MICROSOMAL ACTIVATION OF ESTROGENS AND BINDING TO NUCLEIC ACIDS AND PROTEINS

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SUMMARY: Natural and synthetic estrogens can be activated by rat liver microsomes to bind covalently to polyguanylic acid, single-stranded DNA, nucleotides and proteins. Incubation of polyG, estrone and liver microsomes (0.5 nmole cytochrome P-448 or P-450) from 3-methylcholanthrene-induced, phenobarbital-induced or control rats showed that the former microsomes gave better net binding of estrogens to polyG than the other two. Estradiol incubated with 3MC-induced microsomes did bind to DNA but marginally to polyG. Mestranol and estrone sulfate, both constituents of oral contraceptive formulations, bound to polyG whereas progesterone and cholesterol did not bind. We present also preliminary data on the characterization of estrogen-nucleic acid interactions using nucleases, proteinase K and high-pressure liquid chromatography.

INTRODUCTION

The metabolism of estrogens involves oxidation-reduction of hydroxy or keto groups in ring D, hydroxylations in positions 2, 15α, 16α, 6α, 6β, 7α, methylations and conjugations to produce sulfates, glucuronides or mercapturic acid derivatives (1,2). All estrogen hydroxylations are thought to be catalysed by cytochrome P-450 monooxygenases.

Protein binding of estrone, estradiol and 17α-ethynylestradiol catalyzed by control or phenobarbital induced rat liver or human liver microsomes has been reported and a role implied for P-450 monooxygenases (3-6); recently, Nelson et al. (7) showed binding of the above estrogens and 2-hydroxyestrogens to liver microsomal protein from control or pregnenolone-16α-carbonitrile induced rats. Belt and Kappus (6) showed that mushroom tyrosinase but not rat liver microsomes could catalyze, in the presence of NADPH, the binding of 17α-ethynylestradiol to DNA, RNA and albumin.

It occured to us that cytochrome P-448 (P-450) monooxygenases could form arene oxides (8) of estrogens, the only natural aromatic steroids, which could subsequently bind to nucleic acids and protein as was shown recently with benzo-

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[a]pyrene arene oxides (9, 17 and refs. therein). We wish to report such binding to nucleic acids and proteins; the binding is stimulated by pretreatment of the rats with 3-methylcholanthrene (3-MC) but not with phenobarbital (PB). We therefore postulate that since 3-MC induces aromatic hydroxylations to a greater extent than PB (15 and refs. therein) and the binding to nucleic acids follows the same pattern, an estrogen arene oxide is probably the activated estrogen intermediate.

MATERIALS AND METHODS

[6,7-^{3}H]estrone (E_1), [6,7-^{3}H]-17β-estradiol (E_2), [6,7-^{3}H]estrone sulfate, [1,2-^{3}H]-cholesterol, [1,2,6,7-^{3}H]progesterone, and [4-^{14}C]mestranol (3-0 methyl-17α-ethynylestradiol) were purchased from New England Nuclear, [G-^{3}H]benzo[a]pyrene (BP) and [U-^{14}C]-dGMP(5') were purchased from Amersham-Searle Corp. Prior to incubation, radiolabelled steroids were mixed with the unlabeled steroids which were purchased from Sigma and Steraloids. 3-Methylcholanthrene was purchased from Sigma and phenobarbital from Mallinkrodt.

Polyguanylic acid (K salt), calf thymus DNA, deoxyribonuclease I, glucose-6-phosphate dehydrogenase were purchased from Sigma, ribonuclease T_1 from Calbiochem, proteinase K from EM Laboratories, Elmsford N.Y., and alkaline phosphatase (BAPF) from Worthington Biochemical Corp.

Long Evans male rats (60-70g) were purchased from Blue Spruce Farms, Altamonte, N.Y. and injected once daily for three days intraperitoneally with 3-MC (25 mg/Kg) or PB (80 mg/Kg) in corn oil or corn oil alone and were starved for 18 hrs before decapitation. Each liver was perfused with 50 ml cold saline solution. Microsomes obtained according to Lu et al. (10), were sonicated in the absence of detergents and applied on a Sephadex G-10 column equilibrated with 0.33 M Tris-Cl pH 7.7; all red fractions which eluted with the equilibrating buffer were combined and stored in liquid nitrogen until use.

