BENZPYRENE HYDROXYLASE ACTIVITY IN ISOLATED PARENCHYMAL AND NONPARENCHYMAL CELLS OF RAT LIVER

ELROY CANTRELL and EDWARD BRESNICK

From the Department of Cell and Molecular Biology, Medical College of Georgia, Augusta, Georgia 30902

ABSTRACT

Previous studies have implicated the reticuloendothelial cells of the liver in certain aspects of steroid metabolism. The similarity in the metabolism of steroids and polycyclic hydrocarbons suggested that the nonparenchymal cells possibly play a role in these areas. The present study presents evidence that at least one of the microsomal NADPH-requirig enzymes, benzpyrene hydroxylase, is present in nonparenchymal cells and, furthermore, is "inducible." In adult rats treated with 3-methylcholanthrene or β-naphthoflavone, the nonparenchymal cells exhibited increases in benzpyrene hydroxylase activity of 17-fold and five-fold, respectively. Treatment with phenobarbital resulted in only a slight increase in enzyme activity. Enzyme activity in parenchymal cells under similar conditions was increased sixfold and fivefold by 3-methylcholanthrene and β-naphthoflavone, respectively, but not by phenobarbital.

INTRODUCTION

Mammalian liver is a heterogeneous tissue composed of approximately 60–70% parenchymal cells and 30–40% nonparenchymal cells. Cells of the reticuloendothelial (RE) system make up the greatest portion of the nonparenchymal population. Although parenchymal cells have been presumed to be the major cell type involved in drug metabolism, evidence that the nonparenchymal cells play a role has accumulated in recent years. Berliner et al. (1) noted that the RE cells of liver and of adrenal cortex were involved in the biotransformation of steroids. Zymosan, a stimulant of the RE system, was found by Sawyer et al. (2) to enhance the clearance of injected doses of corticosterone and to increase the A-ring reduction of steroids by liver homogenates. The latter biotransformation is found principally in RE cells (3). Evidence that acetylation of sulfonamides was confined to the nonparenchymal cells and was absent from parenchymal cells (P cells) of liver was provided by Govier (4). The finding of DiCarlo et al. (5) that a relationship existed between the stimulation of phagocytic activity of the RE system and the metabolism of barbiturates further implicated the nonparenchymal cells in drug metabolism. While it is clear that hepatic parenchymal cells are a principal site of metabolism of polycyclic hydrocarbons, many tissues in the body have been shown to oxidize these compounds (6, 7). Yet, the oxidation of polycyclic hydrocarbons by the nonparenchymal cells of liver has not been demonstrated. Additional information was obviously required regarding the enzymatic activity of nonparenchymal cells so that the function of these cells in liver might be more clearly understood. A large number of reports

(reviewed in 7–9) have shown that the drug-metabolizing enzymes are induced by (*a*) polycyclic hydrocarbons, e.g., 3 methylcholanthrene (3MC) and (*b*) barbiturates, e.g., phenobarbital. It is not clear from these studies, however, if the induction is only characteristic of the parenchymal cell population in liver.

The aims of the present study were (*a*) to isolate parenchymal and nonparenchymal cells from the same liver preparation, to establish the presence of benzpyrene (BP) hydroxylase in nonparenchymal cells, and (*c*) to ascertain the relative responses of the enzyme in parenchymal and nonparenchymal cells to the administration of inducers, i.e. 3MC, β-naphthoflavone (BNF), and phenobarbital (PB).

MATERIALS AND METHODS

Materials

Collagenase (type 1), hyaluronidase (type 1), and BP were obtained from Sigma Chemical Co., (St. Louis, Mo.); pronase B was purchased from Calbiochem (Los Angeles, Calif.). Eastman provided 3MC, while BNF was obtained from Aldrich Chemical Co., Inc., (Milwaukee, Wis.). 8-Hydroxybenzo (a) pyrene (8-hydroxy–BP) which was used in the calibration of the enzyme assay was kindly supplied by Dr. H. V. Gelboin of the National Cancer Institute.

