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IN VITRO SYNTHESIS OF 16α-HYDROXYESTRONE BY FEMALE RAT LIVER MICROSOMES: ITS POSSIBLE ROLE IN THE ETIOLOGY OF BREAST CANCER

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Summary—Liver homogenates from female rat strains (Sprague–Dawley, Wistar and Fisher) were incubated in a NADPH regenerating medium in the presence of labelled and unlabelled estrone. Liver microsomes isolated from male rats and female mice were used as positive controls. Using HPLC and paper chromatography, under the experimental conditions used it was found that liver homogenates from female rats were able to convert estrone to various metabolites such as 16α-hydroxyestrone. In a mutagenicity assay (Ames test), with 16α-hydroxyestrone as test substance, two strains (TA98 and TA1538) of the five strains tested showed a 2–3-fold increase in the number of his^+ revertants relative to the control values. Estrone did not cause any mutagens in the test used.

It is concluded that female rats are able to synthesize 16α-hydroxyestrone *in vitro*. Whether this compound is risk factor for breast cancer remains unclear.

INTRODUCTION

Estrone (E_1) is quantitatively the most important metabolite of estradiol (E_2) throughout the menstrual cycle and has been found in high concentrations in breast cyst fluid as well as in mammary tumours [1]. 16α-Hydroxyestrone (16α-OH-E_1) is an intermediate product in the conversion of E_1 to estriol (E_3) [2]. It shows uterotrophic activity, decreases LH secretion and has a very low binding affinity for the rat uterine cytosol estrogen receptor. Furthermore, it shows a very low binding affinity for sex hormone binding globulin [3, 4]. Its most striking property is the formation of covalent bonds with amino groups of biological macromolecules [5–7]. A close correlation between estradiol 16α-hydroxylation and mammary tumour incidence was reported both for men and for animals. Women with breast cancer showed 50% more 16α-hydroxylation compared to a control group free of overt disease [3]. Mice strains with a high incidence of mammary tumours (C_3H) demonstrated elevated 16α-hydroxylation *in vivo* compared to strains with a low tumour incidence ($C_{57}Bl$) [8].

A discrepancy was found for the presence of 16α-hydroxylase activity in female rats. In contrast to male rats, Bradlow *et al.*[9] did not find activity in female Sprague–Dawley rats whereas other investigators [10, 11] reported low activities of 16α-hydroxylase in *in vivo* experiments with these female rats.

In this study we report the *in vitro* formation of 16α-OH-E_1 from E_1 using liver microsomes from various strains of female rats. Liver microsomes from male Sprague–Dawley and Wistar rats and of female mice ($C_{57}Bl$ and C_3H) were used as positive controls. The genotoxic potential of 16α-OH-E_1, which might be indicative of its carcinogenicity, was studied in the Ames test with various strains of *Salmonella typhimurium* and a liver homogenate fraction (S9) of both male and female, Aroclor-induced Wistar rats as metabolic activation system.

EXPERIMENTAL

Animals

Adult female $C_{57}Bl$ and C_3H mice (weighing about 16 g) were obtained from The Netherlands Cancer Institute (Antonie van Leeuwenhoekhuis), Amsterdam, The Netherlands. Adult male and female Sprague–Dawley rats (weighing about 325 and 200 g, respectively) and female Fisher rats (weighing about 200 g) were obtained from Charles River, Kent, England. Adult male and female Wistar rats (Cpb:WU, Wistar random, weighing about 350 and 200 g, respectively) were obtained from TNO Central Institute for Breeding of Laboratory Animals, Zeist, The Netherlands.

Chemicals

All chemicals used were of HPLC or Analar grade. Estrone, 16α-hydroxyestrone, estradiol and estriol were purchased from Sigma Chemicals Co. (St Louis, Mo., U.S.A.). [2,4,6,7-^{3}H(*N*)]estrone (sp.

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act. 3.9 TBq/mmol) was purchased from Amersham, Houten, The Netherlands, and purified by HPLC before use. 16-Keto-estradiol was kindly donated by Dr J. H. H. Thijssen, Academic Hospital, Utrecht, The Netherlands.

