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INHIBITION BY 17-α-ETHINYL ESTRADIOL OF BENZO[*a*]-PYRENE METABOLISM IN ISOLATED ADULT RAT HEPATOCYTES

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Abstract—The effects of 17-α-ethinyl estradiol [EE] were studied on benzo[*a*]pyrene (BP) metabolism and covalent binding to DNA and on some enzymes involved in the activation/inactivation pathway. Suspensions of hepatocytes freshly isolated from adult rats either untreated or pretreated with phenobarbital or methylcholanthrene were used as an experimental model closer to the *in vivo* situation than subcellular fractions. The formation of water-soluble and of organic metabolites of benzo[*a*]pyrene was inhibited by EE at 10^{-4} M in hepatocytes from untreated rats and at 10^{-3} M in those isolated from rats pretreated with phenobarbital. Dihydrodiols were the main class of metabolites affected by EE. The pretreatment of rats with 3-methylcholanthrene rendered hepatocytes insensitive to the effect of EE. The effect of EE on BP metabolism was well correlated to its inhibitory effect on BP metabolite binding to DNA in untreated and phenobarbital-treated rats. These data could not however be related to a quantitative decrease in cytochrome P-450 of hepatocytes incubated in the presence of EE. Epoxide hydratase, UDP-glucuronyl-transferase and glutathione *S*-transferase activities were lowered by 36, 27 and 19% respectively in the presence of 10^{-4} M EE.

The impairment of BP metabolism by EE might be due to a functional alteration specific for cytochrome P-450, or to a non-destructive mechanism and/or to a competition of both chemicals for a number of enzymatic pathways common to their metabolism.

Synthetic estrogens are widely used as human therapeutic and contraceptive agents. In the past few years, various adverse effects of the common estrogenic component of some oral contraceptives, i.e. 17-α-ethinyl estradiol (EE)† have been reported. They involve general liver pathology and, in a few cases, the appearance of benign hepatic tumors in women exposed for a long time to this drug [1]. Experimental hepatocarcinomas were obtained in rats orally treated with EE in association with norethindrone [2]. The role of contraceptive steroids as cocarcinogens for dimethylbenzanthracene-induced mammary tumors in the hamster [3], and as promoters of hepatocarcinogenesis in rats [4] was also demonstrated. These reports emphasized the possible interaction between synthetic estrogens and chemical carcinogens, both substances to which some people could be exposed. The mechanism of an interactive effect might be mediated through an action of steroids on liver enzymes that metabolize carcinogens, as well as estrogens themselves [5, 6]. The effect of a mixture of progestagens and estrogens previously tested on various drug-metabolizing enzymes depended on the nature of these steroids, on experimental *in vivo* and *in vitro* conditions, and on the animal species studied [3, 7], and the question has largely remained unsolved. One of the most clearly demonstrated effects of EE in the liver was a destruction of the cytochrome P450 involved in the mechanism of mixed-function oxidases [8, 9].

In the present study, we investigated the effects of EE on the metabolism of BP in isolated adult rat hepatocytes. BP is a polycyclic aromatic hydrocarbon, widely distributed in our environment [10] and it has been shown to require metabolic activation to cause toxic, mutagenic and carcinogenic effects in various species and tissues. BP is metabolized by the microsomal mixed-function oxidases to reactive electrophilic intermediates that bind to DNA, and to detoxified products [11, 12]. Initially, the NADPH-dependent monooxygenase forms epoxides which can spontaneously rearrange into phenols or be further converted into dihydrodiols by epoxide hydratase. These dihydrodiols may then be recycled through the monooxygenase system to yield highly reactive diol-epoxides. These various BP intermediates can form conjugates with either glucuronic acid, sulfate or glutathione, and it is believed that these conjugations are true detoxication reactions.

