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EFFECTS OF VARIOUS INDUCERS ON DIETHYLSTILBESTROL METABOLISM, DRUG-METABOLIZING ENZYME ACTIVITIES AND THE AROMATIC HYDROCARBON (Ah) RECEPTOR IN MALE SYRIAN GOLDEN HAMSTER LIVER*

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Summary—In order to elucidate the role of metabolic activation of the synthetic estrogen, diethylstilbestrol (DES), in the mechanism of liver tumor formation in male Syrian golden hamsters observed after combined treatment with DES and 7,8-benzoflavone (7,8-BF), the metabolism of DES and the concentrations and activities of various drug-metabolizing enzymes were studied in hamster liver microsomes after various pretreatments. The levels of the hepatic aromatic hydrocarbon (Ah) receptor were also determined.

Pretreatment with 7,8-BF increased both *P*450 and cytochrome b_5 levels, whereas phenobarbital (PB) and 3-methylcholanthrene (MC) induced *P*450 but not cytochrome b_5. 7,8-BF pretreatment increased 7-ethoxyresorufin-*O*-deethylase (EROD) 3-fold and 7-pentoxyresorufin-*O*-dealkylase (PROD) 2.5-fold, whereas aromatic hydrocarbon hydroxylase (AHH) and 7-ethoxycoumarin-*O*-deethylase (ECOD) activities were only slightly induced by 7,8-BF. MC pretreatment increased EROD 8-fold and PROD activity 7-fold, whereas PB pretreatment enhanced AHH 4.5-fold and PROD activity 4-fold. In contrast to PB, pretreatment with 7,8-BF and MC reduced the oxidative metabolism of DES in hepatic microsomes, but the pattern of metabolites was identical with that in untreated controls. Treatment of hamsters with the inducers changed the hepatic Ah receptor level. PB and MC-pretreatment resulted in an increase of the receptor level 1.5-fold and 1.3-fold, respectively, whereas 7,8-BF-pretreatment leads to a 1.5-fold decrease. The dissociation constant K_d is 170 nM for the reaction of 7,8-BF with the hamster Ah receptor compared to 70 nM for 5,6-BF and 38 nM for 2,3,7,8-tetrachlorodibenzofuran (TCDF). The K_d-value is 3.6 nM for TCDF with the rat receptor protein.

It is concluded from these data that metabolic activation of DES is not involved in the mechanism of hepatocarcinogenesis in this animal tumor model.

INTRODUCTION

The combined treatment of male Syrian golden hamsters with the synthetic estrogen diethylstilbestrol (DES) and 7,8-benzoflavone (7,8-BF) gives rise to a high incidence of hepatocellular carcinoma, whereas no such tumors are found with DES nor 7,8-BF alone [1]. Treatment with DES alone causes a 100% incidence of renal tumors [2]. The mechanism of DES carcinogenesis and of the pronounced shift in the organotropism are unknown. A possible explanation could be that 7,8-BF modulates hepatic DES metabolism thereby rendering the liver susceptible to tumor formation.

In order to elucidate the role of metabolic activation of DES in tumor formation, we have studied the metabolism of DES and the concentrations and activities of various drug-metabolizing enzymes in liver microsomes from untreated controls and 7,8-BF pretreated animals. For comparison, the same studies were carried out after pretreatment with phenobarbital (PB) and 3-methylcholanthrene (MC). In addition, we have measured the level of the hepatic aromatic hydrocarbon (Ah) receptor and the affinity of the inducers to the Ah receptor. This regulatory protein is involved in the induction of

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Abbreviations: AHH, aryl hydrocarbon hydroxylase; 7,8-BF, 7,8-benzoflavone; DES, diethylstilbestrol, 3,4-bis-(*p*-hydroxyphenyl)-hex-3-ene; DIES, dienestrol, 3,4-bis-(*p*-hydroxyphenyl)-hexa-2,4-diene; PROD, 7-pentoxyresorufin-*O*-dealkylase; ECOD, 7-ethoxycoumarin-*O*-deethylase; EROD, 7-ethoxyresorufin-*O*-deethylase; HPLC, high performance liquid chromatography; MC, 3-methylcholanthrene; PB, phenobarbital; TCDD, 2,3,7,8-tetrachlorodibenzo(*p*)dioxin; TCDF, 2,3,7,8-tetrachlorodibenzofuran.

drug-metabolizing enzymes. The mechanism is well documented in the case of *P*450IA1 (for cytochrome *P*450 nomenclature see [3]). It has been suggested that in addition to its role in enzyme induction, the Ah receptor is involved in mediating toxic responses of halogenated aryl hydrocarbons and plays a role in the process of carcinogenesis [4].

