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SYNTHESIS OF ESTRADIOL FATTY ACID ESTERS BY HUMAN BREAST TUMORS: FATTY ACID COMPOSITION AND COMPARISON TO ESTROGEN AND PROGESTERONE RECEPTOR CONTENT

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Summary—The estradiol-17β-fatty acid esters are non-polar metabolites of estradiol, formed in many tissues, including human breast tumors. It has been shown in the rat that the synthesis of these esters is greatest in those tissues that respond to estrogen stimulation. Thus the possibility was explored that the biosynthesis of the estradiol esters in human breast tumors occurs mainly in those tumors that are estrogen sensitive; and thus that the synthesis of this family of non-polar metabolites of estradiol could be used as an additional marker for the identification of hormonally dependent tumors. However, the conversion of estradiol to the esters did not correlate with other indicators of estrogen responsiveness, the progesterone or estrogen receptors. Interestingly, the composition of the fatty acids in the estradiol-17-esters synthesized in the human tumors was markedly different from those originally identified in the bovine uterus. In the bovine uterus, the esters were predominantly unsaturated, 85%, while in this study the saturated esters were the major component. Since after systemic administration the saturated estradiol-17-esters have been found to be much longer-lived than the unsaturated esters, the biosynthesis of the relatively high proportion of saturated esters by human breast tumors may indicate a significantly prolonged duration for the estrogenic signal produced by endogenously formed estradiol esters. These esters formed and sequestered within the tumor cell, may serve as a preformed store of estradiol, which after enzymatic hydrolysis, can locally stimulate growth of tumors that are estrogen responsive.

INTRODUCTION

The lipoidal derivative of estradiol (LE_2) is a non-polar metabolite of estradiol, that is synthesized in various tissues, and is hydrolyzed to estradiol with alkaline treatment [1]. LE_2 biosynthesized in bovine uterus has been isolated and identified as a heterogeneous family of C-17 fatty acid esters of estradiol [2]. More recently it has been shown that LE_2 circulates in human blood [3]. These naturally occurring esters are structurally analogous to the synthetic estrogens which are used pharmacologically, e.g. estradiol propionate etc. [4]. They also share the property of long-lived action, producing prolonged stimulation of estrogen responsive tissues [5].

The *in vitro* biosynthesis of the estradiol fatty acid esters had some unique characteristics. For example, the distribution of fatty acids comprising LE_2 synthesized in the bovine uterus is unlike other major esters in this tissue: consisting almost exclusively of polyunsaturated carboxylic acids, with estradiol-17-arachidonate predominating [2]. Furthermore, it was found in incubations of various tissues from rats, that those tissues which are estrogen responsive synthesize the largest amounts of LE_2 [1]. It has also been shown that the synthesis of the C-17 steroidal esters occurs in human breast tumors [1]. Additionally, it was reported that the rate of this biochemical reaction varies dramatically from tumor to tumor [6]. Since LE_2 synthesis occurs predominantly in estrogen responsive tissues, it was proposed that the observed variations in synthesis by the breast tumors might reflect the sensitivity of each tumor to estrogens.

Various enzymes and proteins are formed in response to estrogen stimulation of breast tumors, for example: progesterone receptor (PR) [7, 8], steroid sulfotransferase [9], and an uncharacterized estrogen induced protein [10]. Thus in a similar manner, the esterification of estradiol might serve as an additional marker to further define hormonally dependent tumors to provide information for selecting therapeutic modalities for breast cancer patients. In order to explore this possibility, LE_2 biosynthesis in human breast biopsy specimens was quantified, the fatty acid composition of the steroid esters was determined, and both parameters were compared to the estrogen (ER) and progesterone receptor content of these tumors.

EXPERIMENTAL

Non-radioactive steroids were obtained from Steraloids (Wilton, NH). [2,4,6,7-^{3}H]Estradiol

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(105 Ci/mmol) and [4-^{14}C]estradiol (57 mCi/mmol) were purchased from New England Nuclear (Boston, MA) and purified by high pressure liquid chromatography (HPLC) on a 4.6 mm × 25 cm LiChrosorb Diol column (Merck) with CH_2Cl_2 at 1 ml per min. Solvents were of analytical or HPLC grade and were obtained from Fisher Scientific Co. (Waltham, MA). Acyl chlorides were from NuChek Prep (Elysian, MN). Eagle's HeLa culture medium was purchased from Gibco (Grand Island, NY).

