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METABOLISM OF ESTRADIOL-17β, 5-ANDROSTENE-3β,17β-DIOL AND TESTOSTERONE IN HUMAN BREAST CANCER CELLS IN LONG-TERM CULTURE

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SUMMARY

The human breast cancer cell line MCF-7 is able to metabolize steroids, which are added in order to study the growth rate of these cells. The following steroids: estradiol-17β, 5-androstene-3β,17β-diol and testosterone were incubated with these cells for 48 h under identical conditions used for growth-rate studies in our laboratory. The metabolites formed were identified. The conversion of estradiol-17β to estrone was 7% and no estriol was formed. The conversion of 5-androstene-3β-17β-diol to dehydroepiandrosterone was 8.1%, to testosterone 1.0% and to 5α-androstane-3β,17β-diol 3.2%. The conversion of testosterone to androstenedione was 6.5%, to etiocholanolone 0.4%, to 5α-dihydrotestosterone 0.7%, to 5-androstene-3β,17β-diol 0.5% and to androstenediol 0.2%. Some of these metabolites, due to their relative binding affinity, may have a biological effect on these cells that contain androgen and estrogen receptors.

INTRODUCTION

A number of investigators have studied the effects of steroid hormones on the human breast cancer cell line MCF-7 [1–4]. The results of these studies are hampered by the potency of these cells to metabolize the steroid added and to produce in this way substances, which are biologically active in relation to the growth rate of cells *in vitro*. Effects observed may therefore partly be due to derivatives formed during incubation. In the course of an investigation on the influence of estradiol, testosterone and 5-androstene-3β,17β-diol [5] on the growth rate of the MCF-7 cell line we studied the metabolism of the steroids added to the culture.

EXPERIMENTAL

Materials

The human breast cancer cell line MCF-7 was provided by Dr M. Rich of the Michigan Cancer Foundation, Detroit, U.S.A. We obtained passage 238 of this cell-line, but the studies reported in this paper are done after passage 256 and further.

Medium A. MEM with Earle's salts, L-glutamine and non-essential amino acids, without sodium bicarbonate, was obtained from Gibco Bio Cult Ltd, Paisley, Scotland. To this medium was added: 10% calf serum (Flow Lab. Ltd, Irvine, Scotland), 2 mM L-glutamine (Gibco), 1 mM sodium pyruvate (Gibco), 0.22% sodium bicarbonate and 254 U/l bovine insulin (Organon, Oss, The Netherlands).

Medium B. The same as medium A but without insulin and calf serum. All non-radioactive steroids were obtained from Ikapharm, Ramat-Gan, Israel.

[1,2-^{3}H]-Testosterone (SA 16 Ci/mmol) and [1,2-^{3}H]-5-androstene-3β,17β-diol (SA 14 Ci/mmol) were obtained from New England Nuclear Corp., U.S.A.; [2,4,6,7,-^{3}H]-estradiol-17β (SA 91 Ci/mmol) was purchased from The Radiochemical Centre, Amersham, England. Their purity was checked by thin-layer chromatography. Celite, type 545, was obtained from Serva Feinchemie, Heidelberg, West Germany. Sephadex LH-20 was obtained from Pharmacia Fine Chemical, Uppsala, Sweden. Plastic petri dishes, 3.5 cm diameter, were purchased from C.A. Greiner and Söhne, Nürtingen, West Germany.

Thin-layer chromatography (t.l.c.): Kieselgel 60 plates, F-254, were purchased from Merck, Darmstadt, West Germany. Gas-liquid chromatography (g.l.c.): GCV-chromatograph from Pye-Unicam, Cambridge, England, using a 150 cm column, 0.4 cm diameter, stationary phase 1% XE-60. Liquid scintillation counting (l.s.c.) was done with a Packard Tricarb liquid scintillation spectrometer (type 2650, Packard Instrument Company, Münich, West Germany) with scintillator 299 TM, Packard-Becker, Groningen, The Netherlands. All other reagents were of analytical quality, obtained from Merck, Darmstadt, West Germany.

