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CATECHOLESTROGEN BINDING SITES IN BREAST CANCER

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Summary—The binding of 2-hydroxyestrone (2OH E_1), a catecholestrogen which is the main end product of the 2-hydroxylation of estrogen, was investigated in breast cancers. 2OH E_1-specific bindings were found in the cytosol ($K_d = 0.54 \pm 0.10$ nM) and in the endoplasmic reticulum ($K_d = 3.36 \pm 1.32$ nM). The dissociation rate constants of complexes between [^{3}H]2OH E_1 and cytosol or membrane binding sites were 3.30 h^{-1} and 8.30 h^{-1} respectively. Qualitative analysis of [^{3}H]2OH E_1 cytosolic complexes demonstrated a specific binding component with a mol. wt of 330,000 Daltons. Specificity experiments showed that nonestrogenic hormones were unable to compete with 2OH E_1 for its binding sites, whereas triphenylethylene derivatives and catecholamines were potent 2OH E_1 competitors. The presence of 2OH E_1 specific bindings suggests a potential role of catecholestrogen in breast cancer.

INTRODUCTION

The main pathways of estradiol metabolism consist of the initial oxidation of the 17β-hydroxy group to the 17 ketone, followed by subsequent hydroxylations at either the C_2 or C_4 (catecholestrogen; CE) or 16α positions (estriol, and 16α-hydroxyestrone) [1]. The CE, and especially 2-hydroxy substituted estrogens, are now thought to be the main metabolites of endogenous estrogens [2]. The enzyme system catalyzing their synthesis is a cytochrome P450-dependent monooxygenase which is mainly localized in the microsomal fraction of the liver. This enzyme is also found in numerous estrogen-sensitive mammalian tissues [3, 4], and especially in both benign and malignant mammary tumors, but not in normal breast tissue [5]. *In vivo* hydroxylation of estradiol at C_2 exceeds that at C_4 [6, 7] and therefore the biological contribution of the 4-hydroxyestrogens may be quite limited. Catechol-*o*-methyltransferase (COMT), the enzyme catabolizing the CE, is also detected in breast tissues and has been found to be significantly more concentrated in the cytosol of malignant tumor cells than in the cytosol of benign tumor cells or normal tissue cells [8]. Thus, the enzymes responsible for synthesis and metabolism of CE are present in some breast tumor specimens, suggesting that in such tissues these metabolites may be formed *in vivo*. Furthermore, some synthetic estrogens, including 17α ethinyl estradiol and diethylstilbestrol, can be metabolized by these enzymes [9] so that CE levels may increase when these drugs are administered under pharmacological conditions.

In the present study, we demonstrate in human breast cancer cells the interaction between 2-hydroxyestrone and estrogen receptors of a magnitude which could be biologically relevant. Moreover, our results provide additional evidence for the separate existence of a specific CE-binding protein, the significance of which remains to be defined.

EXPERIMENTAL

Reagents and buffers

17β-[2,4,6,7,16,17-^{3}H]estradiol, [^{3}H]E_2, 160 Ci/mmol; 16α-[^{125}I]iodoestradiol: [^{125}I]E_2, 1500 Ci/mmol; 2-[6,7-^{3}H]hydroxyestrone, [^{3}H]2OH E_1, 40–50 Ci/mmol; adenosyl-L-methionine, *S*[methyl-^{14}C], 59.8 mCi/mmol were purchased from New England Nuclear (Boston, Mass.). Cortisol, dihydrotestosterone, progesterone, estradiol, estrone, 2-hydroxyestrone (2OH E_1), 4-hydroxyestrone (4OH E_1), 2 hydroxyestradiol (2OH E_2), 4 hydroxyestradiol (4OH E_2), estriol (E_3), diethylstilbestrol (DES), 16 epiestriol were from Steraloids Inc. (Pawling, N.J.). Tamoxifen 1-(4-(2-dimethylaminoethoxy)phenyl)1,2-diphenylbut-1-ene) (Tam) and 4 hydroxytamoxifen (4OH Tam) were kindly supplied by ICI Pharmaceuticals (Macclesfield, England). The steroids were kept in ethanolic solution except for CE which are susceptible to autooxidative decomposition and were therefore diluted in methanol–acetic acid (98:2) containing 0.78% w/v ascorbic acid, their purity was checked by isocratic high performance liquid chromatography [10]. The standard protein calibration kit was purchased from Pharmacia (Uppsala, Sweden). Adrenaline and noradrenaline were purchased from Sigma Chemical Company (St Louis, MO, U.S.A.).