The most potent metabolite in cell transformation, covalent binding to DNA, mutagenic and carcinogenic activity, appears to be the 7,8-dihydro-9,10-epoxy-7,8,9,10-tetrahydroBP [13–15]. The balance between the formation of reactive metabolites and the conjugative detoxication pathways is crucial in determining toxic effects of BP, and it is therefore necessary to get a drug-metabolizing system in which we can investigate both of these toxifying and detoxifying pathways. Thus, we have chosen isolated adult rat hepatocytes in suspension as an experimental model, because they are known to actively metabolize BP in a way that much more closely resembles

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† Abbreviations: EE, 17-α-ethinyl estradiol; BP, benzo[*a*]pyrene; PB, phenobarbital; MC, 3-methylcholanthrene; UDP, uridine diphosphate.

the *in vivo* situation than do isolated subcellular fractions [16–18]. In particular, the biochemical and internal structural organization at the cellular level seem to be intact, even if these cells have been shown to be permeable to some small molecules and do not exhibit close normal liver cell morphology [19].

The first step of our work was to determine if EE did or did not modify the pattern of BP metabolism in freshly isolated hepatocytes, either from untreated rats or from rats previously treated with PB and MC. These data were then related to the direct effect of EE on the cytochrome P-450 content of hepatocytes. To clarify the effect of EE on the activation/inactivation balance some enzymatic pathways involved in these mechanisms were investigated. The binding of BP metabolites to the endogenous DNA of hepatocytes incubated in the presence of EE was then measured.

MATERIALS AND METHODS

Chemicals. [G-^{3}H]BP (21–65 Ci/mmole) and [7,10-^{14}C]BP (21.7 mCi/mmole) were obtained from the Radiochemical Centre (Amersham, U.K.). They were diluted with unlabelled BP (Aldrich Chemicals, Gillingham, U.K.) to appropriate concentrations and sp. acts and further purified according to the method of De Pierre *et al.* [20]. The unlabelled reference compounds 4,5-dihydro-4,5-dihydroxy-BP (4,5-dihydrodiol), 7,8-dihydro-7,8-dihydroxy-BP (7,8-dihydrodiol), 9,10-dihydro-9,10-dihydroxy-BP (9,10-dihydrodiol), BP-3,6-dione (3,6-quinone), 3-hydroxy-BP and 9-hydroxy-BP were supplied by the IIT Research Institute (Chicago, IL). EE was obtained from ICN Pharmaceuticals (Irvine, CA). Collagenase was obtained from Boehringer (Mannheim, F.R.G.), bovine serum albumin from Armour Pharmaceutical Inc., and HEPES 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid) from Merck.

Animals and treatments. Male Sprague–Dawley rats (6–8 weeks old) from Iffa Credo (St Germain sur l'Arbresle, France) were allowed food and water *ad lib.* MC-treated rats were given intraperitoneally 20 mg/kg body weight of MC (Fluka) dissolved in olive-oil once daily for 3 days. They are killed 24 hr after the last injection. For PB treatment, drinking-water was replaced by a solution of phenobarbital-sodium (Merck), 1 g/l. water for 10 days. This solution remained available to rats until they were killed. In each case (untreated, MC and PB-treated), rats were fasted 18 hr before the isolation of hepatocytes which was done between 9 and 10 a.m.

Isolation of hepatocytes. Rat hepatocytes were isolated by a two-step perfusion based on the method developed by Seglen [21, 22] with some modifications. Rats were anesthetized with ether. The liver was pre-perfused for 3–4 min at a rate of about 45 ml/min with a perfusate containing 2.38 g (0.01 M) HEPES, 8 g NaCl, 0.2 g KCl, 0.1 g Na_2HPO_4 per litre (pH 7.65). In a second step, the liver was perfused for 10 min at a low rate (20–25 ml/min) with the same perfusate added with $CaCl_2$ (0.75 g/l.) and collagenase (0.025%). During this two-step perfusion, the perfusates were maintained at 37°. Following digestion, the liver was transferred to a plastic Petri dish and the cells were dispersed in approximately 20 ml of the first perfusate (without $CaCl_2$ and collagenase). The suspension thus obtained was filtered through gauze, and the resulting filtrate was centrifuged for 1 min at 600 rpm. The pellet of cells was then washed 3 times with the first perfusate and once with the incubation medium (see later). The final pellet was resuspended in incubation medium, and the hepatocytes were counted using a hemocytometer. Their viability was determined by trypan blue exclusion and was generally greater than 80%.

