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Contribution of α_2-adrenoceptors to the mitogenic effect of catecholestrogen in human breast cancer MCF-7 cells

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ABSTRACT

Catecholestrogens are estrogen metabolites formed by hydroxylation of 17β-estradiol and estrone at either the C-2 or C-4 position, rivaling the parent estrogens in concentration. The objective of the present work was to assess if their catechol group could make them induce proliferation of human breast cancer cells via α_2-adrenoceptors. In competition studies in human breast cancer MCF-7 cells, high concentrations of 2-hydroxy-estradiol (2-OH-E_2), 2-hydroxy-estrone (2-OH-E_1) and 4-hydroxy-estrone (4-OH-E_1) competed for [^{3}H]-rauwolscine binding, whereas 4-hydroxy-estradiol (4-OH-E_2) did not. The contribution of α_2-adrenoceptors and estrogen receptors (ERs) in proliferation enhancement was analyzed with specific antagonists. The specific α_2-adrenergic antagonist yohimbine partially reversed the effect of catecholestrogens except 4-OH-E_2. The selective ER downregulator ICI-182780 or fulvestrant partially or totally reversed the effect of all hydroxylated catecholestrogens. When analyzing the effect of the combination of both antagonists in MCF-7, the contribution of the α_2-adrenoceptors and ERs for 2-OH-E_2, 2-OH-E_1 and 4-OH-E_1 was mixed, whereas for 4-OH-E_2, the only receptor implied was an ER. In MDA-MB-231 cells (ER-α negative) the proliferation stimulation by these three catecholestrogens and reversal by the adrenergic antagonist was also observed. It can be concluded that α_2-adrenoceptors contribute at least in part to the mitogenic effect of 2-OH-E_2, 2-OH-E_1 and 4-OH-E_1.

1. Introduction

Breast cancer is by far the most frequent cancer of women (23% of all cancers), with an estimated 1.15 million new cases in 2002, ranking second overall when both sexes are considered together [1]. The 411,000 annual deaths by this disease represent 14% of female cancer deaths. Breast cancer is the most prevalent cancer in the world today; there are an estimated 4.4 million women alive who have had breast cancer diagnosed within the last 5 years [1].

Estrogens were discovered over 75 years ago, and, shortly thereafter, their carcinogenic activity was shown in animal experiments (cited in [2]). Since the 1960s, abundant epidemiologic evidence has been collected that implicates estrogens as prime risk factors for the development of human breast cancer. Estrogens are thought to have a role in the promotion and progression of hormone-dependent tumors. Levels of estrogens within these tumors are higher than in the normal breast tissue and the peripheral circulation, indicating that these hormones may be synthesized *in situ* [3]. Catecholestrogens are estrogen metabolites formed by the aromatic hydroxylation of 17β-estradiol (E_2) and estrone (E_1) at either the C-2 or C-4 position. They are generated by the activity of different intracellular hydroxylases, all of which are cytochrome P450 monooxygenase-associated enzymes [4]. In mammalian species, catecholestrogen formation from E_2 is quantitatively the most important metabolic pathway of this endogenous sex hormone, rivaling the parent estrogens in concentration [4]. Carcinogenesis is usually viewed as a stepwise process beginning with genotoxic effects (initiation) followed by enhanced cell proliferation (promotion) [2]. Initiation effects of catecholestrogens were largely investigated by other groups. It has been reported [5] that the levels of the ratios of depurinating DNA adducts to their respective estrogen metabolites and conjugates were significantly higher in high-risk women and women with breast cancer than in control subjects. Also [6], treatment of immortalized human breast epithelial cells MCF-10F with 4-hydroxyestradiol (4-OH-E_2) or 2-hydroxyestradiol (2-OH-E_2) induces their neoplastic transformation *in vitro*, even in the presence of the antiestrogen ICI 182780. Russo et al. have described [7] that estrogens act as carcinogens in the human breast. Using human breast epithelial cells in a well-controlled environment, they demonstrated that E_2 and its metabolite 4-OH-E_2 induce transformation of MCF-10F cells *in vitro* (the cells form colonies in agar methocel, lose the capacity to differentiate by forming 3-dimensional structures). With

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respect to proliferation, Schutze et al. [8,9] reported that 2-OH-E_2 and 4-hydroxyestrone (4-OH-E_1) induce cell proliferation, increase secretion of a 160-kDa protein and pS2 mRNA levels in MCF-7 cells.