Preparation of microsomes

Animals (3 from each strain, species or sex) were anaesthesized with ether and then decapitated. The livers were removed immediately, minced and homogenized in ice-cold 0.01 M phosphate-buffered saline (pH 7.0) with a Potter-Elvehjem glass-Teflon homogenizer. An aliquot of the homogenates, corresponding with 1 g of liver, was centrifuged at 20,000 *g* for 20 min; the resultant supernatants were centrifuged in a Beckmann ultracentrifuge for 50 min at 105,000 *g* (30,000 rpm) to give microsomal pellets. The microsomes were resuspended in 1 ml 0.01 M phosphate-buffered saline and used immediately for the incubation experiments.

Incubation of liver microsomes with estrone

Incubation was performed as described previously [12] with some minor modifications. Briefly, liver microsomes (0.2 ml) were incubated in 2 ml phosphate-buffered saline solution containing 10 μM E_1 and 2 μCi [2,4,6,7-^{3}H(*N*)]E_1 for 60 min at 37°C in the presence of 100 μl of a NADPH generating system (2.4 mM NADP, 10 mM glucose-6-phosphate, 2 U glucose-6-phosphate-dehydrogenase/ml). Incubation mixtures without liver microsomes served as blanks. The reaction was terminated by chilling in ice-water.

Extraction and purification of estrogens

Immediately after incubation, 8 ml of a 1:1 mixture of ethanol and acetone were added to the microsome suspensions and thoroughly mixed. After centrifugation at 800 *g* for 10 min at 4°C, supernatants were decanted and evaporated at 40°C under a mild stream of nitrogen. The residues were dissolved in 20% methanol. Thereafter reversed phase C18-cartridges, activated by subsequent washing with 2.5 ml 100% methanol and 2.5 ml distilled water, were used for purification of the extracts. After transferring to a C18-cartridge and subsequently washing with 2 ml distilled water and 2 ml 50% methanol, the hormones were eluted with 2 ml methanol. The methanol fractions were evaporated to dryness at 40°C under a mild stream of nitrogen. The residue was dissolved in 25 μl isopropyl alcohol by vortexing and 225 μl *n*-hexane was added.

High-performance liquid chromatography (HPLC)

HPLC was performed as described recently [13] with slight modifications: A Lichrosorb Diol column (Merck, Darmstadt, F.R.G.; 125 × 4.6 mm) was used with isopropyl alcohol in hexane (1:9; v/v) as eluent and a flow rate of 1.2 ml/min. Base line separations between E_1, E_2, 16α-OH-E_1 and E_3 were obtained (Fig. 1A). Fractions with the same retention time as 16α-OH-E_1 were collected. One-third of the liquid fraction was used for counting tritium activity. The remaining part was evaporated to dryness and used for further identification by paper chromatography.

Paper chromatography

Paper (Whatman No. 1) was impregnated with a formamide:acetone solution (1:4; v/v) and dried between filter-paper. HPLC-fractions, possibly containing 16α-OH-E_1, were chromatographed together