Method

10^5 Cells were cultured in a petri dish, containing 2 ml of medium A, in a humified incubator with 5% CO_2, 95% air atmosphere. After 24 h this medium was

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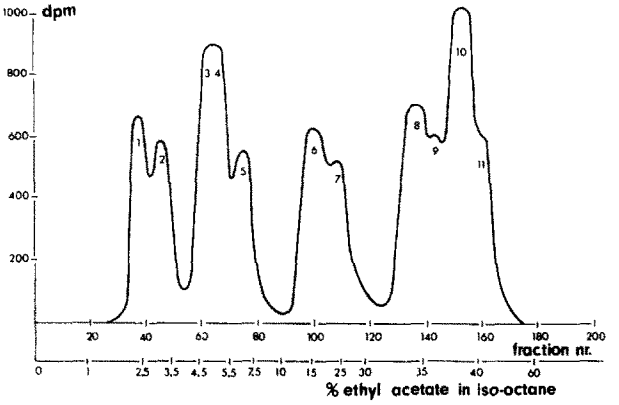

Fig. 1. Elution pattern of labelled reference steroids on a Celite column (diameter 1.8 cm, length 30 cm) containing 36 g of a mixture of Celite–ethylene glycol (10:6, w/v). 1, Androstenedione; 2, androsterone; 3, etiocholanolone; 4, 5α-DHT; 5, DHA; 6, estrone; 7, testosterone; 8, 5α-androstane-3α-17β-diol; 9, 5α-androstane-3β,17β-diol; 10, androstenediol; 11, estradiol.

removed and 2 ml of medium B was added. After 12 h this medium was removed and 2 ml of medium B was added containing 8.4×10^6 d.p.m. [^{3}H]-testosterone (1.1×10^{-6} M), or 4.8×10^6 d.p.m. [^{3}H]-androstenediol (1.0×10^{-6} M), or 3×10^6 d.p.m. [^{3}H]-estradiol (0.8×10^{-8} M). After 48 h of incubation, cells and medium were separated by centrifugation and the metabolites were determined in the medium. This incubation scheme was similar to that used in stimulation experiments in our laboratory (Vossenberg *et al.*, to be published). To the medium of the testosterone and androstenediol incubation, 100 μg of the following reference steroids were added: androstenedione, androsterone, etiocholanolane, androstenediol, testosterone, 5α-dihydrotestosterone, dehydroepiandrosterone, 5α-androstane-3α,17β-diol, 5α-androstane-3β,17β-diol, estrone and estradiol-17β. To both media 5 ml of ethanol was added and they were evaporated to dryness.

Metabolites of the testosterone and androstenediol incubation were separated on a column (diameter 1.8 cm, length 30 cm) containing 36 g of a mixture of Celite–ethylene glycol (10:6, w/v). At the bottom of the column was a water trap (3 g of a mixture of Celite–water, 2:1). Elution was done by iso-octane with an increasing concentration of ethyl acetate. From each fraction of 2.5 ml an aliquot of 200 μl was taken to determine the radioactivity.

A profile of the reference steroids was also constructed (Fig. 1). Fractions containing radioactivity were collected and chromatographed on t.l.c., using one of the following systems:

1, Benzene–methanol (9:1, w/v). 2, Benzene–ethyl acetate (2:1, v/v). 3, Cyclohexane–ethyl acetate (3:7, v/v).

Because androstenediol and the androstanediols are difficult to separate on t.l.c., another column (0.8 cm diameter, with 10 g of a mixture of Celite–ethylene glycol, 10:6, w/v) was used. Elution was done by iso-octane with an increasing amount of ethyl acetate. An elution profile of reference steroids is shown in Fig. 2.

The recovery of the added steroid was measured by g.l.c. after separation on column or t.l.c. The amount of radioactivity was measured by l.s.c. For the final identification each steroid was recrystallized (system: ethanol–water) after addition of 40 mg carrier until constant specific activity was achieved.

For the separation of the estrogens a Sephadex LH-20 column was used (0.5 cm diameter, 16 cm length). Elution solvent: chloroform–benzene–methanol (3:6:1, by vol.).

Radioactivity was measured in each 2-ml column fraction.