Isolation of Cells

P cells and nonparenchymal cells were prepared from 200 g male rats by a combination of the method of Berry and Friend (10) (for the P cells) and the method of Mills and Zucker-Franklin (11) (for the nonparenchymal cells). Rats were injected intraperitoneally with 3MC or BNF in corn oil (20 mg/kg) 48 hr before sacrifice or intraperitoneally with phenobarbital (35 mg/kg, twice daily) for 3 days before sacrifice. Control rats were treated with vehicle alone. On the days of sacrifice each animal was anesthetized with ether and injected intravenously with 1000 units of heparin to facilitate perfusion. The liver was quickly excised and the portal vein was catheterized. The liver was perfused with Krebs-bicarbonate solution, which was gassed with O_2:CO_2 (95%:5%) at 25°C until blanched completely. The perfusion apparatus was then adjusted so that the gassed perfusate could be recirculated through the liver. A concentrated solution of hyaluronidase and collagenase was added to the perfusion medium to obtain a final concentration of 50 mg% of each in a volume of 250 ml. The perfusion was continued for 30–40 min, after which time the liver was removed from the apparatus and diced in a dish over ice. A portion of approximately 1 g was taken for the preparation of parenchymal cells and was gently shaken in 10 ml of the hyaluronidase and collagenase solution for 60 min under O_2:CO_2. After straining through 64-mesh nylon screen, the P cells were collected and washed with Hanks' balanced salt solution (BSS) followed by centrifugation at 30 *g*. The P cells were suspended in 50 mM Tris, pH 7.5, containing 3 mM $MgCl_2$ (Tris-Mg) and homogenized in a glass-Teflon homogenizer at 4°C. Nonparenchymal cells were present in the P-cell preparation to the extent of less than 5%.

The remaining tissue was stirred vigorously on a magnetic stirrer in 50 ml of 0.1% pronase in Hanks' BSS for at least 1 hr at 25°C, a procedure which caused disruption of most of the P cells (11). The nonparenchymal cells were washed in Hanks' BSS by centrifugation at 300 *g*. After two to three washes in 40 ml of Hanks' BSS, the pellet tended to sediment in three layers. The top gelatinous matrix containing a few small cells could be easily removed with a Pasteur pipette. The nonparenchymal cells along with a few erythrocytes were found in the middle layer. P cells, when present, sedimented as a tan button at the bottom of the tube and could be removed with a pipette. P cells were present in the nonparenchymal population to the extent of less than 0.05%.[1] The cells were suspended in Tris-Mg and homogenized as described above. The nonparenchymal cells were identified by their size and by the presence of carbon particles [after injecting a rat intravenously with colloidal carbon (4 mg/100 g) 24 hr before sacrifice.] After their isolation, most of the nonparenchymal cells were found to contain phagocytized particles of carbon. A few cells did not take up carbon, perhaps due to the difference in phagocytic threshold in vivo.

BP Hydroxylase Activity

Enzyme activity was determined under reduced light conditions by a modification of the method of Nebert and Gelboin (12). Triplicate 0.5 ml portions of homogenate (0.5–1.0 mg protein) were placed into test tubes with 0.1 ml NADPH (1.0 mg) on ice. BP was added to all tubes (0.1 μmole) and the tubes were

[1] In later preparations, it was found that nonparenchymal cells could be obtained in even higher purity, without loss of activity, by treating the washed pellet with three to four strokes in a Dounce homogenizer in a volume of 10 ml followed by resuspending in 0.1% pronase. The thick gelatinous matrix could be broken up by addition of 0.1 mg of deoxyribonuclease I with shaking. Nonparenchymal cells could then be sedimented and washed in Hanks' BSS. Erythrocytes may be eliminated by suspending the pellet in cold 5 mM $MgCl_2$, sedimenting, and resuspending in buffer.

placed in a shaking incubator for 15 min at 37°C. The reaction was terminated by placing the tubes in ice water and adding 0.5 ml cold acetone. Acetone was added to the blanks before incubation. Hexane, 2.0 ml, was added to each tube which was then placed on a vortex mixer for 20 sec. The tubes were centrifuged in a table-top centrifuge and 1.9 ml of each organic phase was transferred to 2.0 ml of 1.0 N NaOH and vortex-mixed for 15 sec. After centrifugation, portions of the aqueous phase of each tube were transferred to cuvettes for the determination of fluorescence in an Aminco-Bowman spectrophotofluorometer with the excitation and emission wavelengths set at 396 and 522 nm, respectively. The fluorescence was compared with that of quinine sulfate (excitation and emission wavelengths, 352 nm, 452 nm) which had been previously calibrated with authentic 8-hydroxybenzo(a)pyrene in order to quantitate the products of the reaction. One unit of activity represents the fluorescence equivalent to a picomole of 8-hydroxy-BP produced per minute per milligram of protein. Protein content of the homogenates was estimated by the method of Lowry et al. (13).