The buffers used were buffer A: Tris–HCl 0.02 M, EDTA 3 mM, DTT 1 mM, NaN_3 0.01%, pH 7.4; buffer B consisting of buffer A with 0.015% w/v ascorbic acid and buffer C: 0.25 M sucrose, 5 mM Tris–HCl, 0.5 mM $CaCl_2$, 5 mM mercaptoethanol, pH 7.4.

All sucrose solutions were made up in 5 mM Tris–HCl, pH 7.4; the sucrose concentrations in percent were based on the total weight of the final solution.

Tissue processing and cell fractionation

The mammary tumor specimens were adenocarcinomas. On collection, fat was removed and samples were divided into two pieces; one was submitted for histological studies and the other was frozen and stored in liquid nitrogen until assays were performed.

All procedures were carried out at 4°C. The frozen tissues were weighed and then pulverized with a tissue pulverizer (Spex Industries Inc., Metuchen, N.J.).

The method for cell fractionation derived from those of Aronson and Touster[11] and Takeuchi and Terayama[12]. It allowed separation of cell surface membrane fragments from either the nuclear or microsomal portion of the tissue homogenate. Three volumes of ice-cold buffer C were added to the pulverized tissue. Homogenization was carried out using a Dounce homogenizer, and the homogenate was filtered through six layered gauze.

Preparation of nuclear and cytosolic fractions and gradient separation of membranes:

After centrifugation of the filtrate at 1000 *g* for 10 min, the pellet, which consisted mainly of nuclei and cell membranes with a small portion of mitochondria contamination, was resuspended in the same initial volume of buffer C and layered over 25 ml of 0.34 M sucrose solution and centrifuged at 1000 *g* for 10 min. The nuclear fraction was again separated three times as before and the combined supernatant solutions were saved and processed as described below.

The nuclear pellet was resuspended in buffer C to which an adequate volume of 67% sucrose was added to make a final 48% sucrose solution ($d = 1.22$). Four ml of this nuclear suspension in heavy sucrose were transferred into a centrifuge tube then 4 ml of 45% sucrose ($d = 1.20$), 4 ml of 41% sucrose ($d = 1.18$) and 4 ml of 37% sucrose ($d = 1.16$) were layered over successively and centrifuged at 27,000 rpm for 2 h. The plasma membranes obtained in these respective layers were called N1, N2, N3, N4, N5.

The pooled supernatants were centrifuged at 40,000 rpm for 30 min. The resulting final supernatant fraction (cytosol) was saved, and the pellet was resuspended in buffer C plus 67% sucrose solution to achieve a final concentration of 48% sucrose. The gradient was then performed exactly as described above, in order to obtain membrane fractions called P1, P2, P3, P4, P5.

Characterization of catecholestrogen binding sites

Association studies

Samples of cytosol or membrane fractions were incubated for various periods of time at 4°C with 5 nM [^{3}H]2OH E_1 in the presence or absence of a 200-fold excess of the equivalent nonlabeled steroid. Cortisol and DHT (2.10^{-6} M each) were added to the labeled solutions in order to prevent steroid bindings on sex binding protein or androgen and glucocorticoid receptors.

Binding assays

Cytoplasmic binding. E_2 binding was measured using dextran-coated charcoal (DCC) assays [13]. Increasing concentrations of [^{3}H]E_2 (0.25 to 10 nM final concentrations) were incubated in duplicate at 4°C for 16 h with cytosol aliquots. The same incubation series with the addition of a 200-fold excess of DES were performed to assess [^{3}H]steroid binding to estrogen specific receptor sites.