MATERIALS AND METHODS

Male Syrian golden hamsters were pretreated with 7,8-BF (0.4% in the diet) for 8 weeks, with PB (80 mg/kg injected daily i.p.) for 3 days and with MC (20 mg/kg injected daily i.p.) for 3 days. Microsomes were prepared and enzyme concentrations and activities determined according to standard methods. Microsomal incubations (1 mg protein/ml) were carried out in triplicate in a total volume of 2 ml 100 mM potassium phosphate buffer pH 7.4 containing 80 μM [^{14}C]DES and a NADPH-regenerating system. Incubations were stopped after 30 min at 37°C and the radioactive products extracted into organic solvents and analyzed by HPLC as described previously [5]. *P*450 and cytochrome b_5 and various isoenzyme activities were measured as described elsewhere [6]. The metabolites were identified through their mass spectra. Ah receptor levels were analyzed using a 10–30% sucrose density gradient with [^{3}H]2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) as ligand according to the method of Tsui and Okey[7]. The affinity to the Ah receptor was measured as the capacity to decrease [^{3}H]TCDD binding (3 nM) in incubations with varying amounts of the competitor ranging from a 5-fold to 50,000-fold excess. Specific binding was determined as the difference between incubations in the presence or absence of a 500-fold excess of 2,3,7,8-tetra-chlorodibenzofuran. Binding constants were calculated according to Cikryt and Göttlicher[8].

RESULTS

Effects of pretreatment on cytochrome b_5 and P450 levels and on various P450-associated enzyme activities

Pretreatment with 7,8-BF increased *P*450 as well as cytochrome b_5 content; in contrast, PB and MC enhanced the amount of *P*450 but not of cytochrome b_5 (Fig. 1). 7,8-BF affected *P*450-associated enzyme activities differently. Aryl hydrocarbon hydroxylase (AHH) and 7-ethoxycoumarin-*O*-deethylase (ECOD) were only slightly enhanced, whereas 7-ethoxyresorufin-*O*-deethylase (EROD) and 7-pentoxyresorufin-*O*-dealkylase (PROD) were induced 3-fold and 2.5-fold, respectively. MC caused a 8-fold stimulation of EROD and a 7-fold increase of PROD activity. PB increased AHH, ECOD and PROD activity by factors 4.5, 2 and 4, respectively (Fig. 1).

Effect of the different pretreatments on microsomal DES metabolism

To study the influence of 7,8-BF, MC and PB pretreatment on hepatic DES metabolism, microsomes of pretreated animals were incubated with [^{14}C]DES and the major metabolites determined by

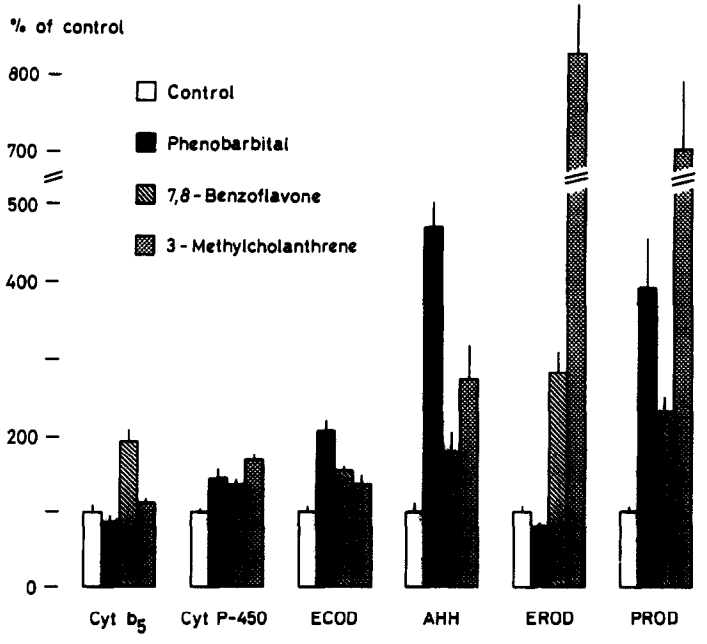

Fig. 1. Effect of pretreatment with phenobarbital (PB), 7,8-benzoflavone (7,8-BF) and 3-methylcholanthrene (MC) on *P*450 and cytochrome b_5 levels and on different *P*450-associated isoenzyme activities in liver microsomes of male hamsters. Data are the mean ±SD of 5 animals and are expressed as % of control. ECOD, 7-ethoxycoumarin-*O*-deethylase; AHH, aryl hydrocarbon hydroxylase; EROD, 7-ethoxyresorufin-*O*-deethylase; PROD, 7-pentoxyresorufin-*O*-dealkylase.