Incubations of nucleic acids with steroids or BP and liver microsomes were performed by an adaptation of the method of King et al. (11): 1 ml incubation mixtures contained 0.5 nmole of cytochrome P-448 or P-450 (protein content 280 µg, 270 µg, and 720 µg for 3-MC, PB and control microsomes, respectively), 20 nmoles radioactive estrogen (200-2000 cpm per pmole), 3 mg of polyG, nucleotide or 1 mg denatured calf thymus DNA and 0.7 mole NADPH and a NADPH-regenerating system [glucose-6-phosphate (G-6-P) and G-6-P dehydrogenase]. After 1 hour incubation at 37°, nucleic acids and proteins were precipitated three times with ethanol-salt as described previously (12) and further characterized by specific nuclease digestions (12,13). In incubations of 5'-GMP and 5'-dGMP, ethanol precipitation of microsomal proteins was followed by removal of the ethanol from the supernatant solution by a stream of nitrogen at 40° and extraction of the aqueous layer three times with 5 ml each of diethyl ether. Radioactivity was determined by scintillation counting in Bray's solution, protein quantitated by the Lowry method using bovine serum albumin as standard, nucleic acids by their 252-260 nm absorption and cytochromes P-450 by the method of Omura and Sato (14).

RESULTS AND DISCUSSION

It had been shown that purified cytochrome P-448 monooxygenase (3-MC induced) is a much better catalyst for the formation of benzo[a]pyrene arene oxides than the cytochrome P-450 system (PB-induced or control) (15 and refs. therein). Con-

TABLE 1. BINDING OF ESTROGENS TO NUCLEIC ACIDS CATALYSED BY RAT LIVER MICROSOMES[a]

Microsomes	Radiolabelled Steroid	Nucleic Acid	pmoles steroid bound per mg nucleic acid[b,c]		
			+NADPH	-NADPH	Difference
3MC-induced	E_1	polyG	288	28	260[c]
	E_2	"	250	233	17
	E_1-sulfate	"	170	8	162
	mestranol	"	123	15	108
	progesterone	"	44	57	----
	cholesterol	"	112	151	----
	BP[d]	"	2374	1288	1086
	E_1	GMP	1369	934	435
	E_1	dGMP	1228	932	296
	E_1	DNA	1277	47	1230[c]
	E_2	"	598	108	490[c]
PB-induced	E_1	polyG	331	243	88
Control	E_1	"	361	280	81

[a] Incubations were as described in Methods; ± NADPH indicates presence or absence during incubation of NADPH and NADPH-regenerating system.

[b] After three precipitations with ethanol (12), the remaining radioactivity is first divided by the specific radioactivity of the steroid before incubation and then by the mg nucleic acid recovered as determined by its 252-260 nm absorption. Range of pmoles steroid bound is ± 10% of the indicated values.

[c] No corrections were made here for protein-bound radioactivity (protein is ca. 10-27% (w/w) of nucleic acid) or for possible loss of tritium due to hydroxylation (see Results and Discussion)

[d] Benzo[a]pyrene (BP)binding is shown for comparison.

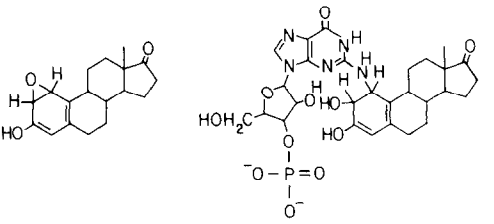
sequently, each of the estrogen-nucleic acid incubation mixtures contained the same amount (0.5 nmole) of the cytochrome and a comparison was made of the binding of estrone to polyG catalyzed by the three monooxygenase preparations. The

results (Table I) show that 3-MC-induced microsomes gave much better net binding to polyG of estrone than PB-induced or control microsomes; these data are consistent with the idea that an estrogen arene oxide, such as the one shown on Figure 1A or its tautomeric keto form, could be the "activated" estrogen intermediate in that binding. Similar incubations of estrone or estradiol with 3MC-microsomes in the absence of added nucleic acid gave net (difference) bindings in the order of 500 and 200 pmoles steroid bound to protein, respectively (cf. Table I values); such increased binding of BP to microsomal protein in the absence of nucleic acid has also been observed (16).