Incubation of hepatocytes with BP. All the assays with BP metabolism were performed according to Burke *et al.* [18]. Freshly isolated hepatocytes were incubated with BP (80 μM) in Krebs–Henseleit buffer [0.12 M NaCl, 5 mM KCl, 1 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 24 mM $NaHCO_3$ (pH 7.4)] containing 2% bovine serum albumin at 37°. For analysis of BP metabolism, 2×10^6 hepatocytes/ml were incubated for 20 min with [^{14}C]BP (20 μCi/μmole added in DMSO, 10 μl/ml of incubate). For analysis of the covalent binding of BP to endogenous DNA, 25×10^6 hepatocytes/5 ml were incubated 30 min with [^{3}H]BP (1–2 Ci/mmole added in DMSO, 10 μl/ml of incubate). In each case, EE was added at zero-time in ethanol, while controls received ethanol only. The final concentration of ethanol in incubates never exceeded 0.15%.

Extraction of BP metabolites. Immediately following incubation, the incubate was added to an equal volume of acetone, the lyzed hepatocytes were centrifuged and the supernatant was extracted 3 times with an equal volume of ethyl acetate containing 0.8 mg/ml of butylated hydroxytoluene (Aldrich Chemicals). The recovery of radioactivity in the two fractions (aqueous and organic) was determined by scintillation counting. The organic phase was dried with anhydrous sodium sulfate, filtered through a 0.2-μm Millipore Teflon filter, and evaporated to dryness. Evaporated samples were stored at −50° under N_2.

High-pressure liquid chromatography of BP metabolites. Just before chromatography, each dry organic extract was redissolved in a small volume (20–40 μl) of dioxane and injected in a Waters Associates high-pressure liquid chromatograph equipped with a Spherosil R-C_{18} column (Prolabo). At the same time, 0.1 μg of each unlabelled reference compound was injected in the chromatograph. This mixture of organic extract and reference compounds was eluted with a linear gradient of methanol/water, varying from 55 to 80% methanol for 60 min, followed by 100% methanol (20–30 min) in order to elute unmetabolized BP. The solvent flow rate was 1 ml/min. Absorbance at 254 nm of the effluent stream was measured, and it was collected as fractions (0.5 ml each) for scintillation counting. More than 90% of the total radioactivity applied to the HPLC column was recovered. The identification of radioactive BP metabolites was based on their co-chromatography with unlabelled reference compounds.

Determination of cytochrome P-450 and P-448. Six to eight million hepatocytes at 2×10^6 cells/ml of Krebs–Henseleit buffer (+ 2% bovine serum albu-

min) were incubated for 20 min at 37° in the presence of increasing concentrations of EE. The cells were then sedimented, washed once in 1 mM EDTA–100 mM Na_2HPO_4 buffer (pH 7.4) containing 20% glycerol, and sonicated for 20 sec. The sonicate was centrifuged for 20 min at 9000 *g* and the supernatant was centrifuged for 45 min at 225,000 *g* to obtain a microsomal pellet which was stored at −80°, under glycerol buffer without loss of activity. Cytochromes P-450 and P-448 were determined by the method of Omura and Sato [23] on a microsomal pellet resuspended in phosphate buffer. Microsomal protein content was determined according to Hartree [24].

Measurement of the binding of BP metabolites to DNA. Following incubation, the hepatocytes from 5 ml of incubation mixture (25×10^6 cells) were centrifuged, and the resulting pellet was treated as described by Burke *et al.* [18] in order to precipitate DNA. The precipitated DNA was then dried under N_2 and redissolved in 1 ml of 0.2 M Tris buffer (pH 7.5) containing 10 mM EDTA. Seven milliliters of an aqueous solution of cesium chloride were then added in order to obtain a final density of 1.63. The mixture was transferred to a polyallomer centrifuge tube and centrifuged at 40,000 rpm for 40 hr at room temperature in a Spinco 50 Ti rotor. Fractions (about 0.5 ml) were collected from the bottom of the tube, 0.5 ml of water was added to each one, and their absorbance at 260 nm was determined. Fractions containing DNA were pooled and their radioactivity was measured by scintillation counting.