CE binding assays were performed with increasing concentrations of [^{3}H]2OH E_1 (0.25–10 nM final concentrations) plus DHT and cortisol (2.10^{-6} M) in buffer B. The incubations were carried out in duplicate at 4°C for 1 h. Nonspecific binding was accounted for by preparing the same incubation series with the addition of a 200-fold excess of nonlabeled 2OH E_1. Bound hormones were obtained after adsorption of free hormones on DCC and counted in a liquid scintillation spectrometer.

Membrane binding. Membrane preparations suspended in buffer B (approx. 1 mg of protein/ml) were incubated at 4°C for 16 h under agitation. Hormone concentrations were the same as in the cytosol. The incubations were terminated by rapid filtration over Whatman GF/B filters and rinsed with 5 × 2 ml of ice-cold buffer B. The radioactivity on the filter was determined by liquid scintillation spectrometry.

The dissociation constants (K_d) and maximal capacities were calculated by Scatchard analyses of specific bound hormones [14]. The results were expressed in fmol/mg protein.

Dissociation studies

Cytosol or membrane preparations were incubated as described above. Specifically bound [^{3}H]2OH E_1 values were determined at $t = 0$, then dissociation experiments were carried out, achieved by addition of a 200-fold excess of cold hormone in the incubate diluted with 300 μl of buffer B. Protein-bound radioactivity was measured at various intervals of time up to 30 min and compared to that at $t = 0$.

Double labeling assay

Twenty-five μl of aqueous solutions of [^{125}I]E_2 and [^{3}H]2OH E_1 (both 5 nM final concentration), were pipetted into the same glass tube alone (total binding) or in the presence of a 200-fold excess DES (E_2 nonspecific binding), or in the presence of a 200-fold excess 2OH E_1 (2OH E_1 nonspecific binding). Then 100 μl of cytosol were added to the solutions. Bound hormone concentrations were determined using a double labeling program [15].

High performance liquid chromatography (HPLC) analysis

HPLC analysis was performed on size exclusion column at 4°C. 200 μl of cytosol were incubated with 100 μl [^{3}H]steroid, with or without a 200-fold excess of the unlabeled counterpart, to give a final concentration of 5 nM. Incubates were injected into a Protein Pak column 300 SW (300 × 7.5 mm) fitted with a 22 mm pre-column (Waters SA, Paris, France) using a model U6K universal liquid chromatography injector (Waters). Proteins were eluted with buffer B containing 10 mM sodium molybdate at a rate of 1 ml per min and each fraction was collected for 30 s.

Other analytical methods

Enzyme assays: 5'Nucleotidase (plasma membrane marker) and glucose 6-phosphatase (microsome marker) activities were measured according to the method of Aronson and Touster[11]. Glucose-6-phosphate dehydrogenase (cell cytoplasm marker) activity was determined as described by Cohen and Rosenmeyer[16]. COMT activity was estimated at pH 7.8 in the cytosol according to Assicot *et al.*[8] and at pH 7.0 in the membrane fractions according to Inscoe *et al.*[17].

Protein concentrations were determined by the method of Lowry[18]. ^{3}H-Steroids were counted using a Beckman LS 6800 liquid scintillation spectrometer with 30–50% counting efficiency. All calculations were carried out using a CBM model 8096 Commodore microcomputer.

RESULTS

Cell fractionation

Gradient separation of the plasma membranes from the nuclear fraction. The plasma membrane layer appeared at the interface between 37 and 41% sucrose (N_2, $d = 1.16$) in the form of a thin compact sheet. The materials banding at the interface between 41–45% (N_3, $d = 1.18$) and 45–48% (N_4, $d = 1.20$) sucrose layers were mainly mitochondria and the other cell particulates but excluding the nuclei which form a pellet (N_5) at the bottom of the centrifuge tube.

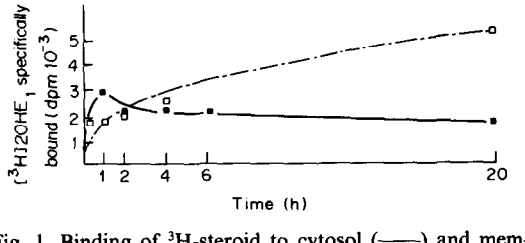

Fig. 1. Binding of ^{3}H-steroid to cytosol (——) and membrane (-·-·-) fractions as a function of time. Binding sites were measured at 4°C using 5 nM ^{3}H-steroid as described in the Experimental section. Values have been corrected for nonspecific binding.