Although catecholestrogens certainly have an estrogenic action (reviewed in [10]), their catechol groups could eventually make them suitable ligands for adrenergic receptors. The α_2-adrenoceptors are members of the G-protein-coupled receptor superfamily that trigger physiological responses to the endogenous catecholamines by modulating the activity of a large panel of effectors including adenylyl cyclase, G protein-coupled inwardly rectifying potassium (K^+) channel, PLCβ, mitogen-activated protein kinases (MAPKs) and PI3K [11]. α_2-Agonists are used clinically in the treatment of hypertension, glaucoma, and attention-deficit disorder, in the suppression of opiate withdrawal, and as adjuncts to general anesthesia [12]. Our group has recently described [13] the presence of α_2-adrenoceptors in several human breast cancer cells, linked to increased proliferation.

The aim of the present manuscript was to assess if part of the mitogenic action of catecholestrogens in human breast cancer cells could be mediated by α_2-adrenoceptors.

2. Materials and methods

2.1. Chemicals

Glutamine, yohimbine-HCL, and rauwolscine-HCL were purchased from ICN Biomedicals Inc. (OH, USA). Methyl [^{3}H]-thymidine (NET 027E; Specific Activity: 20 Ci/mmol) and Methyl [^{3}H]-rauwolscine (NET-722, Specific Activity: 71 Ci/mmol) were from New England Nuclear-PerkinElmer (MA, USA). Antibiotics, culture media, trypsin, insulin and Fetal Calf Serum (FCS, "South American") were from Invitrogen Life Technologies (Carlsbad, CA, USA). The other compounds were purchased from Sigma–Aldrich, (St. Louis, MO, USA). Culture media were either from Invitrogen or from Sigma–Aldrich. Liquid scintillation solution was Optiphase Hisafe 3, from PerkinElmer (Boston, MA, USA). The catecholestrogen were first from ICN Biomedicals Inc. (OH, USA) and then from Sigma–Aldrich.

2.2. Cell cultures

MCF-7 and MDA-MB-231 human breast cancer cells were obtained from the "American type culture collection, ATCC" (Manassas, VA, USA). MCF-7 cells were routinely cultured as already described [14] in phenol red free [15] Dulbecco's Modified Eagle's Medium:Ham F12 (1:1) supplemented with 10% FCS, 2 mM glutamine, 2 μg/ml bovine insulin, 100 Ul/ml penicillin, 100 mg/ml streptomycin and 15 mM HEPES. Cells were sub-cultured once weekly after trypsinization (0.25% trypsin–0.025% EDTA) and seeded at a concentration of 80,000 cells/25 cm^3 flasks. Medium was changed twice weekly. MDA-MB-231 cells were incubated in Leibovitz's L-15 medium with 2 mM L-glutamine and 10% FCS and sub-cultured in the same way as MCF-7 cells.

2.3. Preparation of the compounds

The adrenergic antagonists were prepared in 0.1 mM ascorbic acid, final concentration. The estrogen antagonist ICI 182780 was prepared in absolute ethanol. The catecholestrogen were dissolved in 0.1 mM ascorbic acid in 10% absolute ethanol and immediately aliquoted and frozen. The adrenergic antagonists and the catecholestrogen were thawed immediately before use and diluted just before incubation. The stability of the compounds was tested routinely by repeating standard proliferation experiments.

2.4. [^{3}H]-rauwolscine binding

Binding assays for α_2-adrenoceptors were carried out as already described [13]. This technique was a modification of [16] and [17]. Briefly, cells were seeded at a concentration of 10,000–50,000 cell/well in 24 well-plates in culture media supplemented with 10% FCS, and incubated at 37 °C for 48–72 h. At near confluence, the culture media were changed to 2% charcoal-stripped FCS RPMI, 2mM glutamine, 100 Ul/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml amphotericin B and 15 mM HEPES, and incubated for 48 h. The cells were washed three times with buffer TME (50 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM EDTA, pH 7.4). The washed cells were incubated for 30 min with a constant concentration of [^{3}H]-rauwolscine with or without increasing concentrations (from 10^{-11} to 10^{-4} M) of catecholestrogens at 37 °C. The α_2-adrenergic antagonists rauwolscine and yohimbine were used for comparison. The reaction was stopped by washing three times with buffer TME and 2 ml scintillation solution were added directly over the cell monolayer. Radioactivity was measured in a liquid scintillation counter. Cell number was determined in parallel incubations.