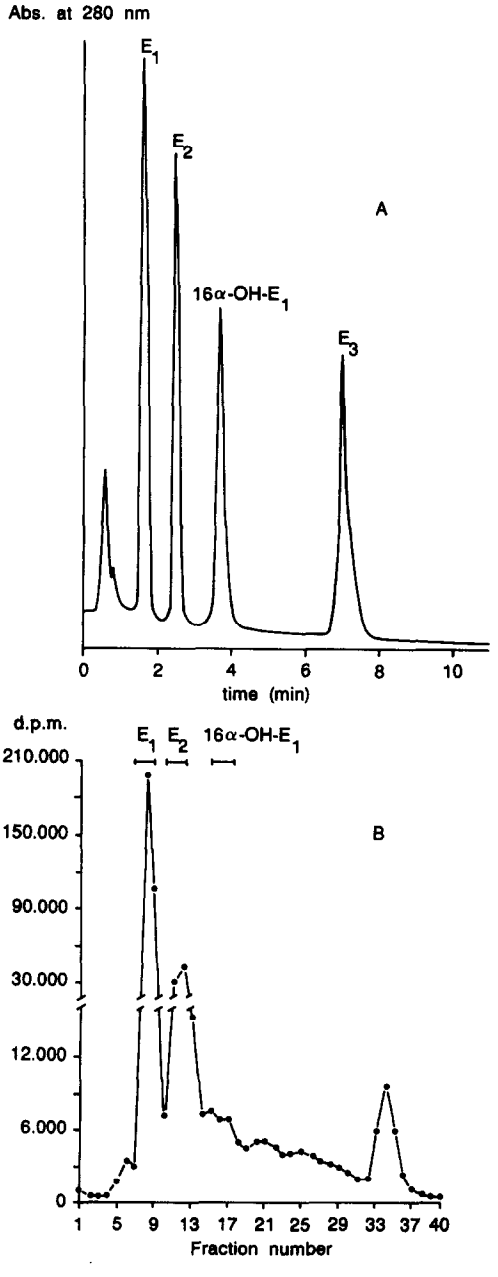

Fig. 1. Elution profiles after HPLC. (A) Standards of estrone (E_1), estradiol (E_2), 16α-hydroxyestrone (16α-OH-E_1) and estriol (E_3). (B) A sample extract obtained after incubation of liver microsomes from female Fisher rats with labelled estrone.

with authentic standards of 16-keto-estradiol (16K-E_2) and 16α-OH-E_1. Elution was performed with chloroform. Detection of standards was done by spraying with sodium carbonate (20% w/v) and Folin Ciocalteu reagens diluted 1:3 in water. The paper was cut into 1 cm pieces; radioactivity was successively counted after addition of scintillation liquid (Atomlight). Peaks of radioactivity with the same retention time as authentic 16α-OH-E_1 were supposed to contain both labelled and unlabelled 16α-OH-E_1.

Mutagenicity assay

Salmonella typhimurium strains TA 1535, TA 1537, TA 1538, TA 98 and TA 100 were obtained from Dr B. N. Ames, University of California. The mutagenicity assay was carried out as described by Ames *et al.*[14] using the plate incorporation procedure with and without a liver metabolic activation system (S-9 mix) of female and male, Aroclor-induced Wistar rats. Briefly, to 2 ml topagar (0.6% agar containing 0.5 mM histidin/biotin) were added in this sequence: 0.1 ml of the appropriate bacterial suspension, 0.1 ml of the appropriate concentration of estrone or 16α-OH-E_1 (dissolved in DMSO) and 0.5 ml of the S-9 mix, if any. The mixtures were then poured out onto minimal glucose agar plates. After a 3-day incubation period at 37°C, revertant colonies were counted. In some experiments test solutions, bacteria and S-9 mix were preincubated for 20 min at 37°C. Thereafter, the mixtures were poured out onto minimal glucose agar plates, the plates incubated for 3 days at 37°C and the revertant colonies counted.

RESULTS

Addition of liver microsomes to a NADPH regenerating medium containing an excess of E_1 results in the formation of polar and non-polar components. During purification of the extracts with reversed-phase C18 cartridges, the relatively most polar components were removed by washing with 50% methanol in water. These polar components equal