Gradient separation of the post-nuclear extract pellet. The membrane fraction (P_2) was a thick band of white material appearing between the 37 and 41% sucrose ($d = 1.16$) which was similar to plasma membrane N_2. The membrane fraction P_4 banding at the interface between 45 and 48% sucrose ($d = 1.20$) contains, according to enzymatic estimation, the bulk of the membranes derived from the endoplasmic reticulum.

Time course of association

The time course of association of [^{3}H]2OH E_1 to binding sites in breast cancer is shown in Fig. 1. In the cytosol, the 2OH E_1 binding was maximal after incubation for 1 h; then the values dropped, presumably in relation to an auto-oxidative decomposition. In the membrane fraction, the binding of 2OH E_1 reached a maximum after incubation for 6 h and the binding material was then quite stable up to 24 h. No binding of E_2 could be observed in the membrane fractions.

Saturation analyses

Table 1 shows that 2OH E_1 had affinity for cytosolic binding sites which was in the same order of magnitude as those of parent compounds. Among all the membrane fractions studied, the only ones which specifically bound this CE, were N_2 and P_2 (plasma membranes), N_5 (nuclei) and P_4 (endoplasmic reticulum). The K_d values were generally higher in the membranes than in the cytosol. It is worth noting that none of these membrane fractions, except N_5 (nuclear fraction) could bind E_2.

Specificity of the CE binding sites

The specificity of binding was studied by investigating 2OH E_1 binding to weak or nonestrogenic target tissues such as human prostate or testis. In none of these cases could specific 2OH E_1 or E_2 binding be demonstrated either in the cytosol or in the endoplasmic reticulum. In the same way, no "specific" binding could be seen using a protein solution of human serum albumin (2 mg/ml).

In breast tissues obtained from patients with hypermastia after plastic surgery, no specific 2OH E_1 or

Table 1. Saturation analyses

	Dissociation constants: K_d (nM)	
Cell fractions	[^{3}H]E_2	[^{3}H]2OH E_1
Cytosol	0.15 ± 0.13*	0.54 ± 0.10
Fraction P4: endoplasmic reticulum	NB†	3.36 ± 1.32
Fraction P2 + N2: plasma membranes	NB	10.6 ± 2.8

Saturation analyses were performed in different fractions of breast cancer tissue using estrogen and estrogen metabolite as tracer as described in the Experimental section. Dissociation constants were expressed in nM.
*Mean ± SD of 5 experiments.
†NB: no binding.

Table 2. Representative bindings of 2OH E_1 to 11 individual cytosol or endoplasmic reticulum fractions from breast tumor cells

Cytosol		Endoplasmic reticulum	
Total binding	Nonspecific binding	Total binding	Nonspecific binding
5479	3154 (57)	25,846	12,034 (46)
10,816	6902 (33)	7344	3741 (51)
1986	1162 (58)	15,513	12,467 (80)
2853	648 (23)	3645	2313 (63)
9649	7162 (74)	18,785	12,384 (66)
15,670	6352 (40)	11,531	7811 (68)
3782	1690 (45)	12,955	5768 (44)
4506	2396 (53)	7599	3085 (40)
3033	1742 (57)	17,990	4463 (25)
4184	2263 (54)	5213	2120 (41)
2374	1628 (68)	2651	832 (31)

The bindings of [^{3}H]2OH E_1 were performed as described in the Experimental section. Results are expressed as dpm/tube. Values in parentheses represent the percentage of nonspecific binding versus total binding.

E_2 binding could be observed in the cytosol but a slight specific 2OH E_1 binding in the endoplasmic reticulum (5–22 fmol/mg protein $n = 7$).