Enzyme assays. Epoxide hydratase was assayed according to the method of Jerina *et al.* [25] using [^{3}H]BP-4,5-oxide as a substrate. The reaction mixture contained 50 mM Tris–HCl buffer, 5 mM $MgCl_2$ (pH 7.5), 2×10^4 hepatocytes and 0.1 mM [^{3}H]BP-4,5-oxide [sp. act. 2.7 μCi/μmole] and was incubated for 20 min at 37°.

UDP-glucuronyl-transferase was assayed according to Singh and Wiebel [26], using 3-hydroxy-BP as a substrate. The reaction mixture contained (in 0.4 ml) 50 mM Tris–HCl buffer, 5 mM $MgCl_2$ (pH 7.6), 3 mM UDP-glucuronic acid (Boehringer), 10^5 hepatocytes and 50 μM 3-hydroxy-BP and was incubated for 20 min at 37°.

Glutathione *S*-transferase activity was determined as described by Habig *et al.* [27] using 1-chloro-2,4-dinitrobenzene [CDNB (Merck)] as a substrate. The reaction mixture consisted of 0.1 M phosphate buffer (pH 6.5), 5 mM reduced glutathione (Merck), 1 mM CDNB, 6×10^4 hepatocytes and water to 2 ml.

RESULTS

Effect of EE on the formation of BP metabolites

Ethyl acetate extractions were performed with the purpose of separating conjugated (water-soluble) metabolites from unconjugated (organic-soluble) BP metabolites. Indeed, it has been shown [28, 29] that a large part of sulfate conjugates are recovered in the ethyl acetate phase: this especially concerns the sulfate conjugates of mono-hydroxylated derivatives (phenols), while sulfate conjugates of quinones, which are more polar, are kept in the aqueous phase. Thus, radioactive metabolites recovered in the aqueous phase following ethyl acetate extractions mainly consisted of glucuronides and glutathione conjugates. The ethyl acetate phase also contained unmetabolized BP, which was separated from organic-soluble derivatives by means of HPLC.

Table 1 shows the results concerning the formation of water-soluble metabolites of BP when freshly isolated hepatocytes were incubated in the presence of increasing concentrations of EE. Up to a concentration of 10^{-4} M EE in the incubation medium, no effect was observed. When cells were isolated from untreated rats, 10^{-4} M EE markedly decreased the recovery of total water-soluble metabolites (−42%), while the inhibitory effect only appeared at 10^{-3} M EE (−34%) when hepatocytes were isolated from PB-treated rats. Using hepatocytes from MC-treated rats, no significant decrease was observed up to 10^{-3} M EE.

Data reported in Table 2 typify the ethyl acetate soluble BP metabolites pattern, as determined by HPLC analysis, from incubation of freshly isolated hepatocytes with [^{14}C]BP. The first eluting fraction (designated as F_1) probably contained polyhydroxylated BP-derivatives [30] and ethyl acetate extractable sulfate conjugates of BP [31]. We designated

Table 1. Effects of EE on the formation of total water-soluble metabolites of BP in freshly isolated hepatocytes from untreated, PB- or MC-treated rats*

Concentration of EE in incubation medium (M)	Total water-soluble metabolites (nmoles/hr/2×10^6 cells)		
	Untreated rats	PB-treated rats	MC-treated rats
Control	12.5 ± 1.63 (7)	19.2 ± 1.37 (6)	28.6 ± 3.60 (5)
10^{-6}	11.3 ± 1.06 (5)	16.1 ± 1.42 (5)	26.2 ± 5.28 (5)
10^{-5}	11.0 ± 1.30 (5)	17.0 ± 1.39 (5)	26.9 ± 3.96 (5)
10^{-4}	7.2 ± 0.77 (6) ($P < 0.05$)	15.8 ± 1.63 (6)	25.2 ± 4.18 (5)
10^{-3}	3.4 ± 0.22 (4) ($P < 0.01$)	12.7 ± 1.15 (4) ($P < 0.05$)	22.6 ± 4.68 (4)

* MC-treated rats were given intraperitoneally 20 mg/kg body weight of MC dissolved in olive-oil once daily for 3 days. PB-treated rats were given 1 g of PB per liter of their drinking water for 10 days. Incubations were performed with 2×10^6 cells/ml for 20 min at 37°. [^{14}C]BP concentration was 80 μM. Results are expressed as the means ± S.E.M. for the number of experiments indicated in parentheses.

