Chem.-Biol. Interactions, 23 (1978) 13—18

COVALENT BINDING OF ETHINYLESTRADIOL AND ESTRONE TO RAT LIVER DNA IN VIVO

WALTER JAGGI, WERNER K. LUTZ and CHRISTIAN SCHLATTER

Institute of Toxicology, Federal Institute of Technology and University of Zurich, CH-8603 Schwerzenbach (Switzerland)

(Received February 2nd, 1978)
(Revision received May 12th, 1978)
(Accepted May 22nd, 1978)

SUMMARY

The covalent binding of [6,7-^{3}H] ethinylestradiol (EE) and [6,7-^{3}H] estrone (E) to liver DNA of 200 g female rats was measured 8 h after the administration of 80 μg (9.2 mCi) estrogen by gavage. The binding is 1.5 for EE and 1.1 for E, expressed as binding to DNA/dose, in units of μmol hormone/mol DNA phosphate/mmole hormone/kg body wt. It is in the same order of magnitude as for benzene and about 10 000 times below the binding of typical liver carcinogens, such as aflatoxin B_1 or *N*,*N*-dimethylnitrosamine.

INTRODUCTION

Since 1973, several reports indicate that adenomas of the liver may occur in women taking oral contraceptives [1], and long-term toxicity studies in the beagle have shown that the administration of synthetic progestogens resulted in a range of mammary lesions [2]. There is no agreement as to the mechanisms whereby these steroids induce such lesions, or whether the lesions found with the beagle are important in assessing the potential safety of a compound for use in other species, such as humans. The hormonal activity of the compounds in question interacts closely with other endocrine secretions so that it is difficult to dissociate the effects of the hormones themselves from the secondary effects of other agents.

In the process of the chemical induction of a tumor, binding of that chemical or one of its metabolites to a biological macromolecule seems to be the first step [3,4]. Good correlations between that binding and carcino-

Abbreviations: E, estrone; EE, ethinylestradiol.

genicity have been found with DNA as target molecules rather than with RNA or protein [5,6]. A binding of ethinylestradiol and other hormones to protein has been measured in vitro and in vivo [7,8] but a binding to nucleic acids was detected only in vitro [9–11].

Our laboratory has specialized in detecting the binding of weak and suspect carcinogens to DNA in vivo [12,13], and in this paper we report our positive findings with tritiated ethinylestradiol (EE) and estrone (E).

6,7-tritiated estrogens were used, because 6 and 7 are relatively inert positions in the metabolism [7] and a binding to DNA in the presence of rat liver microsomes occured with a retention of that label [11].

MATERIALS AND METHODS

Materials

Reagents without specified distributor were of the highest purity available from Merck, Darmstadt, FRG.

[6,7-^{3}H]-E was radiosynthesized by reducing 6-dehydroestrone (3-hydroxy-1,3,5(10),6-estratetraen-17-one, 0.154 mmol, Steraloids Inc. Wilton, N.H.) according to Merrill and Vernice [14] with 0.14 mmol carrier-free tritium gas (8 Ci, The Radiochemical Centre, Amersham, U.K.) in 1 ml ethyl acetate containing 25.6 mg 10% Pd/C catalyst (Fluka AG, Buchs, Switzerland). After 3 h, the remaining educt was reduced with 0.19 mmol hydrogen gas.

[6,7-^{3}H]-EE was prepared by treating 17.9 mg tritiated E with 38.2 mg lithiumacetylide (Lithiumacetylid-Aethylendiaminkomplex pract., Fluka AG) and separated from the E after treatment with 15.4 mg Girard's reagent P (Fluka AG) according to Cooley and Harris (15).

Purification: Immediately before use, the radioactive hormones were chromatographed preparatively on silica gel thin layers (DC Alufolien Kieselgel 60F_{254}, 0.2 mm, Merck) with chloroform/ethyl acetate, 4 : 1. The hormones were eluted from the appropriate locations (as compared with inactive hormones from Serva, Heidelberg, FRG) with ethanol and ether. The solvent was blown off with nitrogen and the hormone was redissolved in 1 ml ethanol, fractions of which were administered to the rats by gavage. Control fractions were used for the determination of the specific activity and were rechromatographed. The radiochemical purity was found to be better than 98.8% for EE (0.6% were E) and 99.6% for E. The specific activity of these compounds was 32.5 Ci/mmol as measured from liquid scintillation counting in a BF 5000 counter (Berthold, Wildbad, FRG) and UV analysis [16].