In human mammary tumors, the ranges of specific binding in 52 tumors assayed were 0–285 fmol/mg protein in the cytosol and 0–320 fmol/mg protein in the endoplasmic reticulum. Representative bindings of 2OH E_1 to breast tumor fractions are specified in Table 2. The data show that the percentages of nonspecific bindings were often high and represented $49 \pm 17\%$ (mean $\pm$ SD, $n = 52$) of total bindings.

The specificity of [^{3}H]estrogen bindings to cytosolic binding sites was determined by measuring the ability of various hormones and compounds having a dihydroxyphenolic moiety such as adrenaline and noradrenaline, to compete for binding with [^{3}H]E_2 or [^{3}H]2OH E_1. Table 3 shows that nonestrogenic hormones such as progesterone present in a 200-fold molar excess did not markedly inhibit the binding of the two tritiated components. Because they were present in the buffer, DHT and cortisol had not been assayed as competitors for [^{3}H]2OH E_1 binding. It is noteworthy that CE competed almost to the same extent as E_2 and DES for [^{3}H]E_2 binding sites, but E_1, E_2, E_3, DES were weak competitors for [^{3}H]2OH E_1 binding sites, and surprisingly two triphenylethylene derivatives tested (Tam and especially 4OH Tam) were efficient competitors for these sites.

In order to focus on possible CE-specific binding sites in the cytosol, binding assays were performed using 5 nM [^{3}H]2OH E_1 after pre-incubation for 3 h at 4°C with 2.10^{-6} M E_2 to saturate estrogen receptor sites. The results are shown in Table 5. The CE were still able to bind to cytosolic sites after incubation

Table 3. Specificity of cytosolic estrogen and cytosolic estrogen metabolite binding sites

Competitor concentration (200-fold molar excess)	% Of inhibition of specific ^{3}H-steroid binding*	
	[^{3}H]E_2	[^{3}H]2OH E_1
E_1	98	35.7
E_2	100	33.3
E_3	88.1	21.4
DES	96	27.4
2OH E_1	94.7	100
2OH E_2	91.8	79.5
2OH E_3	78.7	82.1
4OH E_1	93.4	87.4
4OH E_2	97	85.5
Progesterone	6.2	0.47
16-epiestriol	93.2	87.4
Dihydrotestosterone	16.1	
Cortisol	5.6	
Tam	91.1	78.6
4OH Tam	93.9	87.1
Adrenaline	0.8	12.1
Noradrenaline	2.6	9.2

Competition of a 200-fold excess of unlabeled compounds for binding of 5 nM [^{3}H] E_2 or 5 nM [^{3}H] 2OH E_1 on cytosol binding sites. See the Experimental section for incubation conditions. Results are expressed as percentage of inhibition of specific ^{3}H-steroid binding.

*Mean of 2 separate experiments on particularly large tumors.

Table 4. Specificity of catecholestrogen binding sites in the P4 membrane fraction (endoplasmic reticulum)

Competitor concentration (200-fold molar excess)	% Of inhibition of specific ^{3}H-steroid binding*
	[^{3}H]2OH E_1
E_2	0.4 ± 0.6
E_3	13.9 ± 2.5
4OH E_1	89.4 ± 5.7
2OH E_1	100
Tam	37.6 ± 22.1
4OH Tam	67.9 ± 10.2
Adrenaline	38.2 ± 15.4
Noradrenaline	25.3 ± 13.2

Competition of a 200-fold excess of unlabeled compounds for binding of 5 nM [^{3}H]2OH E_1 on membrane binding sites. See the Experimental section for incubation conditions. Results are expressed as percentage of inhibition of specific ^{3}H-steroid binding.

*Mean $\pm$ SD of 5 separate experiments.

Table 5. Cytosolic catecholestrogen binding sites

% Of remaining specific binding after E_2 treatment	
^{3}H-steroids assayed	
[^{3}H]2OH E_1	[^{3}H]E_2
77 ± 19.8*	11.5 ± 7.8

Cytosol was incubated at 4°C for 3 h with 2.10^{-6} M unlabeled E_2 before assays of specific bindings using 5 nM of appropriate ^{3}H-steroids. See the Experimental section for incubation conditions. Results are expressed as the percentage of the remaining specific binding after E_2 treatment. *Mean $\pm$ SD of 5 separate experiments.