Table 1. Effect of pretreatment with 7,8-benzoflavone, phenobarbital and 3-methylcholanthrene on the oxidative DES metabolism in hepatic microsomes. Data represent % of radioactivity and are the mean ± SD of 5 animals

Metabolite	Control	Pretreated with Pheno-barbital	7,8-Benzo-flavone	3-Methyl-cholanthrene
Unknown polar metabolites	7.4 ± 0.5	7.4 ± 0.7	6.3 ± 1.4	6.8 ± 1.8
1-hydroxy-Z,Z-DIES	ND	4.1 ± 0.4	ND	3.1 ± 0.5
3'-hydroxy-E-DES	4.9 ± 0.3	3.5 ± 0.1	5.9 ± 0.5	4.3 ± 0.5
E-DES	16.1 ± 1.4	16.5 ± 1.6	23.9 ± 4.6	32.9 ± 2.6
3'-hydroxy-Z-DES	8.7 ± 0.6	10.3 ± 0.6	8.2 ± 1.1	7.3 ± 1.1
Z,Z-DIES	2.9 ± 0.3	3.7 ± 0.7	2.6 ± 0.6	1.8 ± 0.4
Z-DES	39.7 ± 1.2	37.2 ± 2.3	37.8 ± 1.6	37.1 ± 1.4
Unknown nonpolar metabolites	11.5 ± 0.9	7.4 ± 0.4	10.5 ± 0.5	2.9 ± 0.3

ND not detected (less than 0.2%).

HPLC. These comprise the Z-isomer of DES, the oxidation product Z,Z-dienestrol (Z,Z-DIES), hydroxylated metabolites of E- and Z-DES and Z,Z-DIES, and some as yet unknown polar and nonpolar products (Table 1). In contrast to PB pretreatment, which did not affect the metabolic DES pattern compared with untreated controls, pretreatment with 7,8-BF or with MC reduced the amount of most oxidative DES metabolites. Isomerization of E-DES to Z-DES was not affected by any of the treatments.

Affinity of the inducers to the hamster hepatic Ah receptor

The induction of P450IA1 is mediated by the Ah receptor. This enzyme is specifically monitored using ethoxyresorufin as a substrate [9]. Our data show (Fig. 1) that EROD is induced by 7,8-BF in hamster. Therefore we have determined the affinity of 7,8-BF and other inducers to the hamster Ah receptor. Figure 2 shows the binding of [^{3}H]TCDD to hamster hepatic cytosol in the sucrose density gradient centrifugation and the effect of a 500-fold excess of 7,8-BF in the incubation mixture on [^{3}H]TCDD binding. The dissociation constants of the inducers to the hamster protein are given in Table 2. PB and DES do not decrease [^{3}H]TCDD binding even when they are present in a 50,000-fold excess. For comparison, the K_d-value of TCDF to the rat hepatic Ah receptor was determined as 3.6 nM under the same experimental conditions.

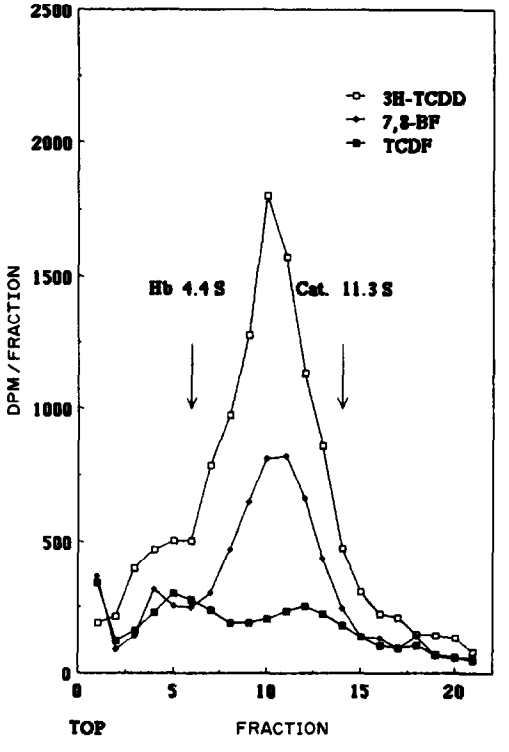

Fig. 2. Binding of [^{3}H]TCDD in the absence and presence of a 500-fold excess of TCDF and 7,8-BF to hamster hepatic cytosolic proteins: sucrose density gradient centrifugation. Catalase (11.3S) and hemoglobin (4.4S) were taken as marker proteins.