Table I and results below, indicate that DNA and nucleotides are also good "traps" of the estrogen intermediate(s). Furthermore, mestranol and estrone sulfate can also bind to polyG and BP (80 nmoles per incubation mixture) showed a 4fold higher net binding to polyG than estrone and very high (50%) non-specific binding in the absence of a source of electrons. Progesterone and cholesterol, as expected, did not bind to polyG.

The extent of ^{3}H-steroid or ^{3}H-BP binding to nucleic acids, shown on Table I, would be underestimated if the steroid lost tritium due to hydroxylation by the microsomal monooxygenases.

Phenol extractions of the incubation mixtures drastically reduced the binding of estrogens to nucleic acids (see below). In order to show that estrogens were bound to polyG covalently, we treated the ethanol precipitates with T_1 RNAse. Whereas there was a 20-40% loss of radioactivity in the ethanol-precipitable material, indicating partial hydrolysis of nucleic acid in the presence of microsomal proteins, the ratio of radioactivity to A^{252} in the pellet remained unchanged (within 10%). In other experiments, incubation mixtures of 3MC-microsomes, polyG and ^{3}H-E_1, were precipitated once with salt-ethanol and the pellets treated with proteinase K (a protease free of nucleases); the hydrolysates were then precipitated twice with ethanol and it was found that the pellets contained no protein but all of the added polyG and 85-90% of initial radioactivity, indicating that most of the metabolized estrogen was bound to polyG. However, similar



A B

Figure 1. Proposed structures of an estrone arene oxide (A) and resulting adduct through the exocyclic amino group of 3'-GMP(B), by analogy with the well characterized structure of the benzo[a]pyrene adduct (9,17).

proteinase K treatment of 3MC microsomes-DNA-E_1 or E_2 incubation mixtures resulted in complete loss of protein, 75% recovery of DNA and 12% recovery of radio activity which would correct the values of Table 1 to 140 pmoles E_1 and 50 pmoles E_2 net bound per mg DNA. Experiments are in progress concerning the specific nuclease sensitivity of these proteinase K treated precipitates.

Reverse phase high-pressure liquid chromatographic experiments (DuPont ODS-columns, 0-100% methanol-water gradients at 50°) are now in progress to analyze the hydrolysates of the above nucleases or GMP, dGMP incubations (Table 1). Our results at the present time show that a) there is no E_1 or E_2 left in the pellets after three ethanol precipitations and b) the peaks of radioactivity have shorter retention times (are more polar) than 2-hydroxyestrogens; after alkaline phosphatase hydrolysis some of these peaks became less polar and some more polar indicating in the former case that the estrogens are possibly bound to mononucleotides or oligonucleotides. This interpretation is strengthened by similar studies using ^{14}C-dGMP and unlabelled estrone. The fact that alkaline phosphatase treatment increases the polarity of some chromatographic peaks is puzzling, but we also observed it in the BP-polyG experiments. This would suggest that estrogen and BP arene oxides may bind to the phosphate groups of nucleic acids forming phosphotriesters as has been observed with DNA alkylating agents (18).

Figure 1B is drawn by analogy from the known BP diol-epoxide-poly G adduct (9,17). One explanation of the observed lability to phenol extraction of the

nucleic acid-estrogen binding could be the acidity of phenol, assisted by the aromatization of the A ring; we expect the BP diol-epoxide-GMP adduct to be less acid-labile than estrogen-GMP.

In conclusion, our results suggest that a) different estrogens are metabolized to varying degree by the P-448 monooxygenase(s), b) activated estrogen intermediates will bind covalently to more than one functional group of the nucleic acids but the stability of these bonds to various chemical treatments may vary and c) from the work of Nelson et al. (7) and of others (4,6) estrogen arene oxides are probably not the exclusive form of estrogen "activation".

The question whether or not estrogens, naturally occuring or prescribed (e.g. estrone sulfate and mestranol) are carcinogenic is still a matter of discussion (19-26). The concept of metabolic activation of some chemical carcinogens, originally proposed by the Millers (27), is also supported by our results with estrogen activation by a specific form (P-448) of microsomal monooxygenases. This enzyme system can be induced by environmental chemicals such as aromatic and polychlorinated hydrocarbons, cigarette smoke, etc. and may play a central role in carcinogenesis.

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