Histochemical Enzyme Demonstration

The highly sensitive method of Wattenberg and Leong (14) was employed to examine the hydroxylase activity in individual cells of the parenchymal and nonparenchymal preparations. Air-dried smears of the cell suspensions were fixed in cold acetone and dried. BP, 0.02 μg/ml in hexane, was added to each slide (2–3 drops per slide) in a cold room. Tris-Mg containing 0.1 mg/ml NADPH was layered over each slide in a shallow tray, the tray was incubated for 2–5 min at 37°C, and the slides were immediately fixed in 10% neutral Formalin, rinsed in 80% ethanol, and dried. It was found that the rinse in 80% ethanol effectively removed most of the unreacted benzpyrene without affecting the amount of hydroxylated products. The slides were kept under reduced light throughout. Immediately before examination, a drop of 1.0 N NaOH was placed on a slide and a coverslip was added. High-speed Ektachrome film recorded the green fluorescence of the cells, indicating the presence of oxidized metabolites of BP. Unincubated controls exhibited no green fluorescence.

RESULTS

90% of the P cells were found to exclude trypan blue (Fig. 1 a). The presence of nonparenchymal cells in this preparation was about 5% as estimated from hematoxylin- and eosin-stained smears. The appearance of the P cells was similar to that of P cells obtained by others (10, 15), and their diameter was approximately 20 μ.

Nonparenchymal cells were obtained in high purity (Fig. 1 b) and were considerably smaller than the P cells; the former had diameters of 10–15 μ. Those nonparenchymal cells that were prepared from rats previously injected with col-

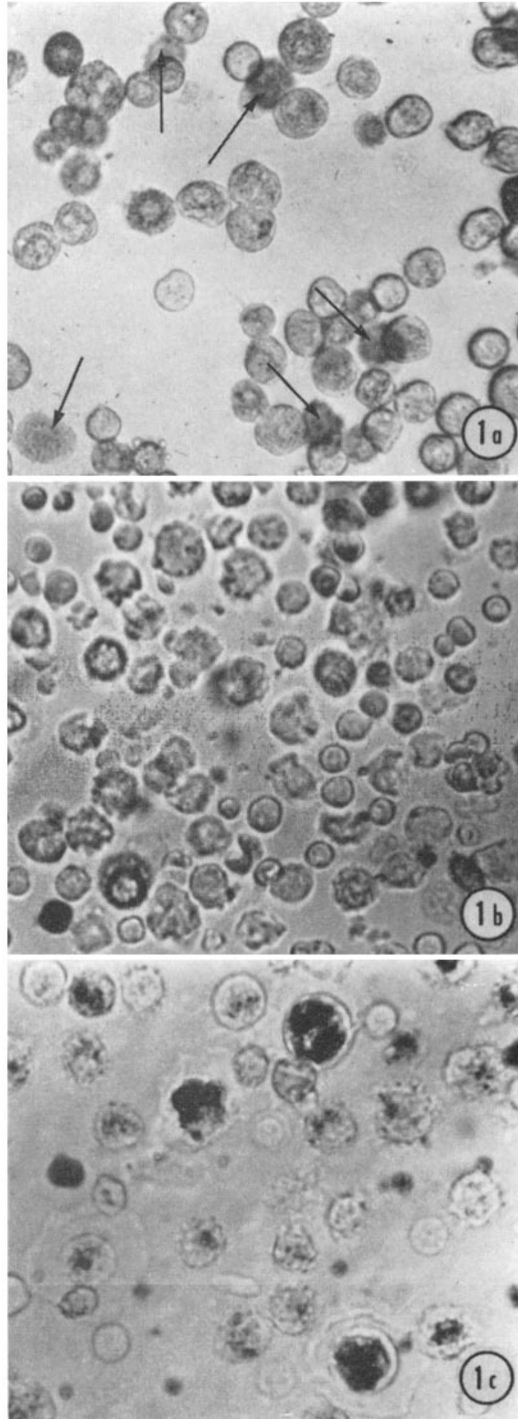

FIGURE 1 Light micrographs of hepatic parenchymal and nonparenchymal cells. Wet mounts of suspensions of cells. Fig. 1 a, Parenchymal cells; the arrows denote those cells which did not exclude the trypan blue. × 250. Fig. 1 b, Nonparenchymal cells. × 400. Fig. 1 c, nonparenchymal cells from rat injected with colloidal carbon. Note different amounts of carbon in the cells. × 500.