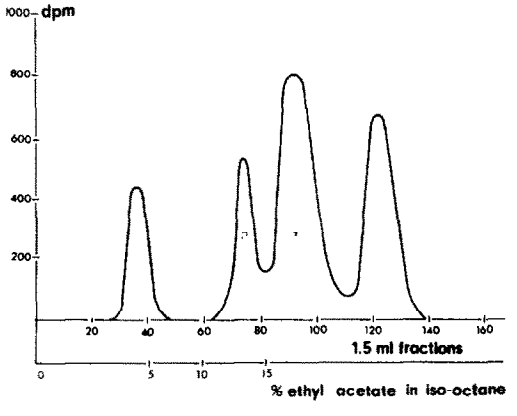

Fig. 2. Elution pattern of labelled reference steroids on a Celite column (diameter 0.8 cm) with 10 g of a mixture of Celite–ethylene-glycol (10:6, w/v). 1, 5α-androstane-3α,17β-diol; 2, 5α-androstane-3β,17β-diol; 3, androstenediol; 4, estradiol.

RESULTS

Incubation with estradiol

The recovery of the incubated radioactivity in the medium was 92% after separation of cells and medium.

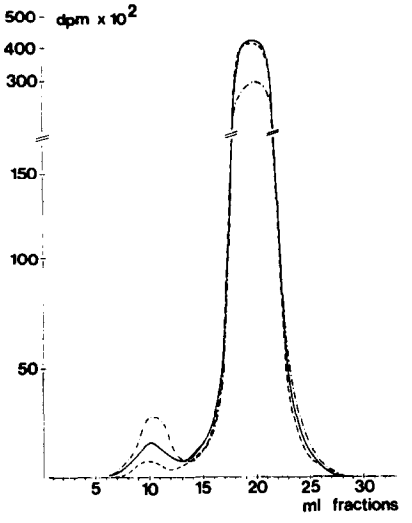

Fig. 3. Profile of recovered radioactivity after incubation of plated MCF-7 cells with 0.8×10^{-8} M. [^{3}H]-estradiol, separated on a Sephadex LH-20 column (diameter 0.5 cm, length 16 cm). Elution solvent: chloroform–benzene–methanol (3:6:1, by vol.). Incubation time: 1 h ---, 24 h —— and 48 h -·-.

Figure 3 shows the elution pattern of the radioactivity on Sephadex LH-20 after incubation with [^{3}H]-estradiol. Only two peaks could be detected after an incubation period of 1, 24 and 48 h. The major peak was unchanged estradiol, the other one was identified as estrone. No estriol was formed during these periods. Based on the radioactivity in the estrone fractions it could be calculated that the conversion to estrone was 2, 4 and 7% after 1, 24 and 48 h respectively.

Incubation with testosterone

The recovery of the incubated radioactivity in the medium was 94% after separation of cells and medium.

Figure 4 shows the elution pattern of the recovered radioactivity after chromatography on the Celite column (1.8 cm diameter). Five peaks were detected. Each peak was purified on t.l.c., the recovery of the added steroid was determined on GLC and radioactivity with l.s.c. The androstanediols were separated on the second Celite column and determined as described. Three-fifths of the amount of each steroid recovered was used for recrystallization. Each fraction, however, was recrystallized three times. Specific activity was calculated from the mean of the last two recrystallizations after reaching a constant specific activity in the mother liquor and crystals after two crystallizations. Results are given in Table 1.

Incubation with androstenediol (=5-androstene-3β,17β-diol)

The recovery of the incubated radioactivity in the medium was 95% after separation of cells and medium.

The elution pattern of the recovered radioactivity is shown in Fig. 5. Four peaks were detected. After purification on t.l.c. and column the recovery of the steroids added and the radioactivity of each steroid was determined. For recrystallization three-fifths part of each steroid extract was used. Specific activity after recrystallization was determined on the last two recrystallizations for the same reason as mentioned for the testosterone incubation. Results are shown in Table 1.

In a control experiment the whole procedure was carried out with testosterone and androstenediol in an incubation medium to which no cells had been added. Testosterone yielded a peak corresponding to peak nr. 3 in Fig. 4; androstenediol a peak corresponding to peak nr. 3 in Fig. 5. These metabolites are not contaminating impurities of the tracer because the elution pattern of the tracer without incubation did not show these peaks.