C-17 Fatty acid esters of estradiol were synthesized as previously described, by esterification of estradiol with the appropriate acyl chloride in pyridine [2]. The resulting estradiol diester was treated with $NaHCO_3$, which selectively hydrolyzes the relatively labile phenolic ester. The C-17 esters synthesized by this procedure were purified by crystallization. Those esters which could not be crystallized were purified by HPLC as above, with CH_2Cl_2–isooctane (3:2, v/v) as the eluent.

Incubations

Human breast tumor specimens were immediately frozen on dry ice in the operating room and were stored for up to 5 days at −70°C. Incubations were performed as previously described [1]. [^{3}H]Estradiol (3.5×10^6 cpm, 34 pmol) dissolved in 30 μl of ethanol was added to 25 ml Erlenmeyer flasks containing 3 ml of cold Eagle's medium, previously saturated with 95% O_2–5% CO_2. Portions of the tumors, 100–350 mg, were weighed, minced into 2–3 mm pieces and placed in the buffer. The flasks were gassed with the O_2–CO_2 mixture, sealed, and then incubated in a shaking water bath at 37°C for 5 h. After the incubation, the tumor fragments were removed from the solution, rinsed with 3 ml of cold Eagles medium, blotted, and placed in 3 ml of methanol containing 50 μg and 1,250 cpm ^{14}C of estradiol-17-stearate. The methanolic suspensions were homogenized with a Polytron homogenizer (Brinkman, Westbury, NY), and passed through filter paper. The retentates were thoroughly washed, first with 40 ml of methanol, and then 10 ml each of acetone, chloroform and benzene. The organic solvents were combined, evaporated, and the resulting residues dissolved in 50 ml of CH_2Cl_2.

LE_2 assay

The CH_2Cl_2 solution of the tumor extracts were evaporated under vacuum, dissolved in benzene and chromatographed on 0.5 × 7.5 cm columns of silica gel 60 (Merck), equilibrated in the same solvent. After 2 ml of benzene were passed through the columns, the estradiol fatty acid esters were eluted with 12 ml of benzene–ethyl acetate (99:1, v/v). Those fractions containing the internal standard were evaporated and purified by thin layer chromatography (TLC) on silica gel. The TLC plates were developed in system A, benzene–isooctane (1:1, v/v), and then in system B, benzene–ethyl acetate (8:, v/v). While the estradiol esters do not migrate from the application point in system A, contaminating non-polar lipids which interfere with the chromatographic separation, migrate close to the solvent front, and in this manner are removed from the subsequent separation in system B. In the latter system (B) estradiol-17-fatty acid esters migrate with an R_f of 0.44, while the more polar steroids, such as estradiol, barely move, $R_f < 0.1$. In order to visualize the carrier steroid, the plates were sprayed with a solution of Primuline [11] and illuminated under long-wave, 366 nm u.v. light. The region on the plates in which the estradiol-17-stearate migrated were scraped and eluted with chloroform–methanol (9:1), v/v). An aliquot of the extracts were reserved for counting and 50% of the remainder was rechromatographed in system B. The remaining portion was saponified with Na_2CO_3 as previously described [1] and then purified in system C, benzene–ethyl acetate (1:1, v/v), in which estradiol migrates with an R_f of 0.45. The ^{3}H/^{14}C ratios of the regions containing the internal standards from all three chromatograms were determined, correcting for overlap of ^{14}C into the ^{3}H window as described by Okita *et al.*[12]. In every case the ^{3}H/^{14}C ratios were in excellent agreement, demonstrating that the esters were radiochemically pure.

HPLC analysis of the biosynthetic estradiol-17-fatty acid esters

The extracts of the second TLC in system B from eight of the tumor specimens (4 ER + PR +, 2 ER + PR −, 2 ER − PR −) contained sufficient radioactivity to analyze the composition of the estradiol fatty acid esters by HPLC. The TLC extracts were evaporated, dissolved in acetonitrile, and analyzed by HPLC on a 4.6 mm × 25 cm Ultrasphere ODS column with acetonitrile at a flow rate of 1 ml per min. In this system most of the estradiol esters are cleanly separated (the retention times are given in Table 1), although the esters $C_{16:1}$, $C_{18:2}$ and $C_{18:3}$ migrated close together and consequently are quantitated as one group.