Results are expressed as percentage of the binding obtained in the absence of unlabelled compounds. EC^{50} values were calculated as the Hill equation, or four-parameter logistic equation, or variable slope sigmoidal equation, using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. K_i values were determined according to the equation described by Cheng and Prusoff [18].

2.5. Proliferation studies

The cells were seeded at 10,000 cells/well in 96-well plates and incubated in DMEM-F12 for MCF-7 or Leibovitz's medium for MDA-MB-231 cells with 2% charcoal-stripped FCS, 2 mM glutamine, 100 Ul/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml amphotericin B and 15 mM HEPES. After 24 h, culture media were changed and the different compounds were added. This procedure was repeated for three days, and at the third day, 0.1 μCi [^{3}H]-thymidine/well were added. The cells were harvested with a Nunc cell harvester 8 and the filters were counted in a liquid scintillation counter.

In the first series of experiments, the catecholestrogens were used at increasing concentrations from 10^{-12} to10^{-6} M. Results are expressed as percentage of the value obtained in the absence of any compound. In a second series of experiments, a constant concentration of catecolestrogen (30 nM) was incubated in the presence or absence of increasing concentrations (from 10^{-9} to 10^{-6} M) of the specific α_2-adrenergic antagonist yohimbine. The selective estrogen receptor (ER) downregulator (SERD) ICI 182780 (also called fulvestrant) was then used in concentrations ranging from 10^{-10} to 10^{-5} M. Finally, a constant concentration of cathecholestrogen (30 nM) was incubated in the presence or absence of the specific α_2-adrenergic antagonist rauwolscine (1 μM), the estrogen antagonist ICI 182780 (1 μM) or a combination of both with the estrogen receptor positive MCF-7 and the ERα-negative MDA-MB-231 cells. Results are also expressed as percentage of the value obtained in the absence of any compound.

2.6. Statistical analysis

Statistical analysis for binding studies was performed by ANOVA followed by Duncan's test [19] whereas the proliferation studies were analyzed by ANOVA followed by Tukey–Kramer test [19]. Differences were considered statistically significant when $P < 0.05$.

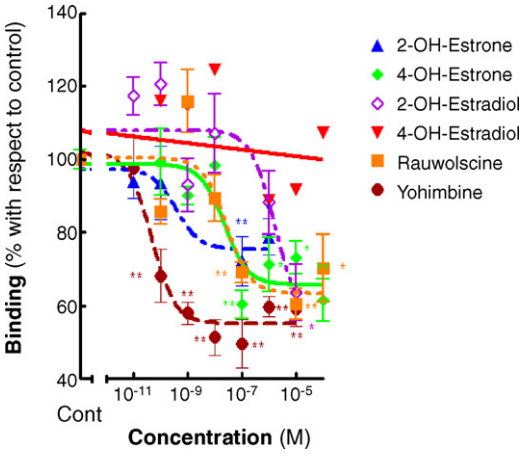

Fig. 1. Competition studies of catecholestrogens as compared to classical adrenergic antagonists for the α_2-adrenergic ligand rauwolscine binding. 2-OH-estradiol (◇), 4-OH-estradiol (▼), 2-OH-estrone (▲), 4-OH-estrone (◆) and the α_2-adrenergic antagonists rauwolscine (■) and yohimbine (●). Competition assays were performed with 2.47 nM [^{3}H]-rauwolscine for 2-OH-E_2 and 4-OH-E_2, 3.1 nM for 2-OH-E_1 and 4-OH-E_1 and 5.01 nM for rawolscine and yohimbine as stated in Section 2. $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}P < 0.001$ represent significant differences between groups, as analyzed by ANOVA followed by Dunett's comparison test. All assays were performed by quadruplicate and repeated with similar results. Mean ± S.E.M. is shown.