Table 6. Double labeling assay

	Specific binding (fmol/ml cytosol)	
	Channel I [^{3}H]2OH E_1	Channel II [^{125}I]E_2
Without pre-incubation	198	625
With pre-incubation with 2.10^{-6} M E_2	91	2.2

Cytosol was incubated together with 5 nM [^{125}I]E_2 and 5 nM [^{3}H]2OH E_1. Specific bindings of each steroid were determined with or without pre-incubation with 2.10^{-6} M E_2. Correct adjustment of the counting channels was set after determination of the energy spectra of iodine-125 and tritium. Channel I: tritium (lower limit 0, upper limit 390 U), Channel II: iodine-125 (lower limit 390, upper limit 600 U). By dynamic adjustment of the channels for each samples, the spill over of iodine-125 in the tritium channel was prevented. Moreover, owing to an automatic quench compensation the ^{125}I-counting efficiency remained fairly high.

with E_2, suggesting that other CE binding sites might exist. A similar type of experiment was performed adding the two tracers simultaneously, 5 nM [^{3}H]2OH E_1 plus 5 nM [^{125}I]E_2, in the incubation medium after pre-incubation with 2.10^{-6} M E_2. Only traces of specific E_2 binding was found in contrast to specific 2OH E_1 binding which was still significant (Table 6).

Competitive effects of the main competitors on [^{3}H]2OH E_1 binding in the endoplasmic reticulum are shown in Table 4. E_2 was unable to compete for CE binding sites in this membrane fraction, however, as in the cytosol, triphenylethylene derivative 4OH Tam was an efficient competitor for these CE binding sites.

In the two competition experiments, in the soluble as well as in the membrane fractions, compounds having dihydroxyphenolic moiety were potent inhibitors for CE binding sites, and inhibition was more significant in the membrane fraction.

To investigate the possibility of [^{3}H]2OH E_1 binding to catechol-binding proteins such as COMT, enzymatic activities were assayed in breast tumor cytosol or endoplasmic reticulum. A significant COMT activity was demonstrated in all samples, and large variations were observed among the tumors (Table 7). Besides, [^{3}H]2OH E_1 binding was estimated in the same samples: no specific binding was found in 21 out of the 38 (55%) cytosol and in 4 out of the 20 (20%) endoplasmic reticulum fractions assayed. Therefore, whatever the fractions studied, no correlation was noted between COMT activity and 2OH E_1 binding.

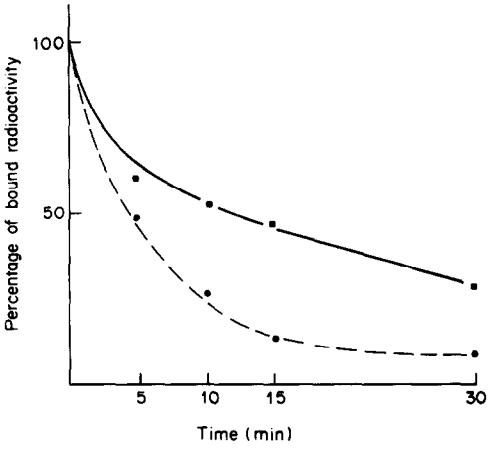

Fig. 2. Rate of dissociation of complexes between ^{3}H (CE) and breast cancer cytosol or membrane binding sites. Cytosol and membrane were labeled by incubation with [^{3}H]2OH E_1, at 4°C prior to dissociation experiments carried out on the diluted materials. See the Experimental section for the specific conditions. Results are expressed in term of percentage of bound radioactivity remaining at each time point. — [^{3}H]2OH E_1 cytosol complex, --- [^{3}H]2OH E_1 P4 membrane complex.

Dissociation studies

Figure 2 shows that in the cytosol approx 50% of bound [^{3}H]2OH E_1 had dissociated from cytosolic sites within 10 min. In the P4 membrane fraction, the dissociation was more rapid and was nearly complete after 15 min. The dissociation rate constants were 3.30 h^{-1} and 8.30 h^{-1} respectively.