Estrogen and progesterone receptors

The receptor content of the tumors was assayed as

Table 1. Composition of the estradiol fatty acid esters synthesized human breast tumors

Fatty acid ester	*R_t	PR+ †% of total	PR− % of total
$C_{20:4}$	21	5.3 ± 0.5	8.0 ± 2.9
$C_{16:1}$	29		
$C_{18:2}$	27	18.0 ± 4.1	17.8 ± 4.9
$C_{18:3}$	28		
$C_{18:1}$	42	26.8 ± 3.3	30.5 ± 5.3
$C_{16:0}$	46	25.8 ± 5.0	20.8 ± 2.5
$C_{18:0}$	71	25.0 ± 5.3	22.5 ± 9.3

Tumors are grouped for progesterone receptor (PR) content as described in the text. *R_t is the HPLC retention time in minutes. Conditions for reversed phase HPLC are given in the text. A typical chromatogram is shown in Fig. 2. †Values are expressed as the mean % ± SD of the total LE_2. PR+, $n = 4$; PR−, $n = 4$.

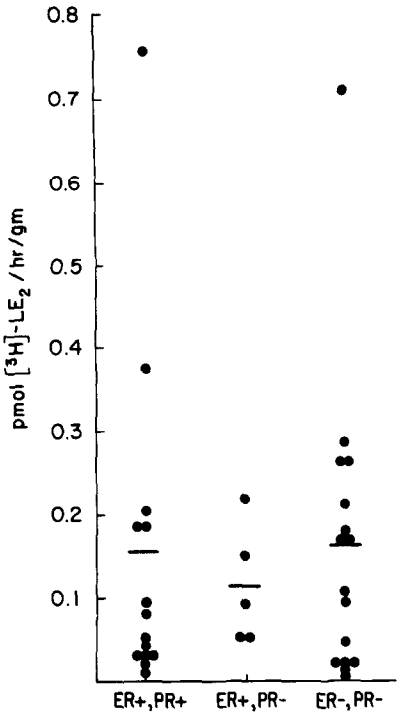

Fig. 1. Biosynthesis of [^{3}H]estradiol-17-fatty acid esters by human breast tumors. Tumor slices were incubated with [^{3}H]estradiol and the LE_2 synthesized was analyzed as described in the text. Tumors were classified by estrogen receptor (ER) and progesterone receptor (PR) content as described in the Experimental section.

previously described [13]. The estrogen receptor was analyzed by incubation of the tumor cytosol with 0.25–2.5 nM [^{3}H]estradiol in the presence and absence of 60 nM diethylstilbestrol to correct for non-specific binding. Likewise, the progesterone receptor was determined through incubations of the cytosol with 0.5–20 nM [^{3}H]promogesterone, including 1 μM dexamethasone, in the presence or absence of 1 μM promogesterone. In both assays the receptor bound steroid was quantified after absorption of the free steroid on micro columns of Sephadex LH-20 [14]. The concentration of the receptor was calculated by Scatchard analysis [15].

As recommended for grouping according to ER content of the tumor [16], positive was considered more than 14 fmol/mg cytosol protein in a post-menopausal patient and more than 4 fmol/mg protein in a premenopausal patient. In the current series 22 patients were postmenopausal and 13 were premenopausal. For the PR results, more than 19 fmol/mg cytosol protein was considered positive [17].

RESULTS

The synthesis of LE_2 was quantified in 35 human breast tumors. Every tumor synthesized the estradiol C-17 fatty acid esters. The amounts synthesized varied widely, from 0.01 to 0.8 pmol per hour per gram of tumor. When the amount of LE_2 synthesized was compared to the ER concentration there was no correlation, $r = -0.19$, $P = 0.36$, nor was there a correlation of LE_2 synthesis with the PR content, $r = 0.04$, $P = 0.84$. In addition the rate of synthesis of the esters was compared in the three classes of tumor, ER+ PR+, ER+ PR− and ER− PR− (Fig. 1). The tumors that contained both ER and PR synthesized 0.152 ± 0.054 pmol of LE_2/h/g (mean $\pm$ SEM); the tumors that were ER+ PR−, synthesized 0.114 ± 0.30; and those tumors that were ER− PR−, synthesized 0.162 ± 0.044. There was no difference between any of these groups (analysis of variance).