Table 2. Effects of EE on the formation of ethyl acetate soluble metabolites of BP in freshly isolated hepatocytes from untreated, PB- or MC-treated rats (HPLC analysis)*

	Untreated rat			PB-treated rat			MC-treated rat		
	Control	10^{-4} M EE	10^{-3} M EE	Control	10^{-4} M EE	10^{-3} M EE	Control	10^{-4} M EE	10^{-3} M EE
F_1	1.30	0.57	0.30	2.61	1.72	0.97	2.06	2.41	2.39
9,10-Diol	0.52	0.21	0.06	1.33	0.21	nd	3.02	2.31	1.15
4,5-Diol	0.15	0.09	nd	1.91	1.22	0.54	0.78	0.52	0.49
7,8-Diol	nd†	nd	nd	nd	nd	nd	0.34	0.26	
Quinones	0.27	0.28	0.14	0.72	0.34	0.51	0.65	0.47	0.41
9-Hydroxy	0.39	0.30	0.33	0.53	0.44	0.66	2.00	2.46	2.76
3-Hydroxy				0.57	0.33	0.52	3.74	3.57	3.68
Total	2.63	1.45	0.83	7.67	4.26	3.20	12.59	12.00	10.88

* Results are expressed as nmoles/20 min/2 × 10^6 cells. Pre-treatment of rats and hepatocyte incubations were conducted as described in Table 1.

† Not detectable.

as "quinones" the group of peaks occurring between the 7,8-diol and 9-hydroxy-BP, for we did not have the appropriate reference compounds to identify them individually. They probably corresponded, not only to the 3,6-dione, but to other BP-quinones like the 1,6- and the 6,12-dione, and also to oxides such as BP-4,5-oxide. Moreover, and despite the addition of butylated hydroxytoluene in the extracting solvent, we cannot entirely exclude the spontaneous oxidization of some phenols into quinones. From a qualitative point of view, control hepatocytes from induced rats metabolized BP in a different way than did control hepatocytes from untreated rats. The major differences are classically related to the different categories of inducers we used [11]. Using control hepatocytes from PB-treated rats, we noticed a preferential increase in 4,5-diol (K-region diol) formation relative to other metabolites, while control hepatocytes from MC-treated rats tended to metabolize BP towards dihydrodiols of the non-K-region (9,10- and 7,8-diol), and produced enhanced amounts of monohydroxylated derivatives (phenols).

Data presented here show that the EE inhibitory effect also affected the total production of these ethyl acetate soluble derivatives. At 10^{-4} M EE in incubation medium, their total amount was decreased by 44% when cells were isolated from both untreated and PB-treated rats, while no significant decrease was observed up to 10^{-3} M EE with hepatocytes from MC-treated rats. This inhibitory effect mainly concerned the formation of dihydroxylated metabolites, and especially the 9,10-diol, which decreased by 60 and 84% with 10^{-4} M EE and hepatocytes from untreated and PB-treated rats, respectively. The decrease of the diols was less important (~ 25%) with hepatocytes from MC-treated rats. In all cases, the increase of EE concentration enhanced the loss of this group of BP metabolites. The formation of monohydroxylated derivatives (9-hydroxy- and 3-hydroxy-BP) was slightly decreased by 10^{-3} M EE (~ 25%) in hepatocytes from untreated and PB-treated rats, and was not modified by EE in hepatocytes from MC-treated rats. Quinones were decreased in all types of hepatocytes. Using hepatocytes from untreated or PB-treated rats, F_1 fraction

Table 3. Effects of EE on the amount of cytochromes P-450 and P-448 in freshly isolated hepatocytes from untreated, PB- and MC-treated rats*

	Untreated rats	PB-treated rats	MC-treated rats
Control	138 ± 15.8 (4)	1543 ± 308 (4)	414 ± 93.8 (4)
10^{-6} M EE	146 ± 9.13 (3)	1510 ± 69 (4)	nd†
10^{-5} M EE	100 ± 12.4 (3)	1449 ± 76 (4)	nd
10^{-4} M EE	122 ± 18.8 (4)	1376 ± 70 (5)	485 ± 71.2 (4)
10^{-3} M EE	129 ± 19.2 (5)	1414 ± 157 (4)	422 ± 96 (4)