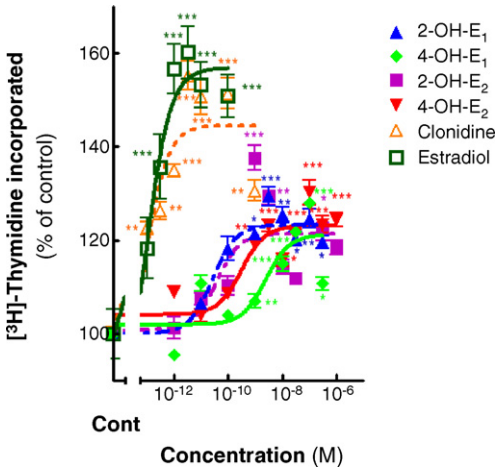

Fig. 2. Proliferation studies of catecholestrogens in MCF-7 cells. 2-OH-estradiol (■), 4-OH-estradiol (▼), 2-OH-estrone (▲), 4-OH-estrone (◆), estradiol (□) and Clonidine (△) Proliferation studies were performed with increasing concentrations of the catecholestrogens as stated in Section 2. Results are expressed as percentage of the value obtained in the absence of any compound. $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}P < 0.001$ represent significant differences between groups, as analyzed by ANOVA followed by Tukey–Kramer comparison test. All assays were performed by octuplicate (except for the 16 controls) and repeated with similar results. Mean ± S.E.M. is shown.

3. Results

3.1. Competition studies

In order to assess if catecholestrogens 2-OH-E_2, 2-hydroxyestrone (2-OH-E_1), 4-OH-E_2 and 4-OH-E_1 were able to bind to α_2-adrenoceptors, competition studies were performed with [^{3}H]-rauwolscine as specific ligand. As can be seen in Fig. 1, high concentrations of 2-OH-E_2 were able to compete for [^{3}H]-rauwolscine binding, whereas no effect was observed for 4-OH-E_2. Both 2-OH-E_1 and 4-OH-E_1 compete at lower concentrations for tritiated rauwolscine binding. The specific α_2-adrenergic antagonist rauwolscine and yohimbine were included in order to compare catecholestrogens with known α_2-adrenergic compounds. In Table 1, the EC_{50} values obtained from Prism calculation are shown. In order to allow a better comparison between these experiments and others, the K_i values were calculated with the Cheng and Prussoff formula [18]. 2-OH-E_1 was particularly efficient to compete for the specific ligand, even better than rauwolscine.

3.2. Proliferation studies

The ability of the different hydroxylated catecholestrogens to stimulate proliferation of MCF-7 human breast cancer cells was confirmed. As shown in Fig. 2, 2-OH-E_2, 4-OH-E_2 2-OH-E_1 and 4-OH-E_1 significantly stimulate proliferation, even at very low concentrations, although the last compound was the least efficient in producing this effect. In Table 2, the EC_{50} values are shown. For comparison, clonidine [13] and estradiol were included in the experiment as positive controls in order to compare the sensitivity of the cells as well as the percentage of stimulation.

The next step in the investigation was to evaluate the implication of the α_2-adrenoceptors in this increase of proliferation. To do so, the specific α_2-adrenergic antagonist yohimbine was used to block the effect. As can be seen in Fig. 3 and Table 3, this compound partially reversed the effect of the catecholestrogens except in the case of 4-OH-E_2. The α_2-adrenoceptors therefore contribute at least in part to the mitogenic effect of 2-OH-E_2, 2-OH-E_1 and 4-OH-E_1, but are not implied in the effect of 4-OH-E_2.

The contribution of the ERs to this mitogenic effect was analyzed by inhibiting them with the estrogen antagonist ICI 182780. As can be seen in Fig. 4, this agent was able to partially or totally reverse the effect of all hydroxylated catecholestrogens, suggesting that in all cases at least one of the ERs is implied in the effect. In Table 3, the EC_{50} values are shown.

Table 1
Comparison of effective concentration 50 (EC_{50}) and inhibition constants of the four catecholestrogens studied and the α_2-adrenergic antagonists rauwolscine and yohimbine from Fig. 1

Compounds		EC_{50}	K_i
2-OH-estradiol	Catecholestrogen	0.633 μM	0.784 μM
4-OH-estradiol	Catecholestrogen	–	–
2-OH-estrone	Catecholestrogen	0.354 nM	0.407 nM
4-OH-estrone	Catecholestrogen	21.9 nM	25.2 nM
Yohimbine	α_2-Adren antag	44.32 pM	50.9 pM
Rauwolscine	α_2-Adren antag	30.11 nM	37.34 nM

The EC_{50} obtained were processed by Cheng and Prusoff [18] analysis in order to allow a better comparison of the results.