The fatty acid composition of the biosynthetic estradiol esters (LE_2) from eight of the tumor extracts was analyzed by reversed phase HPLC. The results of a typical chromatogram is shown in Fig. 2. The

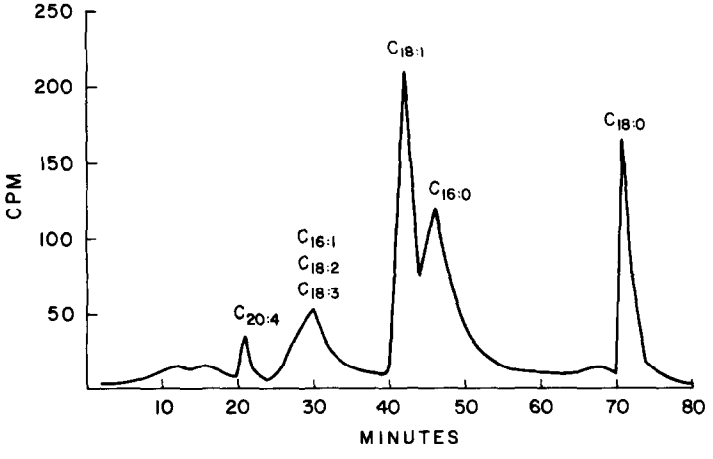

Fig. 2. HPLC analysis of [^{3}H]Estradiol-17-fatty acid esters synthesized by a human breast tumor. The [^{3}H]ester fraction was obtained by TLC and then separated on the reversed phase HPLC Ultrasphere ODS column with acetonitrile at a flow rate of 1 ml/min. The extract is from an ER− PR− tumor.

composition of the fatty acid esters comprising LE_2 is shown in Table 1. It is clear from these experiments, that the saturated esters, palmitate, $C_{16:0}$, and stearate, $C_{18:0}$, make up approx 50% of the total ester fraction, and that oleate, $C_{18:1}$, is the most prevalent ester, comprising about 30% of the total LE_2. Of the five fatty acid ester fractions isolated, arachidonate, $C_{20:4}$, was found in the lowest concentration, 5–8% of LE_2, The data in Table 1, shows the composition of LE_2 in two populations of the breast tumors, those with and without the progesterone receptor. Assuming that the progesterone receptor is a marker for estrogen responsiveness of the tumor, there is no difference in the fatty acid composition of the two groups.

DISCUSSION

Our earlier finding [1] that human breast tumors were capable of synthesizing LE_2 from estradiol, taken in concert with our studies showing that in the rat this enzymatic process took place predominantly in estrogen sensitive tissues, suggested that the formation of these estradiol esters might be useful as an indicator of hormonally responsive tumors. This possibility was also posed by other studies [6] which showed a wide variation in the amount of LE_2 synthesized in human breast tumors. However in the present experiments, when the amount of LE_2 synthesized by human breast cancer specimens was compared to two markers routinely used as a means of predicting hormonal dependency of breast tumors, estrogen receptor [16], and progesterone receptor [17], there was no correlation. LE_2 is synthesized equally in tumors regardless of their ER and PR content (Fig. 1, Table 1). Consequently, it appears that the capacity of breast tumors to produce LE_2 is not estrogen regulated.

Eight of the purified extracts had sufficient radioactivity remaining to determine the composition of the esters. The tumors from which these extracts were obtained were from the following classes: 4 were ER+ PR+; 2 were ER+ PR−; and 2 were ER− PR−. Because of the limited number of samples that could be analyzed in this fashion, the composition of the esters was compared using the marker for estrogen responsiveness, the progesterone receptor (in fact the values for LE_2 composition are similar for all four PR− specimens). As can be seen in Table 1 the composition of the fatty acids in LE_2 was the same in both the PR+ and PR− groups. Thus neither the quantity nor the composition of LE_2 was related to known markers of estrogen sensitivity in breast tumors.

Interestingly, the composition of the estradiol-17-esters synthesized in the human breast tumors is very different from the esters found in the bovine uterus [2]. In that study the predominant fatty acid in LE_2 was arachidonate, comprising almost 30%, and the total unsaturated fatty acids account for 86% of the esters. The two saturated acids, stearate and palmitate together were only 14% of the uterine LE_2. As shown in Fig. 2 and Table 1, the LE_2 synthesized in human tumors was composed of predominantly saturated esters, 51%, while arachidonate was only 5%.

If similar results to those presented in this paper are obtained from *in vivo* studies then it is likely that the estradiol esters synthesized in humans are saturated. The fatty acids that comprise LE_2 are important in determining the biological potency of this non-polar estrogen through the enzymatic release of estradiol. It was previously shown *in vitro* that the fatty acids in LE_2 are the determining factor in the rate of hydrolytic release of the active hormone, estradiol [18]. Further studies *in vivo* have confirmed this, showing that estradiol-17-stearate is converted into estradiol at a much slower rate than the unsaturated ester, estradiol-17-arachidonate. Consequently, the saturated esters synthesized in human breast tumors produce estrogenic effects of greatly prolonged duration.