* Hepatocytes were isolated from rats treated as described in Table 1 and 6 × 10^6 at least were incubated in the presence of increasing concentrations of EE. Microsomes were isolated, cythochromes P-450 and P-448 were determined according to Omura and Sato [23] and expressed as pmoles/mg microsomal protein. Each determination was made in duplicate from the number of rats indicated in parentheses. Each value is the mean ± S.E.M.

† Not determined.

was also decreased by EE, while this was not the case when hepatocytes were isolated from MC-treated rats, for which a slight increase of the F_1 fraction was even present.

Effect of EE on cytochrome P-450 and P-448

The various effects of EE observed on the formation of BP metabolites could be mainly related to its direct action on the hemoprotein, cytochrome P-450. To clarify this point the amount of microsomal cytochrome from hepatocytes incubated in the absence and in the presence of EE was determined. According to the nature of the chemical inducer, cytochrome P-450 was measured from untreated and PB-treated rats, and cytochrome P-448 from MC-treated rats. Results are presented in Table 3. In hepatocytes isolated from untreated rats, a small decrease in microsomal cytochrome P-450 appeared when the cells were incubated in the presence of EE, but it did not however reach the level of significance. When cytochrome P-450 was induced by PB pretreatment of rats, the addition of EE to the hepatocyte incubation medium did not modify the amount of hemoprotein, whatever the concentration of EE. The level of cytochrome P-448 induced by MC pretreatment remained also unchanged by the presence of EE in the medium. We can therefore conclude that various concentrations of EE were entirely inefficient in modifying the amount of microsomal cytochrome of hepatocytes isolated from untreated and induced rats.

Effect of EE on enzymatic activities involved in the activation/inactivation balance.

Since the formation of all the metabolites of BP is dependent on activation and inactivation enzymatic pathways present in liver cells, we investigated the influence of EE on some enzymatic activities of hepatocytes. Hepatocytes from untreated rats were incubated in the presence of increasing concentrations of EE and epoxide hydratase, UDP-glucuronyl-transferase and glutathione *S*-transferase were determined.

As shown in Table 4, the addition of 10^{-4} M EE to the incubation medium inhibited both epoxide hydratase and UDP-glucuronyl-transferase to about the same extent (−36% for epoxide hydratase and −27% for UDP-glucuronyl-transferase). Increasing concentrations of EE up to 10^{-3} M enhanced this inhibitory effect. Glutathione *S*-transferase activity also decreased, but to a lesser extent (−19% at 10^{-4} M EE).

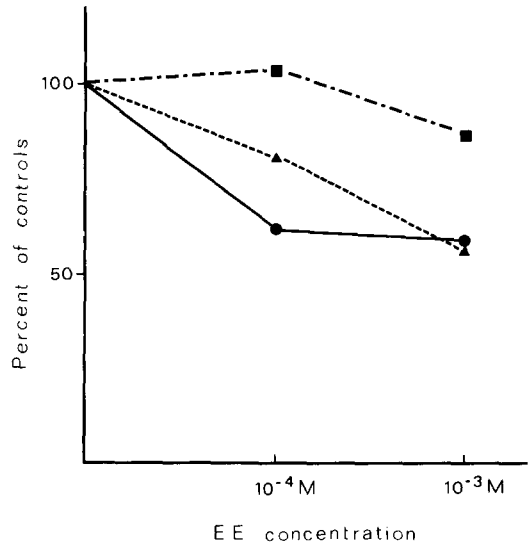

Fig. 1. Effects of EE on the binding of [^{3}H]BP metabolites to hepatocyte DNA from untreated, PB- and MC-treated rats. Hepatocytes were isolated from untreated rats (●), and from rats which received either three i.p. injections of MC (20 mg/kg—one injection per day) (■) or PB in the drinking water (1 g/l.) for 10 days (▲). Incubations were performed with 25 × 10^6 cells/5 ml for 30 min at 37°. [^{3}H]BP concentration was 80 μM. Results are expressed as % of the controls incubated without EE in the medium. Control values expressed as pmoles of BP/mg DNA were 0.90 ± 0.15 for untreated rats, 5.6 ± 0.89 for PB-treated rats and 18 ± 0.60 for MC-treated rats.