Table 2
The effective concentration 50 (EC_{50}) of the four catecholestrogens and the positive controls clonidine and estradiol studied in Fig. 2

Compounds	EC_{50}
2-OH-estradiol	50.85 pM
4-OH-estradiol	0.358 nM
2-OH-estrone	26.18 pM
4-OH-estrone	2.427 nM
Clonidine	0.430 fM
Estradiol	0.336 pM

Table 3
The effective concentration 50 (EC_{50}) of the four catecholestrogens studied in Fig. 3 (Yohimbine) and 4 (ICI 182780)

Compounds	Yohimbine: EC_{50}	ICI 182780:EC_{50}
2-OH-estradiol	21.24 nM	0.891 pM
4-OH-estradiol	–	89.630 nM
2-OH-estrone	412.90 nM	219.50 nM
4-OH-estrone	1.607 nM	0.324 pM

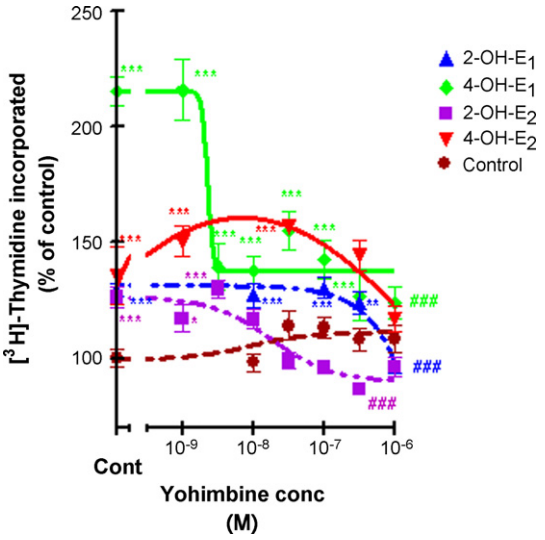

Fig. 3. Inhibition of the proliferation induced by catecholestrogens by the specific α_2-adrenergic antagonist yohimbine in MCF-7 cells: 2-OH-estradiol (■), 4-OH-estradiol (▼), 2-OH-estrone (▲) and 4-OH-estrone (◆). The control values (●) are shown as increasing concentrations of yohimbine in the absence of any catecholestrogen. Proliferation studies were performed with a constant concentration of the catecholestrogen (30 nM) and increasing concentrations of yohimbine as stated in Section 2. The value obtained in the absence of any compound is considered 100% and the remaining values were calculated with respect to this one. Values are the mean ± S.E.M. of 8 wells for treated and 16 for untreated cultures. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}P < 0.001$ represent significant differences between groups, as analyzed by ANOVA followed by Tukey–Kramer comparison test. All assays were performed by octuplicate (except for the 16 controls) and repeated with similar results. Mean ± S.E.M. is shown.