DISCUSSION

In discussing our results we assume that the quantity of endogenous steroid precursors in the medium was negligible and that therefore all metabolites found were derived from the steroid added. This assumption is justified by the technique used, in which the cells were cultured in serum free medium 12 h before and during incubation. In this study we have investigated only the metabolites formed in the medium. It appeared that in the three incubations done more than 92% of the total radioactivity was extractable from the medium. In one experiment we extracted the radioactivity from the medium as well as from the incubated cells. The elution profile from the Celite column was exactly the same as for cells and the medium. To identify the metabolites formed

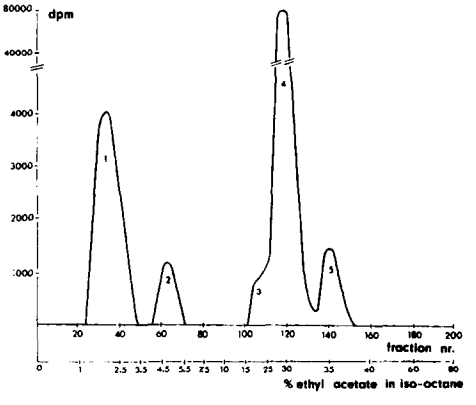

Fig. 4. Elution pattern of recovered radioactivity after incubation of plated MCF-7 cells with 1.1×10^{-6} M [^{3}H]-testosterone during 48 h. Separated on a Celite column (diameter 1.8 cm, length 30 cm) containing 36 g of a mixture of Celite–ethylene glycol (10:6, w/v). 1, Androstenedione and androsterone; 2, etiocholanolone and 5α-DHT; 3, not identified; 4, testosterone; 5, androstenediol and 5α-androstane-3α,17β-diol.

Table 1. Conversion of steroids added, after 48 h incubation at 37°C with 10^{-5} of plated MCF-7 cells

Peak nr.	t.l.c. syst. or column		Steroid detected	SA before recrystallization (d.p.m./mg)	Recrystallization 1	2	3	% Conversion
Testosterone incubation 1.1×10^{-6} M								
1	1	a	Androstenedione	591	568	570	553	6.5
		b	Androsterone	140	97	102	105	0.2
2	2	a	Etiocholanolone	176	100	124	103	0.4
		b	5α-DHT	130	—	132	127	0.7
3	1		None	—	—	—	—	—
4	1	a	Testosterone	840	842	837	842	70.3*
		b	Estrone	85	11	3	0	—
5	c	a	5α-Androstane-3α,17β-diol	162	177	153	148	0.5
		b	5α-Androstane-3β,17β-diol	40	7	5	2	—
		c	Androstenediol	104	84	78	86	0.2
	3		Estradiol	11	4	0	0	—
Androstenediol incubation, 1.0×10^{-6} M								
1	2		DHA	683	—	708	699	8.1
2	1		Testosterone	497	431	461	407	1.0
3	1		None	—	—	—	—	—
4	c	a	Androstenediol	287	277	273	281	81.0*
		b	5α-Androstane-3α,17β-diol	250	33	17	2	—
		c	5α-Androstane-3β,17β-diol	161	146	122	129	3.2
	3		Estradiol	302	121	0	0	—

* = not converted.

by micro-recrystallization we used the media because they contained more than 92% of the radioactivity.

The discussion will relate to possible receptor mediated effects caused by metabolites which may confuse the interpretation of cell growth studies. The MCF-7 cell line contains receptors for estrogens, androgens, glucocorticosteroids and progesterone [1–3, 10]. Only estrogen and androgen receptors will be discussed here.

Estradiol was converted 7% to estrone in 48 h and no other metabolites could be detected. This is in partial agreement with Lippman *et al.*[3]. They found however a 20% conversion but do not state the number of cells used for incubation.

The estrone, formed from conversion of estradiol, is able to compete with estradiol for specific binding sites in cytoplasmic extracts from MCF-7 cells. Its relative binding affinity (RBA) is about 15% as compared to estradiol, calculated from the data of Lippman *et al.*[1]. The biological effect of estrone will be mediated by the estradiol-receptor and therefore identical to the effect of estradiol. Metabolism of testosterone and androstenediol showed a more complex pattern. Neither testosterone or its metabolites, nor androstenediol or its metabolites were aromatized. Similar results for androstenedione have been reported by d'Agata *et al.*[6]. It appears therefore that the MCF-7 cell line does not contain an aromatizing enzyme system. The observed conversion of testosterone to androstenedione will not lead to a biological effect. Androstenedione itself does not bind to the androgen or estrogen receptor [7]. The formation of androsterone and etiocholanolone is more difficult to interpret in terms of biological effects as no data are available about their relative binding to the androgen or estrogen receptor. The conversion of testosterone to androstenediol may influence the interpretation of stimulation experiments with testosterone. Investigations on the ligand specificity of the estrogen receptor in human myometrial and malignant mammary cancer tissue [9] have shown that androstenediol has a RBA of 2.4% compared to estradiol. A 0.2% conversion of testosterone to androstenediol (in a molar concentration of 1.1×10^{-6}) may therefore, in combination with the above mentioned RBA, result in a biological effect comparable to the influence of