Effect of EE on the binding of BP metabolites to hepatocyte DNA

The critical role of covalent binding of BP metabolites to DNA in the carcinogenic process prompted us to investigate the consequence of the inhibition of BP metabolism by EE on the covalent binding of [^{3}H]BP metabolites to the DNA of hepatocytes isolated from untreated and induced rats. The results shown in Fig. 1 indicated that EE clearly inhibited the binding of BP metabolites to hepatocyte DNA from untreated rats. The concentration of 10^{-4} M EE in the incubation medium, which inhibited the

Table 4. Effects of EE on epoxide hydratase, UDP-glucuronyl-transferase and glutathione *S*-transferase activities in freshly isolated hepatocytes from untreated rats*

Concentration of EE in incubation medium (M)	Epoxide hydratase (pmoles/min/2 × 10^4 cells)	UDP-glucuronyl-transferase (pmoles/min/10^5 cells)	Glutathione *S*-transferase (nmoles/min/6 × 10^4 cells)
Control	44.5 ± 7.06 (6)	70 ± 3.0 (3)	34.4 ± 1.97 (7)
10^{-5}	42.8 ± 2.44 (6)	61 ± 2.0 (3)	32.3 ± 1.09 (6)
10^{-4}	28.3 ± 1.23 (6)	51 ± 2.9 (3)	28.0 ± 1.15 (9)
	(P < 0.05)	(P < 0.05)	(P < 0.05)
10^{-3}	19.7 ± 1.05 (6)	25 ± 0.9 (3)	nd†
	(P < 0.01)	(P < 0.01)	

* Enzyme assays were performed as described in Materials and Methods. Results are expressed as the means ± S.E.M. for the number of experiments indicated in parentheses.

† Not determined.

formation of both aqueous and organic solubme BP metabolites, inhibited [^{3}H]BP/DNA binding by 40%. Using hepatocytes from PB-treated rats the inhibitory effect of EE was present though much less pronounced than that obtained with untreated rats. The inhibition was only 20% at 10^{-4} M EE and reached 40% at 10^{-3} M EE. In the presence of hepatocytes from MC-treated rats, no significant effect of the addition of high concentrations of EE on [^{3}H]BP/DNA binding was observed.

DISCUSSION

Using suspensions of hepatocytes freshly isolated from untreated rats, the pattern of BP-metabolites was modified by EE in several ways. First, the addition of 10^{-4} M EE in the incubation medium resulted in a general inhibition of BP metabolism as pointed out by a decrease in water and organic-soluble metabolites. This overall effect was more pronounced when the EE concentration was increased to 10^{-3} M.

The reduced amount of water-soluble metabolites and of the F_1 fraction, ahead of HPLC pattern, both representing conjugated metabolites, might be related to the inhibitory effect of EE on two enzymes involved in detoxication, i.e. UDP-glucuronyl-transferase and glutathione *S*-transferase activities respectively decreased by 27 and 19% by 10^{-4} M EE. The inhibitory effect of EE on these processes was yet must less efficient in hepatocytes from induced rats than in hepatocytes from untreated rats. The only significant effect of 10^{-3} M EE concerned a decrease of water-soluble metabolites and the F_1 fraction in hepatocytes from PB-treated rats while in hepatocytes from MC-treated rats a slight decrease in water-soluble metabolites and a moderate increase of the F_1 fraction did not appear truly significant. The classical induction of both enzymes, UDP-glucuronyl-transferase and glutathione *S*-transferase, in PB and MC-treated rats [32–35] might counteract the inhibitory effect of EE on these enzymes. Moreover, the difference in sensitivity due to the pretreatment of rats might be explained by the heterogenous structure of UDP-glucuronyl-transferase, one form of the enzyme being more easily inducible by MC while the other is preferentially inducible by PB [34, 35]. Another possibility implies a specific effect of EE on the formation of BP-hydroxylated metabolites by cytochrome P-450 mediated monooxygenase and will be discussed later.