The present studies point to the possibility that high concentrations of estradiol-17-fatty acid esters may be present in many human breast cancers. The formation of these non-polar estradiol esters by the tumor, may be part of a self-stimulating mechanism by which performed but inactive estrogens can be sequestered within the hydrophobic lipids in the cell, and then activated by enzymatic hydrolysis. Estradiol, released by hydrolytic cleavage [20, 21], is then available for the local stimulation of estrogen sensitive tumors.

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REFERENCES

1. Schatz F. and Hochberg R. B.: Lipoidal derivative of estradiol: the biosynthesis of a nonpolar estrogen metabolite. *Endocrinology* **109** (1981) 697–703.
2. Mellon-Nussbaum S. H., Ponticorvo L., Schatz F. and Hochberg R. B.: Estradiol fatty acid esters: the isolation and identification of the lipoidal derivative of estradiol synthesized in the bovine uterus. *J. biol. Chem.* **257** (1982) 5678–5684.
3. Janocko L. and Hochberg R. B.: Estradiol fatty acid esters occur naturally in human blood. *Science* **222** (1983) 1334–1336.
4. Deghenghi R. and Givner M. L.: The female sex hormones and analogs. In *Burger's Medicinal Chemistry* (Edited by M. E. Wolf). John Wiley, New York (1979) P. 931.
5. Larner J. M., MacLusky N. J. and Hochberg R. B.: The naturally occurring C-17 fatty acid esters of estradiol are long-acting estrogens. *J. steroid Biochem.* **22** (1985) 407–413.
6. Abdul-Hajj Y. J.: Formation of estradiol 17β-fatty acyl esters in mammary tumors. *Steroids* **40** (1982) 149–156.
7. Horwitz K. B., McGuire W. L., Pearson O. H. and Segaloff A.: Predicting response to endocrine therapy in human breast cancer: A hypothesis. *Science* **189** (1975) 726 727.
8. Horwitz K. B. and McGuire W. L.: Estrogen control of progesterone receptor in human breast cancer. Cor-

relation with nuclear processing of estrogen receptor. *J. biol. Chem.* **253** (1978) 2223–2228.
9. Penwim T., Adams J. B. and Ho K. P.: Estrogen sulfurylation as an alternative indicator of hormone dependence in human breast cancer. *Steroids* **39** (1982) 47–52.
10. Ciocca D. R., Adams D. J., Bjercke R. J., Edwards D. P. and McGuire W. L.: Immunohistochemical detection of an estrogen-regulated protein by monoclonal antibodies. *Cancer Res.* **42** (1982) 4256–4258.
11. Wright R. S.: A reagent for the non-destructive location of steroids and some other lipophilic materials on silica gel thin-layer chromatograms. *J. Chromat.* **59** (1971) 220–221.
12. Okita G. T., Kabara J. J., Richardson F. and LeRoy G. V.: Assaying compounds containing ^{3}H and ^{14}C. *Nucleonics* **15** (1957) 111–114.
13. Schwartz P. E., LiVolsi V. A., Hildreth N., MacLusky N. J., Naftolin F. N. and Eisenfeld A. J.: Estrogen receptors in ovarian epithelial carcinoma. *Obstet. Gynec.* **59** (1982) 229–238.
14. Ginsburg M., Greenstein B. D., MacLusky N. J., Morris I. D. and Thomas P. J.: An improved method for the study of high-affinity steroid binding: oestradiol binding in brain and pituitary. *Steroids* **23** (1974) 773–792.
15. Scatchard G.: The attraction of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.* **51** (1949) 660–672.
16. Jensen E. V., Smith S. and DeSombre E. R.: Hormone dependency in breast cancer. *J. steroid Biochem.* **7** (1976) 911–917.
17. Clark G. M. and McGuire W. L.: Progesterone receptor and human breast cancer. *Breast Cancer Res. Treat.* **3** (1983) 157–163.
18. Janocko L., Larner J. M. and Hochberg R. B.: The interaction of the C-17 esters of estradiol with the estrogen receptor. *Endocrinology* **114** (1984) 1180–1186.
19. Larner J. M. and Hochberg R. B.: The clearance and metabolism of estradiol and estradiol-17-esters in the rat. *Endocrinology* **117** (1985) 1209–1214.
20. Abul-Hajj Y. J. and Nurieddin A.: Significance of lipoidal estradiol in human mammary tumors. *Steroids* **42** (1983) 417–426.
21. Katz J., Raju U. and Levitz M.: Properties of an esterase in MCF-7 cell line that hydrolyzes fatty acid esters of steroids. *67th Ann. Mtg Endocr. Soc.* (1985) Abstr. 767.