Qualitative analysis of cytosolic complexes

Because of the rapid dissociation of the CE complexes, the experiments were carried out within the shortest time possible i.e. 15 min. The chromatographic (HPLC) studies of CE complexes revealed one specific high-molecular weight component, eluting just after the void volume. A mol. wt around 330,000 Daltons was calculated by the method of Siegel and Monty[19], this CE complex is apparently analogous to the 8–9 S form observed with E_2 complexes using the sucrose gradient method (Fig. 3a). The preparation exhibited moreover a large peak of unbound ligand in fractions from 26 on.

When cytosol was preincubated with 2.10^{-6} M cold E_2 before incubation with [^{3}H]2OH E_1, the specific

Table 7. Correlations between COMT activity and 2OH E_1 binding in breast tumor fractions

Tissue fraction	No. of tumors	COMT activity* (range)	[^{3}H]2OH E_1 binding† (range)	Coefficient of correlation
Cytosol	38	8.4–107	0–138	$r = 0.105$, NS‡
Endoplasmic reticulum	20	11–337	0–148	$r = 0.18$, NS

*COMT activity is expressed as pmole of radioactive 2-methoxyestrone formed per 20 min per mg protein.
†[^{3}H]2OH E_1 binding is expressed as fmol per mg protein.
‡NS: not significant.

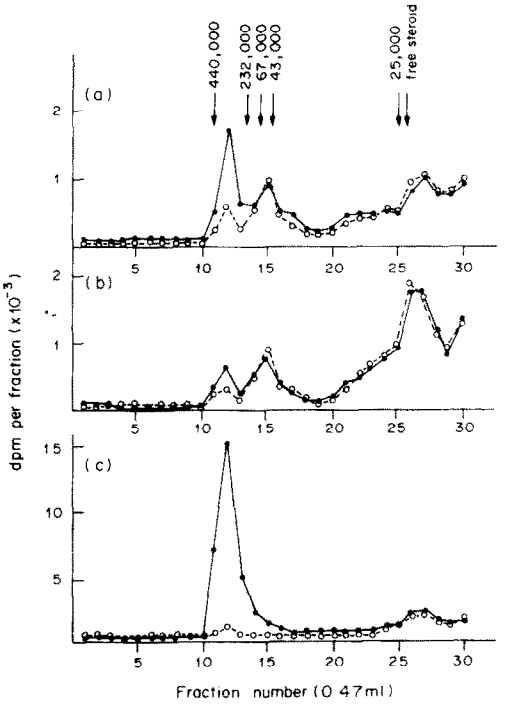

Fig. 3. HPLC analyses of cytosolic 2OH E_1 and E_2 binding components. Analyses were carried out with the same cytosol on Protein Pak 300 column using buffer B plus 10 mM sodium molybdate as eluant at a flow-rate of 1 ml/min. See the Experimental section for the specific conditions. ●——● total binding, ○——○ non specific binding. a, 2OH E_1 binding component; b, 2OH E_1 binding component after saturation with 2.10^{-6} M cold E_2; c, E_2 binding component.

high molecular weight component was reduced but not abolished (Fig. 3b).

E_2 complex was analysed under the same conditions. Virtually all the specific binding was exhibited by high molecular weight species (mol. wt 330,000 Daltons). Lower molecular-weight species were rarely observed perhaps due to the rapid separation afforded by HPLC (Fig. 3c).

DISCUSSION

This paper is focused on binding of 2OH E_1, the most prevalent E_2 metabolite in breast cancer. We first investigated the specificity of binding and reported no 2OH E_1 binding in nonestrogenic target tissues (prostate, testis), either in the cytosol or in the endoplasmic reticulum. In the cytosol of normal breast tissue, we were likewise unable to demonstrate any 2OH E_1 binding as well as no E_2 binding assayed as a comparison. This finding also reported by others [20, 21] may be due to the relatively high concentration of adipose cells and connective tissues as well as the comparatively low number of epithelial cells in these specimens. In the endoplasmic reticulum, however, we measured a slight 2OH E_1 binding and no E_2 binding.