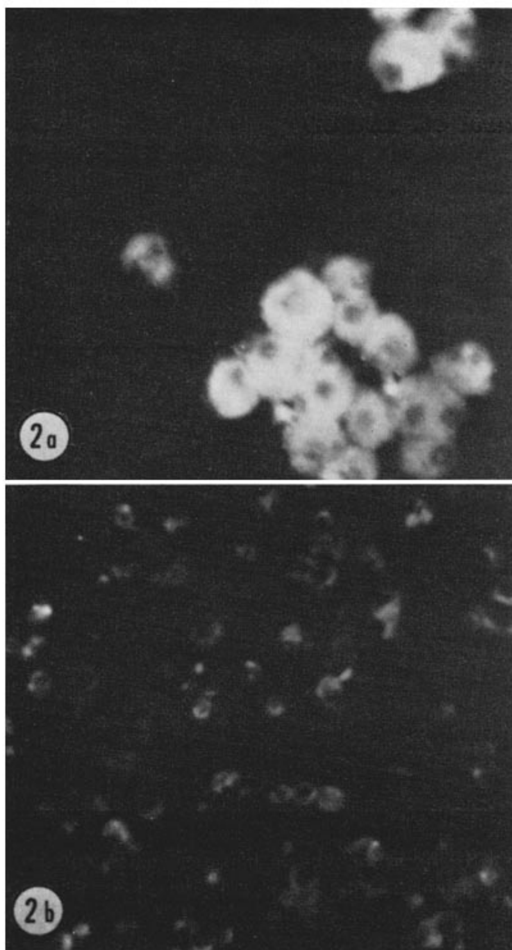

FIGURE 2 Demonstration of fluorescence of BP hydroxylase in P cells (Fig. 1 a) and nonparenchymal cells (Fig. 1 b). P cells and nonparenchymal cells were prepared as described in the text and were assayed for BP hydroxylase by the histofluorescence technique (12). The bright green fluorescence indicates the presence of oxidized metabolites of BP. × 400.

loidal carbon have an appearance similar to that reported by Rous and Beard (16). While their preparation selected only cells of low phagocytic threshold, cells with even small amounts of carbon are seen in the preparation shown in Fig. 1 c.

The activity of BP hydroxylase in the cell preparations was compared by two techniques, histofluorometry and spectrophotofluorometry. In Fig. 2, it can be seen that both cell preparations were able to oxidize BP as denoted by the fluorescence. Activity in the P cells seemed much greater than that in the nonparenchymal cells, as suggested by the relative intensities. Cell preparations fixed in Formalin without incubation (unincubated controls) exhibited no green fluorescence (the photograph was totally black).

BP hydroxylase was measured in homogenates of the P cells and nonparenchymal cells under conditions where the activity was linear with respect to time (15 min) and was proportional to protein concentration (up to 2 mg per tube). The quantitative comparison of enzyme activity in both cell types is presented in Table I. As suggested by the histofluorometric demonstration, P cells were found to possess greater activity than the nonparenchymal cells, i.e., approximately 13-fold greater.

Having demonstrated the presence of BP hydroxylase in both cell populations, we considered it of interest to ascertain if both responded equally well (if at all) to the administration of "inducers", i.e., 3MC, BNF, and PB. These results are also shown in Table I. Both cell types showed greater activity when the animals were treated with 3MC or BNF before isolation of cells. Activity in P cells was increased by six-fold and four-fold after injection of 3MC and BNF to rats, respectively. Nonparenchymal cells appeared more responsive to the inducing agents, with increases of 17-fold and five-fold, respectively. Phenobarbital was not

TABLE I

Effect of 3MC, BNF, and PB on BP Hydroxylase Activity in P Cells and Nonparenchymal Cells

	BP hydroxylase (units)	
Group	P Cells	Nonparenchymal cells
Control	24 ± 8*	1.8 ± 1.0
3MC	175 ± 66 (<0.03)‡	32.2 ± 8.9 (<0.005)
BNF	103 ± 15 (<0.001)	11.1 ± 2.7 (<0.006)
PB	28 ± 9 (<0.4)	3.1 ± 1.7 (<0.3)

Both cell types were isolated and were assayed for BP hydroxylase activity as described in the text. Rats had been pretreated with either 3MC or BNF (20 mg/kg) 48 hr previously, or with PB (three times daily for 3 days, 35 mg/kg). Control rats were pretreated with corn oil alone. Enzyme activity is expressed as units, where one unit represents the fluorescence equivalent to a picomole of 8-hydroxy-BP/min per mg protein.