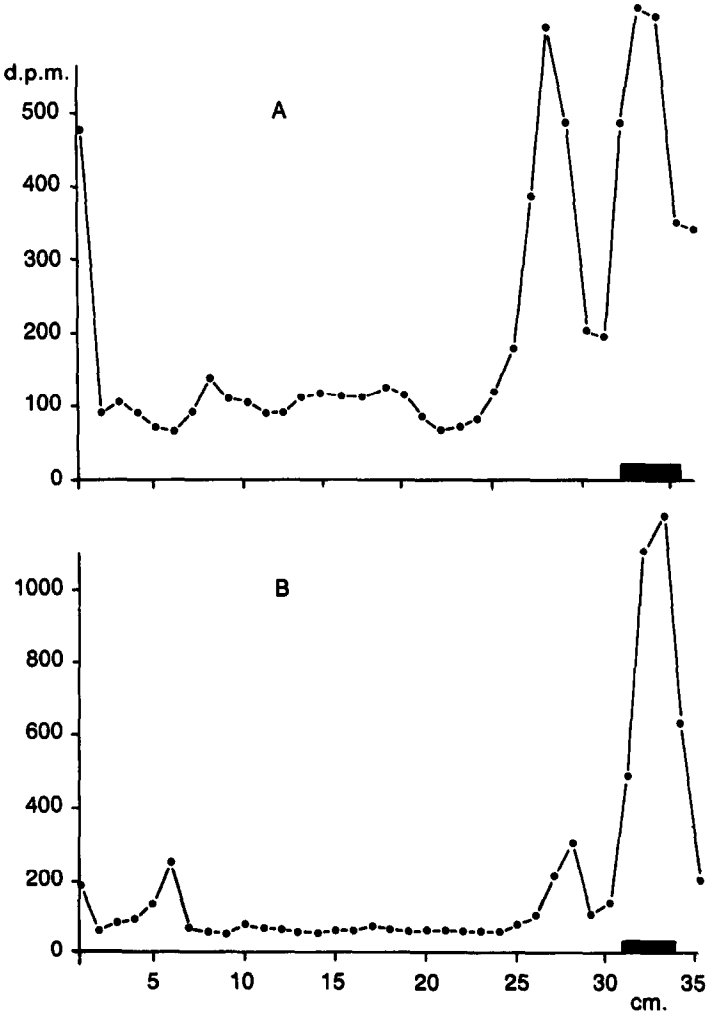

Fig. 2. Elution profiles after paper chromatography of the HPLC 16α-OH-E_1 fraction of samples obtained after incubation of liver microsomes from female Wistar rats (A) and female Fisher rats (B) with labelled estrone. ■ = Spot of authentic 16α-hydroxyestrone.

Table 1. Radioactive counts (dpm) corrected for the procedural loss, on the spot of 16α-OH-E_1 after paper chromatography ($n = 3$)

	Female	Male
Rats		
Wistar	6800 ± 1400	13,000 ± 2500
Sprague–Dawley	680 ± 75	5500 ± 1400
Fisher	12,750 ± 940	—
Mice		
C57Bl	5100 ± 420	—
C3H	1570 ± 480	—

(—) Not analysed.

15–20% of the original quantity of labelled compound. After reversed-phase C18 chromatography 60–80% of the original radioactivity added is recovered. During HPLC radioactivity is distributed among the different fractions. In Fig. 1B a typical HPLC profile is shown, obtained after incubation of liver microsomes from female Fisher rats with E_1. Of the total activity added, 14% was recovered in the E_1 fraction, 4% in the E_2 fraction and 1% in the 16α-OH-E_1 fraction.

The HPLC fractions containing the 16α-OH-E_1 were collected and rechromatographed on the paper chromatography (PC) system. With this system a nearly quantitative separation between 16α-OH-E_1 and 16K-E_2 can be obtained, as both compounds elute in the same HPLC fraction. The radioactivity profiles obtained after PC for female Wistar and Fisher rats are shown in Fig. 2. For both strains radioactivity was found on the 16K-E_2 as well as on the 16α-OH-E_1 spot, equalling 0.01–0.001% of the total radioactivity originally added. For female Wistar rats, the amount of radioactivity in the 16K-E_2 and the 16α-OH-E_1 position was about the same. For Fisher rats, most of the radioactivity applied was recovered on the spot with the R_f value of 16α-OH-E_1. In Table 1 the counts measured in the 16α-OH-E_1 fraction after HPLC and PC are presented for the various species/strains/sexes. These values are corrected for the loss during the clean-up procedure (50%) and for HPLC and PC aliquot factors (1.25 and 1.5, respectively).

The results of the Ames test showed a slight, but reproducible increase in the number of his$^+$ revertants with strains TA 1538 and TA 98 only in the presence of S-9 mix and with 16α-OH-E_1 as test substance (Table 2). Maximum values (2–3 times the control values) were obtained at dose levels of 500–2500 μg/plate. No significant increase in the number of his$^+$ revertants was observed in the absence of S-9 mix. There was no effect of the type of liver homogenate used (either male or female; results not shown). With estrone as test substance, the Ames test did not show any mutagenicity with or without the presence of a S-9 mix (Table 3).

DISCUSSION

The production of 16α-OH-E_1 by the hydroxylation of estrone was studied *in vitro* in a NADPH regenerating system containing liver microsomes from various species. In the system described, a small amount of 16α-OH-E_1 (<0.01%) is synthesized from estrone. A great part is not metabolized, reduced to estradiol (Fig. 1B) or metabolized to other compounds [15]. 16K-E_2 is one of the compounds synthesized from estrone. In the HPLC system used, this metabolite elutes in the same fraction as 16α-OH-E_1. With PC and chloroform as the eluent a clear separation between 16K-E_2 and 16α-OH-E_1 can be obtained.

The disadvantage of an *in vitro* model is the absence of a normal physiological environment with the presence of compounds influencing the 16α-hydroxylase activity [16]. However, with an *in vivo* experiment a direct production of 16α-hydroxyestrone is difficult to demonstrate due to the low concentrations synthesized.