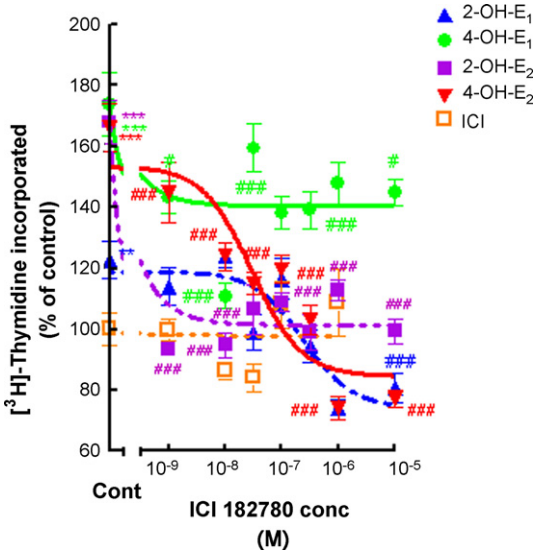

Fig. 4. Inhibition of the proliferation induced by catecholestrogens by the selective estrogen receptor downregulator ICI 182780 in MCF-7 cells: 2-OH-estradiol (■), 4-OH-estradiol (▼), 2-OH-estrone (▲) and 4-OH-estrone (●). □ is the control of increasing concentrations of ICI 182780 in the absence of any catecholestrogen. Proliferation studies were performed with a constant concentration of the catecholestrogens (30 nM) and increasing concentrations of ICI 182780 as stated in Section 2. The value obtained in the absence of any compound is considered 100% and the remaining values were calculated with respect to this one. Values are the mean ± S.E.M. of 8 wells for treated and 16 for untreated cultures. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}P < 0.001$ represent significant differences between groups, as analyzed by ANOVA followed by Tukey–Kramer comparison test. All assays were performed by octuplicate (except for the 16 controls) and repeated with similar results. Mean ± S.E.M. is shown.

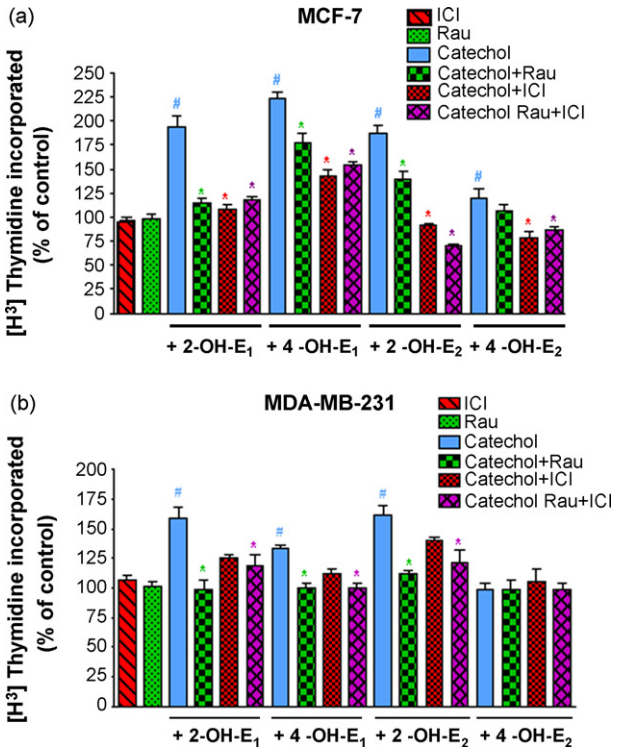

Fig. 5. Effect of the specific α_2-adrenergic antagonist rauwolscine and/or the selective estrogen receptor downregulator ICI 182780 on cell proliferation induced by catecholestrogens in MCF-7 cells (a) and MDA-MB-231 cells (b). The experiments were performed with a constant concentration of the corresponding catecholestrogen (30 nM) and a constant concentration of the antagonist (1 μM). $^{\#}p < 0.001$ with respect to the control in the absence of any compound (except in the case of 4-OH-E_2, in which p value was <0.05 in (a)) $^{*}p < 0.001$ with respect to the incubation in the presence of the corresponding catecholestrogen in (a); $p < 0.05$ in (b), as analyzed by ANOVA followed by Tukey–Kramer comparison test. The experiment was repeated with similar results. Mean ± S.E.M. is shown.

In order to better clarify the contribution of α_2-adrenoceptors and ERs in the mitogenic effect of the four hydroxylated catecholestrogens, Fig. 5a shows the reversion of the mitogenic effect of each of these compounds by the α_2-adrenergic antagonist rauwolscine and the estrogen antagonist ICI 182780 both separately and together in MCF-7. In the case of 2-OH-E_2, 2-OH-E_1 and 4-OH-E_1, again, the contribution of the α_2-adrenoceptors was evident, whereas in the case of 4-OH-E_2, the only receptor implied was an ER. However, no additive effect was found in the reversion, suggesting that the effects could be linked. Furthermore, the mitogenic effect of the four catecholestrogens was investigated in MDA-MB-231 cells which lack the expression of ER-α (but not β [20]). In this case (Fig. 5b), again, 2-OH-E_2, 2-OH-E_1 and 4-OH-E_1 showed a significant increase of proliferation. This effect was reversed by the simultaneous incubation of the specific α_2-adrenergic antagonist rauwolscine. No significant reversion of the effect was found with the estrogen antagonist ICI 182780. When 4-OH-E_2 was analyzed, neither the stimulation of proliferation nor the reversion by the α_2-adrenergic antagonist rauwolscine or by the estrogen antagonist ICI 182780 were observed in these cells.