In breast cancer, the present data show specific 2OH E_1 cytosolic binding sites. Their dissociation rate ($3.30\ h^{-1}$) was very rapid as has been shown for CE binding in the uterus [22]. Their dissociation constants (0.54 ± 0.10 nM) were within the same order of magnitude of those reported in the cytosol of rat hypothalamus [24, 25]. 2OH E_1 was found to be a potent competitor for E_2 cytosolic receptors and, conversely, E_2 was a weak competitor for 2OH E_1 binding sites in comparison with CE and even with the triphenylene derivative 4 OH Tam. In the nucleus, we had previously demonstrated that CE were potent competitors especially for the second nuclear binding sites with low affinity for E_2 [23].

In the membrane fractions, we demonstrate CE binding sites which cannot be accounted for by cytosolic contamination since cytoplasmic enzymatic marker was absent in these fractions. 2OH E_1 specific binding was found in P4 membrane fraction which corresponds to the endoplasmic reticulum; the K_d values ($K_d = 3.36 \pm 1.32$ nM) were in a similar range to that usually observed with hormone receptors and the dissociation rate was very fast ($8.30\ h^{-1}$). Moreover, antiestrogens and catecholamines but not estrogens were competitors for these CE binding sites.

The fractions $P_2 + N_2$, corresponding to the plasma membranes, were not considered further in subsequent studies because of their high K_d values.

The precise biochemical mechanism by which CE exert their effects is not fully understood. Most of the experimental data suggests that CE occupy estrogen receptors of the brain [26] and the uterus [27], and are translocated to the nucleus [28].

Depending on the system studied, their biological activities have been shown to be estrogenic, nonestrogenic and even antiestrogenic. Kirchhoff *et al.* have proposed [29] that the antiestrogenic properties of the 2OH estrogens were the result of a highly reduced intrinsic activity of nuclear estrogen receptors complexed with CE. The specific antiestrogenic properties of dihydroxysubstituted steroids have been studied by Schneider *et al.* [30–31]. They demonstrated that a shift of the hydroxy groups from the para position to the meta position in DES leads to a compound with antiuterotropic and mammary tumor inhibiting properties. Recently, in the human breast cancer cell line MCF-7, 2OH E_1 added to culture medium has been shown to suppress tumor cell proliferation both under control and estradiol stimulatory conditions [32].

In addition to a binding to cytosolic estradiol receptors, the present results demonstrate that CE could be found associated with binding sites that were distinct from the estrogen receptors. It can be argued that the binding may be related to some extent to the enzyme COMT which is present in breast tissue [8]. Indeed, CE are known to be good substrates for COMT with affinity constants (i.e. K_m 2OH $E_1 = 20\ \mu M$) which are the tenth of that reported for catecholamines (i.e. K_m noradrenaline $= 200\ \mu M$)

[33]. However, this does not rule out the possibility of other types of binding sites, e.g. the specific CE binding sites described by Schaeffer *et al.*[34] in the membranes of rat pituitary cells. In fact, the present data do not agree with a sole binding of 2OH E_1 on enzymatic sites because no correlation between COMT activity and 2OH E_1 binding was noted, in addition the measured K_d for 2OH E_1 binding are much lower that the K_m reported in the literature for CE and analogues. These findings are particularly relevant for the endoplasmic reticulum, where estradiol receptors do not account for CE binding sites.

If CE act as antiestrogens, analogies with triphenylethylene derivatives may be suggested. Many reports [35, 36, 37] have now provided evidence of subcellular distribution and ligand binding specificity of triphenylethylene derivatives different from those of the estrogen receptors. Moreover, it has been shown that breast tumoral cell lines devoid of estrogen receptors were able to bind antiestrogens [38]. In fact, although the inhibitory action of triphenylene derivatives on cancer growth has been suggested to be mediated by competition for the estrogen receptor [39], the exclusive role of the estrogen receptor in *in vitro* systems is still controversial [40, 41, 42]. Recently, an interaction of triphenylethylene derivatives with dopamine receptor binding has been described in membrane preparation from rat brain [43].

Our results demonstrate the interaction between CE and estrogen receptors in human mammary tumors and suggest that the action of CE, which is still ill-defined, could also be mediated through mechanisms other than those linked to estradiol receptors.

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