A 2-fold (Wistar) to 8-fold (Sprague–Dawley) higher amount of 16α-OH-E_1 is produced by liver microsomes from the male control rats compared with those of the female strains (Table 1). This relatively higher 16α-hydroxylase activity was also found in male Sprague–Dawley rats during metabolism studies of 4-[^{14}C]androstene-3,17-dione by other investigators [10, 11]. The elevated 16α-hydroxylase activity in male rats can be explained by circulating androgen and by the male-type pattern of blood GH levels [16]. In this experiment, an extremely low production of 16α-hydroxyestrone was found in

Table 2. Number[a] of his$^+$ revertants per plate of the Salmonella/microsome mutagenicity test (Ames test) with 16α-hydroxyestrone in the presence of S-9 mix from male Wistar rats (Arocolor-induced)

Dose (μg/plate)	Salmonella strains				
	TA1535	TA1537	TA1538	TA98	TA100
0	22 ± 3	15 ± 2	33 ± 3	48 ± 10	144 ± 13
62	19 ± 1	16 ± 6	—	48 ± 3	148 ± 24
185	23 ± 5	14 ± 2	—	59 ± 6	140 ± 6
500	—[b]	—	68 ± 5	68 ± 12	—
555	24 ± 1	23 ± 6	—	88 + 9	162 ± 11
1000	—	—	87 ± 12	80 ± 5	—
1500	14 ± 3	13 ± 3	95 ± 12	110 ± 10	—
2000	—	—	69 ± 5	106 ± 17	—
2500	—	—	47 ± 4	95 ± 2	—

[a]Mean of triplicate values ± SD.
[b]Test not performed at that dose/plate.

Table 3. Number[a] of his$^+$ revertants per plate of the Salmonella/microsome mutagenicity test (Ames test) with estrone

Dose	TA1535		TA1537		TA1538		TA98		TA100	
(μg/plate)	−S9[b]	+S9	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
0	39 ± 2	21 ± 3	14 ± 2	20 ± 3	15 ± 3	48 ± 1	26 ± 7	66 ± 10	143 ± 4	149 ± 3
37	43 ± 4	17 ± 5	12 ± 5	14 ± 5	14 ± 3	46 ± 7	25 ± 3	65 ± 10	158 ± 24	159 ± 10
111	45 ± 5	20 ± 5	8 ± 5	12 ± 1	15 ± 2	44 ± 4	32 ± 6	54 ± 4	169 ± 15	159 ± 29
333	36 ± 8	26 ± 4	11 ± 3	12 ± 2	16 ± 4	57 ± 9	31 ± 5	62 ± 8	131 ± 4	170 ± 8
1000	36 ± 3	15 ± 4	13 ± 5	9 ± 1	16 ± 3	45 ± 2	31 ± 3	61 ± 5	155 ± 1	142 ± 7
3000	34 ± 6	18 ± 1	14 ± 6	14 ± 5	21 ± 3	45 ± 7	25 ± 6	62 ± 5	151 ± 11	140 ± 20

[a]Mean of triplicate values ± SD.
[b]With (+) or without (−) the presence of S-9 mix derived from male Wistar rats (Arocolor-induced).

the Sprague–Dawley female strain compared with the female Wistar and Fisher strains (Table 1), suggesting strain dependence of 16α-hydroxyestrone production.

In the mutagenicity assay (Ames test) only two strains (TA 98 and TA 1538) of the five strains tested showed a slight increase in the number of his$^+$ revertants at a non-physiological high concentration of 16α-OH-E_1. It is doubtful, whether this is of any significance to support the postulated role of 16α-OH-E_1 as risk marker for breast cancer [3, 8]. However, administration of estrone to rats by s.c. implantation [17], resulting in an estimated absorption of 6–7 μg estrone per day, caused mammary tumours in various female strains tested. As estrone itself seems not to be mutagenic [18; Table 3], this may be explained by an increased concentration of 16α-hydroxyestrone resulting from the conversion of estrone.

Contrary to preliminary data [19], we now conclude that liver microsomes from female rats are able to synthesize 16α-hydroxyestrone *in vitro*. The results of the Ames test do not support a strong direct role of 16α-OH-E_1 as initiator in the carcinogenesis process. Whether or not 16α-OH-E_1 is risk factor for breast cancer remains unclear.

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