Animals

Female rats (SIV-50, Sprague—Dawley-derived) were obtained from the Kantonales Tierspital, Zürich, Switzerland. They were fasted the night

TABLE I

INCORPORATION OF RADIOACTIVITY INTO LIVER DNA OF 4 FEMALE RATS, 8 H AFTER A GAVAGE OF TRITIATED STEROID HORMONE

		EE		E	
Dose administered	μg	79	91	56	94
	mCi	8.7	10.0	6.7	11.3
	mmol/kg	0.00139	0.00154	0.00087	0.00145
DNA isolated	mg	3.3	3.7	7.1	7.3
	gross cpm	296	441	278	605
	μmol/molDNA P	$1.86 \cdot 10^{-3}$	$2.55 \cdot 10^{-3}$	$8.18 \cdot 10^{-4}$	$1.79 \cdot 10^{-3}$
Binding index	$\frac{\mu mol/mol\ DNA\ P}{mmol/kg}$	1.34	1.65	0.94	1.24
	mean	1.5		1.1	

before treatment but had free access to drinking water. The weight ranged from 190—240 g. At 11:00 h, the hormone was administered with dose and radioactivity as given in Table I. The animals were held in open metabolism cages for the collection of urine. After 6 h, they were given 5 ml tap water by gavage in order to increase urine production. At 19:00 h, the animals were killed by stunning and cervical dislocation, were bled by heart puncture and the liver was excised.

Isolation of DNA.

DNA was purified according to Markov and Ivanov [17] and redissolved in 4 ml 0.014 M sodium phosphate buffer, pH 6.8. An aliquot was diluted for UV analysis and the bulk quantity was mixed with 10 ml Insta-Gel (Packard, Downers Grove, Ill.) for liquid scintillation counting. The amount of DNA was determined by assuming an absorbance of 190 for a 1% solution at 260 nm. The absorbance ratio 260 nm/230 nm was better than 2.27 in all DNA samples so that a maximal protein contamination of 0.1% can be expected [17].

Control experiments.

Background radioactivity of DNA was measured from a rat which was held in parallel to the treated ones. The gross count amounted to 21.7 ± 0.6 cpm from 2.5 mg DNA.

Non-enzymatic binding of the hormones to DNA was checked by incubating 3 g of liver, homogenized and denatured in 25 ml lysing medium (0.24 M sodium phosphate, pH 6.8, 8 M urea, 0.01 M EDTA, 1% sodium dodecyl sulfate) with 2.7 mCi hormone for 4 h at room temperature. The net count after isolation of that DNA was 12 cpm from EE and from E.

Incorporation of tritium from tritiated water (HTO) into liver DNA was

determined 12 h after administrating 10 mCi HTO/kg to control rats by stomach tube. The specific radioactivity amounted to 21 dpm/mg DNA.

RESULTS

Table I summarizes the experiments performed on the binding of tritiated EE and E to rat liver DNA, 8 h after oral administration of the hormone.

The control experiment on non-enzymatic incorporation of radioactivity into DNA showed that the radioactivity recovered from the main experiments must be covalently bound. Such a binding can occur from a hormone molecule, one of its metabolites or from the incorporation of tritium from tritiated water. Water could be formed by metabolic oxidation of the tritiated carbon atoms 6 or 7, or by the release of a tritium atom during the radioactive decay of its neighbor. Analysis of the urine collected after the administration of the hormones showed that from EE only 1% of the total radioactivity was excreted in the 8-h urine. Four percent of that radioactivity lyophilized as tritiated water. With estrone, the corresponding figures were 3% excreted, 3% of which was tritiated water. The specific activity of water in urine was therefore in the order of 10^6 dpm/ml, and we can assume that the total body water had a similar specific activity. The control experiments on the incorporation of tritium from tritiated water into DNA were performed with a specific activity of the body water of about $3 \cdot 10^7$ dpm/ml. The corresponding specific activity in the hormone experiments was thirty times lower so that, theoretically, less than 0.7 dpm/mg DNA could be due to that mode of DNA labelling. This is so little as compared to the specific activity of DNA from the main experiment that our binding values must represent the labelling of DNA with a hormone derivative.

DISCUSSION

We have demonstrated a covalent binding of EE and E to rat liver DNA in vivo. Carcinogenic activities of these — and probably other — estrogens could therefore be based also upon their chemical reactivity towards nucleic acids and do not necessarily depend upon the hormonal activity. The introduction of the ethinyl substituent into the naturally occurring estrone enhances the binding capacity slightly, probably due to the fact that the metabolic degradation at position 16 is slower in the case of the synthetic analogue and leads to an increased formation of other, chemically more reactive, metabolites. This is in agreement with the in vitro experiments [7] where the binding of EE to protein is about twice that of estradiol.

The binding index found with the hormones is similar to that of benzene (1.7, see ref. 12), but about 4 orders of magnitude below those of typical liver carcinogens such as aflatoxin B_1 [18] or *N,N*-dimethylnitrosamine [19]. For a comparison of different chemicals the binding index gives the number of molecules which are bound to DNA from an administration of

a unit dose of 1 mmol/kg. If the binding from a specific exposure has to be estimated, e.g. for a risk assessment, the effective dose of that specific chemical to which an individual is exposed must also be accounted for. Typical exposures to benzene which eventually lead to leukemia in humans amount to grams/day [20]. The exposure to EE as a contraceptive, on the other hand, is only about 50 μg/day.