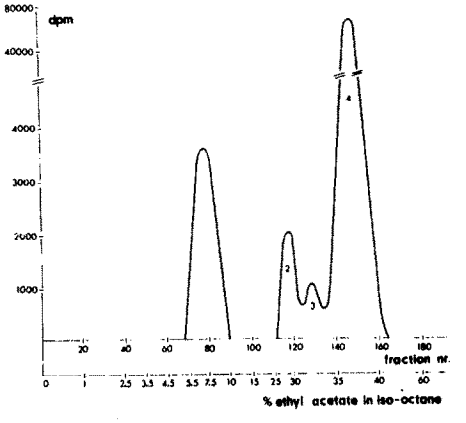

Fig. 5. Elution pattern of recovered radioactivity after incubation of plated MCF-7 cells with 1.0×10^{-6} M [^{3}H]-androstenediol during 48 h. Separated on a Celite column (diameter 1.8 cm, length 30 cm) containing 36 g of a mixture of Celite–ethylene glycol (10:6 w/v). 1, DHA; 2, testosterone; 3, not identified; 4, androstenediol and 5α-androstane-3β,17β-diol.

0.5×10^{-10} M estradiol, a concentration which is able to enhance the thymidine incorporation as shown by Lippman *et al.*[1] and by ourselves (Vossenberg *et al.*, to be published). In the same way it can be calculated that the conversion of testosterone to 5α-androstane-3α,17β-diol: 0.5%, RBA, 0.4% (9), will be equivalent to 2×10^{-11} M estradiol, which is a concentration too low to influence thymidine incorporation.

Conversion of testosterone to 5α-DHT will extend the biological effects of testosterone mediated by the androgen receptor [8]. It is unlikely that 5α-DHT in a concentration of 7×10^{-9} M (0.7% conversion of 10^{-6} M testosterone) will have an effect mediated by the estrogen receptor.

Zava and McGuire found that 5α-DHT can deplete the estrogen receptor from the cytosol only at concentrations above 10^{-7} M [12]. In our own experiments 5α-DHT in a concentration of 10^{-8} to 10^{-5} M did not stimulate the thymidine incorporation in MCF-7 cells (unpublished observations).

The observation of androstenediol to DHA, which is 5% after 24 h and 8.1% after 48 h, has a decreasing effect on the molarity of the steroid added. DHA itself has a extremely low RBA: 0.004% [9] for the estrogen receptor, which makes it unlikely that this conversion will result in any estrogen receptor mediated effect. No data are known about the androgen receptor nor DHA.

Conversion of androstenediol to testosterone of 1% may give an androgen receptor mediated effect comparable to 10^{-8} M testosterone. The conversion of androstenediol to 5α-androstane-3β,17β-diol, which is 3.2% may result in a 2.2×10^{-10} M estradiol effect, because its RBA for the estrogen receptor is 0.7% of the estradiol value.

The discrepancy between the expected 95% and the total recovery found (for the testosterone incubation 80% and for the androstenediol incubation 93%) can be explained by the formation of conjugates during incubation. In the system we used they were not determined. This explanation is in agreement with the results of Raju *et al.*[11] who published recently that metabolites of androstenedione and estriol were partly conjugated during incubation with the MCF-7 cells.

From this study, the general conclusion can be drawn that mammary cancer cells may metabolize steroid hormones and especially androgens in such a way, that biologically effective metabolites are formed. The influence of this process on the course of incubation experiments may be small, but not negligible.

We do believe that similar conditions will be found in studies with other cell lines. Circumstances will have to be chosen which limit these unwanted reactions, or alternately one has to include these possibilities in the interpretation of results.

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