Among organic-soluble metabolites of BP, the dihydrodiols were the main category of metabolites affected by the addition of EE in the medium. In hepatocytes from untreated rats, the inhibition rate produced by 10^{-4} M EE was 55% for dihydrodiols and only 23% for phenols. Using hepatocytes from PB-treated rats, a similar percentage of inhibition by 10^{-4} M EE was found for dihydrodiols and phenols; in addition the formation of quinones was inhibited. The pretreatment of rats by MC deeply modified the sensitivity of BP metabolism to the action of EE. The total amount of organic-soluble metabolites was not affected by 10^{-4} M EE and the production of diols and quinones was only slightly reduced (by about 25%). Therefore hepatocytes from untreated rats and PB-treated rats presented a similar behaviour whith respect to the action of EE on BP metabolism and were distinguishable from hepatocytes from MC-treated rats which appeared insensitive to the effect of EE.

This difference due to the origin of hepatocytes was also encountered in experiments dealing with binding of BP metabolites to endogenous DNA of hepatocytes. In the presence of EE, the amount of metabolites bound to DNA was decreased in hepatocytes from untreated and PB-treated rats. In contrast, the binding to DNA was not affected by EE in hepatocytes from MC-treated rats. A good correlation was thus established between the inhibitory effect of EE on BP metabolism and on BP metabolite binding to DNA.

The differential effect of EE on untreated, PB- and MC-treated rats might be related to the constitutive or inducible nature of the cytochrome involved in BP metabolism. White and Müller-Eberhard [8] previously reported that EE was able to induce a loss of cytochrome P-450 from untreated and PB-treated rats, and that cytochrome P-448 from MC-treated rats was insensitive to the action of steroids. The determination of microsomal cytochrome P-450 and P-448 in hepatocytes incubated in the presence of EE did not however reveal any effect of EE on each type of cytochrome, since the slight decrease observed in hepatocytes from untreated rats did not reach the level of significance. From these data we cannot conclude that there is a degradation of any type of cytochrome, which conflicts with previously reported data obtained with microsomes incubated *in vitro* in the presence of EE [8, 9]. *In vivo* administration of a high dosage of EE to rats was also known to induce a loss of microsomal cytochrome P-450 which appeared even more effective in the liver of PB-treated rats than in untreated rat liver [8, 36]. Hepatocytes isolated from untreated or PB-treated rats therefore represented a peculiar case on account of the insensitivity of their hemoprotein to the action of EE. The reason for such a particularity is presently unknown.

Since, in our experimental conditions, the impairment of BP metabolism by EE cannot be attributed to a net loss of cytochrome P-450, it might be due to an alteration of the function of cytochrome by a non-destructive mechanism different from that described by Ortiz de Montellano and Kunze [9]. The constant level of cytochrome in the presence of EE did not yet exclude the possibility that some enzymatic activities could vary since both parameters did not always present a parallel evolution as exemplified by cholesterol-7α-hydroxylase activity [37]. Another explanation of BP metabolism inhibition by EE would be supported by a competition between both substrates for a number of enzymatic pathways common to their metabolism and several levels of interaction might be considered. The competition for cytochrome P-450 dependent monooxygenase probably occurred since the overall BP metabolism was decreased in untreated and PB-treated rats. This would mean that EE could act like natural steroids which are known to interact with BP hydroxylase [38, 39]. A second type of competition between EE and BP for glucuronyl and glutathione conjugation

would contribute to the important decrease in water-soluble BP metabolites.

Lastly, the inhibition of epoxide hydratase by 10^{-4} M EE (36%) and by 10^{-3} M EE (55%) might be involved in the large decrease in dihydrodiols, the main class of BP metabolites affected by EE and precursors of diol-epoxides which bind to DNA. Epoxides deriving from steroid estrogens could be formed by hepatic microsomes and were found to be efficient substrates for epoxide hydratase [6, 40]. The hypothesis of competition between EE and BP is strengthened by the fact that most EE inhibitory effects appeared in the presence of very similar concentrations of both substrates in the incubation medium, that is 80 μM for BP and 100 μM for EE.

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