Effect of pretreatment on hepatic Ah receptor level

Pretreatment of hamster with the inducers changed the hepatic Ah receptor level as shown in Table 3. PB and MC lead to an elevation of the receptor level whereas 7,8-BF decreased the hepatic concentration.

DISCUSSION

The experimental induction of liver tumors by certain estrogens in the male Syrian golden hamster treated concomitantly with 7,8-BF has been proposed as an animal model for human liver tumors in women

Table 2. Dissociation constants K_d of the inducers with the hamster hepatic Ah receptor

Compound	K_d (nM)
TCDF	38
5,6-BF	70
7,8-BF	170
PB	NA
DES	NA

K_d-values were calculated according to Cikryt and Göttlicher[8]; NA, no affinity ($K_d > 50\ \mu M$).

Table 3. The effects of various inducers on the hamster hepatic Ah receptor levels

Compound	Cytosolic Ah receptor (fmol/mg protein, mean ± SD, n = 4–5)	Control	Error probability (Student's t-test)
Control	85 ± 5	100	—
PB	130 ± 31	159	$P < 0.05$
7,8-BF	57 ± 12	67	$P < 0.01$
MC	103 ± 5	130	$P < 0.001$

taking oral contraceptives [1]. The mechanism of tumor formation is as yet unknown. Our data presented here indicate that pretreatment of the male Syrian golden hamster with 7,8-BF results in a marked effect on drug metabolizing enzymes in the liver. In contrast to MC and PB, which increase *P*450 levels but not b_5, treatment with 7,8-BF increases the concentration of both enzymes. With respect to the induction of monooxygenase activities, 7,8-BF behaves like an MC-like inducer *in vivo* because of the EROD induction which is specifically associated with *P*450IA1 and *P*450IA2 [10].

To clarify the induction mechanism, we have determined the affinity of 7,8-BF to the hamster Ah receptor *in vitro* by sucrose density centrifugation. The binding constant for this protein and 7,8-BF is not much different from the K_d-values with the isomer 5,6-BF and TCDF (Table 2). The EROD induction by 7,8-BF confirms that this compound is an Ah receptor agonist *in vivo* in hamster if the animals are pretreated with this compound alone. Blank *et al.*[11] have shown that 7,8-BF acts as an Ah receptor antagonist in murine cultured splenic lymphocytes when added to the cells together with TCDD. These authors demonstrated that 7,8-BF (K_i 15 nM) and 5,6-BF (K_i 1.5 nM) bind to rat Ah receptor. We have determined a K_d of 3.6 nM for TCDF to the rat protein. A comparison of the binding constants for these compounds in rat and hamster show that the binding to the hamster protein is one order of magnitude lower. This finding is interesting with regard to the lower susceptibility of the hamster towards polychlorinated aromatic hydrocarbons.

The elevation of the hamster hepatic Ah receptor level by the inducers PB and MC was expected. It has been shown that PB pretreatment doubled the receptor level [12] and MC pretreatment caused a 1.3-fold increase [13] in the rat. Our data show that 7,8-BF pretreatment decreased the hepatic Ah receptor level in hamsters. 7,8-BF was administered orally in the diet for several months in contrast to the much briefer application period of the other inducers. Therefore we can not exclude the possibility that the hepatic levels of the compounds are different and that the cytosol preparation from 7,8-BF pretreated animals contains receptor bound 7,8-BF which could not be displaced completely by labelled TCDD.

Although 7,8-BF acts like an inducer, this induction is not reflected in an enhanced oxidative DES metabolism in hepatic microsomes. In fact, pretreatment with 7,8-BF reduced DES metabolism, an effect which parallels that of MC. This suggests that *P*450 isoenzymes inducible by 7,8-BF are not primarily involved in DES metabolism and that modulation of hepatic DES metabolism is not the reason for the observed hepatocarcinogenicity. Instead, it is conceivable that 7,8-BF and not the estrogen acts as the initiating agent in the hamster liver.

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