The damage expected from the natural estrogens is similar to the one found from the use of the ethinyl derivative: A woman produces an average of a few 100 μg/day while towards the end of a pregnancy, this production increases by more than a factor of 100.

This quantitative discussion of binding and presumptive carcinogenic risk is still very tentative. Additional data are required on the mutagenic potency of a specific type of damage to the DNA as well as a measure of the repair of the DNA in the target organ. Nevertheless, EE and E should be equal in this respect so that on the basis of our findings we can deduce that E and its ethinyl derivative should not differ much with regards to a suspected chemical carcinogenicity.

REFERENCES

1 H.A. Edmondson, B. Henderson and B. Benton, Liver-cell adenomas associated with use of oral contraceptives, New Engl. J. Med., 294 (1976) 470.
2 M. Briggs, The beagle dog and contraceptive steroids, Life Sci., 21 (1977) 275.
3 J.A. Miller, Carcinogenesis by chemicals: an overview. G.H.A. Clowes memorial lecture, Cancer Res., 30 (1970) 559.
4 P.N. Magee, A.E. Pegg and P.F. Swann, Molecular mechanisms of chemical carcinogenesis, in: Handbuch der allgemeinen Pathologie vol. VI/6, Springer, Berlin, 1975, p. 329.
5 P. Brookes and P.D. Lawley, Evidence for the binding of polynuclear aromatic hydrocarbons to nucleic acids of mouse skin: relation between carcinogenic power of hydrocarbons and their binding to deoxyribonucleic acid, Nature (Lond.), 202 (1974) 781.
6 E. Huberman and L. Sachs, DNA binding and its relationship to carcinogenesis by different polycyclic hydrocarbons, Int. J. Cancer, 19 (1977) 122.
7 H.M. Bolt, H. Kappus and R. Käsbohrer, Metabolism of 17α-ethinylestradiol by human liver microsomes in vitro: Aromatic hydroxylation and irreversible protein binding of metabolites, J. Clin. Endocrinol. Metab., 39 (1974) 1072.
8 H. Kappus, H.M. Bolt and H. Remmer, Irreversible protein binding of metabolites of ethinylestradiol in vivo and in vitro, Steroids, 22 (1973) 203.
9 H.M. Bolt and H. Kappus, Irreversible binding of ethinylestradiol metabolites to protein and nucleic acids as catalyzed by rat liver microsomes and mushroom tyrosinase, J. Steroid Biochem., 5 (1974) 179.
10 J.C.M. Tsibris and P.M. McGuire, Microsomal activation of estrogens and binding to nucleic acids and proteins, Biochem. Biophys. Res. Commun., 78 (1977) 411.
11 G.M. Blackburn, L. Orgee and G.M. Williams, Oxidative bonding of natural oestrogens to DNA by chemical and metabolic means, J.C.S. Chem. Comm., 1977 No. 11 386.
12 W.K. Lutz and Ch. Schlatter, Mechanism of the carcinogenic action of benzene: irreversible binding to rat liver DNA, Chem.-biol. Interact., 18 (1977) 241.
13 W.K. Lutz and Ch. Schlatter, Saccharin does not bind to DNA of liver or bladder in the rat, Chem.-biol. Interact., 19 (1977) 253.

14 E.J. Merrill and G.G. Vernice, Synthesis of 17α-ethinylestradiol-6,7-^{3}H and 17α-ethinylestradiol-6,7-^{3}H-3-cyclopentyl-1-^{14}C-ether, J. Labelled Compds., 6 (1970) 266.
15 G. Cooley and I.A. Harris, Preparation of ethinyloestradiol-4-^{14}C, J. Labelled Compds., 5 (1969) 8.
16 E.G.C. Clarke (ed.), Isolation and identification of drugs, The Pharmaceutical Press, London, 1969, pp. 335 & 454.
17 G.G. Markov and I.G. Ivanov, Hydroxyapatite column chromatography in procedures for isolation of purified DNA, Anal. Biochem., 59 (1974) 555.
18 D.H. Swenson, J.-K. Lin, E.C. Miller and J.A. Miller, Aflatoxin B_1-2,3-oxide as a probable intermediate in the covalent binding of aflatoxin B_1 and B_2 to rat liver DNA and ribosomal RNA in vivo, Cancer Res., 37 (1977) 172.
19 P.J. O'Connor, M.J. Capps and A.W. Craig, Comparative studies on the hepatocarcinogen *N,N*-dimethylnitrosamine in vivo: reaction sites in rat liver DNA and the significance of their relative stabilities, Br. J. Cancer, 27 (1973) 153.
20 E.C. Vigliani, Leukemia associated with benzene exposure, Ann. New York Acad. Sci., 271 (1976) 143.