) and outer (OM) nuclear membranes.

in the nuclei almost doubled control values following phenobarbital and benzo[α]pyrene injection (Table I).

Tests for Contamination of the Nuclear Fraction

The outer nuclear membrane is frequently observed to be continuous with the endoplasmic reticulum, fragments of which may thus become absorbed to the surface of the nuclei during homogenization. To test for such contamination, we conducted a cross-mixing experiment in which the nuclear fraction from phenobarbital-induced mice containing cytochrome P-450 was mixed with the supernatant fraction obtained from the homogenate of benzo[α]pyrene-treated animals, which have cytochrome P-448 in the microsomes. After incubating for 10 min at 0°, the nuclei were reisolated from the mixture as described previously, and P-448 and P-450 content was determined. Conversely, nuclei from benzo[α]pyrene-treated mice containing cytochrome P-448 were mixed with enriched P-450 microsomes from the original supernatant fraction of phenobarbital-treated animals. Again, nuclei were isolated and assayed for cytochrome content. The CO-difference spectrum of both nuclei preparations was found to be unaltered. As a further test, purified microsomal fractions and liver homogenates (minus the first nuclei and debris pellet) were individually tested by cross-mixing and re-isolation, confirming that the outer nuclear envelope remained uncontaminated by microsomal membranes.

Effect of Triton Treatment

Because Triton X-100 in excess can induce loss of activity in microsomal membranes, the effects of treatment were assessed. As

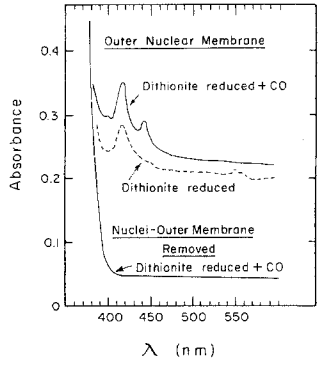

Figure 3: Absorption spectrum of outer nuclear membranes and CO-difference spectrum of a nuclear suspension stripped of the outer membrane by 0.5% Triton X-100 treatment.

Triton X-100 concentration per unit protein was increased, light scattering and demethylase activity decreased, indicating membrane alteration (Fig. 4). The CO-difference spectrum showed that with an increase in Triton concentration, P-450 in microsomes and in nuclei was converted to P-420. Based on these findings and on electron microscopic examination of nuclear preparations following Triton X-100 treatments, the conditions of outer nuclear membrane removal were determined as indicated in Methods.

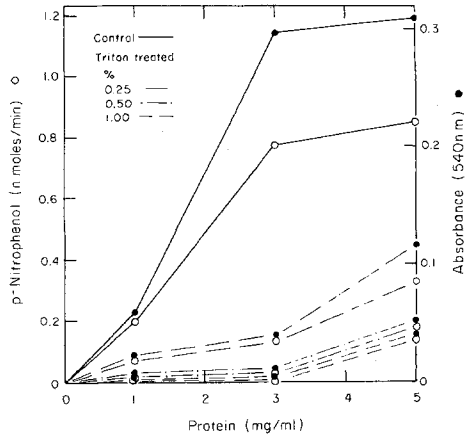

Figure 4: Effect of Triton X-100 on microsomal enzyme activity by assay of oxidative demethylation of p-nitroanisole to p-nitrophenol and on the structure of the membranes as assayed by absorbance change at 540 nm. Triton X-100 was added to 2 ml of mouse liver microsomal suspensions and assayed after mixing.

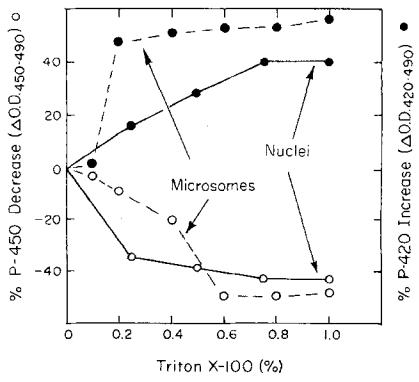

Figure 5: The effect of Triton X-100 on microsomal and nuclear P-450 and P-420 CO-difference spectra.

Localization in Outer Nuclear Envelope

Electron microscopic examination confirmed that the membrane was stripped from the nucleus leaving the inner membrane intact (Fig. 2). Absorption spectrum of the nuclei stripped of outer membrane showed no P-450 or P-448 which, however, were recovered in a 100,000 g pellet (Fig. 3).

Discussion

This study confirms the presence of the cytochrome P-450 in mouse liver nuclei as in rat liver, and demonstrates that the enzyme system capable of metabolizing chemical carcinogens is inducible by phenobarbital and benzo[α]pyrene, the former as evidenced by P-450 and the latter by P-448 formation. In addition, we have shown that this enzyme system is in close proximity to nuclear DNA (specifically in the outer nuclear membrane as was postulated by Gelboin) and that carcinogens covalently bound to DNA in situ may arise from an intermediary metabolite formed by the aryl hydrocarbon hydroxylase enzyme system.

Other investigations have reported differences between microsomal and nuclear membranes in terms of the amount of P-450 present and the specific activity of the enzyme system [12-16, 23]. We believe these differences may be attributable to methodological factors. For example, the nuclear membranes are subjected to more stress than the microsomal membranes in that the osmotic differential to which these organelles are subjected (ie, sucrose gradients) the exposure to larger volumes of isolation medium per unit of membrane protein, and the environment to which they are exposed during their separation and purification are quite different. With respect to the latter, the ionic strength of the medium in which nuclear "ghosts" or nuclear membranes have been isolated by others varies, and, in some cases, may have caused membrane alteration. (Our assessment of the effects of Triton X-100 demonstrated how easily alterations can be effected in the CO-binding cytochrome content of the nuclear envelope.) In any event, the resulting purified membranes very likely would not contain all of the components of the in situ membrane. For example, as shown here, the labile membrane-bound form of P-450 is easily converted to the membrane-free form, P-420, by membrane alteration. Consequently, the amounts of cytochrome P-450 and P-448 may vary from preparation to preparation, simply as a function of the relative harshness of treatment during purification. These factors may account for the published observations that purified nuclear preparations do not always manifest the presence of P-450 in difference spectra [16].

Although the percent protein [24, 14] and esterified cholesterol [25] content is higher in the nuclear than in the microsomal fraction, the

significance of these factors—which may enhance or inhibit the formation and/or transport of a polycyclic hydrocarbon as a reactive intermediate for the DNA—warrants consideration.

Acknowledgements

We are grateful to Professor K. B. DeOme, Cancer Research Genetics Laboratory, University of California, Berkeley, for supplying the BALB/c mice and chemical carcinogens, and to the Cancer Co-ordinating Committee, University of California, for partial support of this research.

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