* Values are expressed as mean ±SEM with five animals per group.

‡ The p values are given in parentheses.

Table II

Effect of Pronase on BP Hydroxylase in P Cells and Cell Fragments

Sample	BP hydroxylase* (units)
P cells	72
P cells + pronase	62
250 *g* supernatant	104
250 *g* supernatant + pronase	0

Whole liver was homogenized in 0.25 M sucrose, and the 250 *g* supernatant was incubated at 25° C for 30 min in the presence or absence of 0.05% pronase, then assayed in duplicate for BP hydroxylase activity. Intact P cells (derived in the livers of 3MC-treated rats) were allowed to stand at 25°C for 30 min in the presence or absence of 0.05% pronase, washed, homogenized, and assayed in duplicate for BP-hydroxylase. Rats were pretreated with 3MC as described in the text.

* One unit represents fluorescence equivalent to a picomole of 8-hydroxy-BP/min per mg protein.

an effective inducer of hydroxylase activity in P cells although the nonparenchymal cell population seemed more responsive; the latter results were of borderline significance.

The question arose as to the contribution of the limited "contamination" of the nonparenchymal cells with intact parenchymal cells or fragments thereof (less than 0.5%). The whole cell contamination could not account for the values obtained in Table I. However, there was a possibility that fragmented parenchymal cells could contribute the BP hydroxylase activity seen in the nonparenchymal cells. This situation could only arise if the fragmented cells were resistant to the action of pronase which was employed in the preparation of the nonparenchymal cell population. Accordingly, a broken cell population, i.e., 250 *g* supernatant of a whole liver homogenate, was incubated in the presence of pronase, and the activity of BP hydroxylase was subsequently assayed. The results, depicted in Table II show that although enzyme activity in intact isolated parenchymal cells was unaffected by this treatment, the activity in fragmented cells was destroyed by the pronase treatment. Consequently, the activity of BP-hydroxylase, reported in Table I, was truly a property of the nonparenchymal cells and not simply due to contamination of the latter preparation with either whole or fragmented parenchymal cells.

DISCUSSION

Nonparenchymal cells of the liver have been implicated in the metabolism of a variety of drugs. Most reactions which have been found in this cell have been of the reductive or conjugative types. Govier (4) reported that the acetylation of sulfonamides and of p-amino-benzoic acid was localized in these cells and that no acetylating activity could be detected in the parenchymal cells. Reduction of the steroid A ring was found by Berliner and Dougherty (3) to be confined to Kupffer cells in liver. Other reactions found in nonparenchymal cells included reduction of pentaerythritol tetranitrate (17), triphenyltetrazolium (18), and ferritin (19). Hydroxylation of the 17-α position of progesterone has been reported to be a function of the RE cells (1). These data suggested that nonparenchymal cells may have microsomal enzyme pathways which could possess different substrate specificity as compared with liver parenchymal cells. Wooles and Borzelleca (20) showed that the RE stimulant, zymosan, caused a decrease in metabolism of barbiturates. A direct effect of zymosan on P cells has not been ruled out, and obviously this approach to the study of the relationship of the RE system to drug metabolism requires further study. In contrast to the inhibition of barbiturate metabolism reported above (20), Sawyer et al. (2) demonstrated that zymosan caused an increase in the clearance of injected doses of corticosterone in rats and an increase in A-ring reduction by liver homogenates.

A more direct relationship between the metabolism of drugs and nonparenchymal cells is suggested by the results reported in this paper. Thus, it has been definitively established by two techniques that BP hydroxylase is present in nonparenchymal liver cells although to a much lesser extent than in parenchymal cells. Furthermore, BP hydroxylase activity can be significantly elevated in the former by previous administration to rats of 3MC or BNF. It may be calculated that less than 1% of the polycyclic hydrocarbon would be metabolized by the enzyme present in the nonparenchymal cells. However, the nonparenchymal cell types may provide the "first line of attack" since their presence in specified regions of the liver would allow their early interaction with and response to circulating polycyclic hydrocarbon.

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