4. Discussion

4.1. Competition studies

The first step of the present investigation was the assessment of binding of the hydroxylated catecholestrogens to

α_2-adrenoceptors. In order to analyze this possibility, the tritiated antagonist rauwolscine was used, because this compound has a greater affinity for α_{2C} and α_{2B} subtype than for the canonical α_{2A} subtype [21], whose expression is absent in both MCF-7 and MDA-MB-231 cells [13]. The results shown in the present manuscript clearly show that 2-OH-E_2, 2-OH-E_1 and 4-OH-E_1 compete for rauwolscine binding at medium concentration, the affinity of 2-OH-E_1 being intermediate between rauwolscine and yohimbine. On the other hand, 4-OH-E_2 exhibited no interaction with this receptor. A long time ago, an interaction of 2-OH-E_2 with the β-adrenoceptors has been described with no interaction with α-adrenoceptors, by competition to dihydroergocriptine binding [22], the ligand available at that time. The β-adrenoceptors have been described in MCF-7 and MDA-MB-231 [23] and their stimulation inhibits cell growth in MDA-MB-231 cells [24,25]. These receptors could eventually account for part of the proliferative effect of the catecholestrogen if acting as antagonists. They could also account for the percentage of binding which is not displaced by the catecholestrogen.

The group of Vandewalle has shown that catecholestrogens were able to bind to cytosolic and endoplasmic reticulum binding sites [26] and also described a membrane binding site for 2-OH-E_1 in several human breast cancer cell lines, MCF-7 among them [27]. Steroid hormones did not compete for binding, but the catecholamines did [26]. Recently, another group has described the interaction of catecholestrogens with a cytosolic binding site, present even in ER-α knockout mice [4]. When analyzing competition in ER-α knock-out animals, norepinephrine displacement of tritiated 4-OH-E_1 and 4-OH-E_2 was important, whereas E_2 had no effect in this model [4]. Although the majority of the α_2-adrenoceptors are bound to plasma membrane, it has been described that the α_{2C} subtype, is mainly cytoplasmic [28]. It would be tempting to think that this cytoplasmic binding site could eventually be an α_{2C}-adrenoceptor.

4.2. Proliferation studies

The first series of proliferation experiments was designed to confirm the action of hydroxylated catecholestrogens in MCF-7 breast cancer cells in culture. The action of these compounds was always mitogenic with low EC_{50} values. It should be stated that in the same model, epinephrine and norepinephrine showed an EC_{50} value of 10 and 14.2 pM [14]. It has recently been described [29] that 2-OH-E_1, 2-OH-E_2, 4-OH-E_2 and 4-OH-E_1 significantly stimulated MCF-7 cell number, although this effect diminished significantly at higher concentrations. Another group has also shown that 2-OH-E_1 and 2-OH-E_2 significantly stimulated cell proliferation at concentrations 10 and 100 nM, respectively, in the same human cell line and this increase was reversed by 1 μM tamoxifen or ICI 182780 [30].

The biological importance of the increase in breast cancer cell proliferation is enhanced by the fact that intratumor concentrations of hydroxylated catecholestrogen are very important. Whereas E_2 and E_1 levels range the 40 and 30 fmol/mg tissue, respectively, 2-OH-catecholestrogen concentration is about 1600 fmol/mg tumor tissue and 4-OH-catecholestrogen 2000 fmol/mg tissue, 2-OH-E_2 and 4-OH-E_2 being significantly higher in cancer tissue as compared to normal breast tissue [31].

The catecholestrogens can enhance ERα-positive MCF-7 cell proliferation by estrogenic or adrenergic stimulation or acting through the specific receptors described though not already identified or cloned, that could be or intracellular or bound to membranes [4,26,27]. To analyze the first two possibilities, catecholestrogen stimulation of proliferation was reversed first by a specific adrenergic antagonist, then by a specific estrogenic downregulator and finally by the combination of both antagonists.

In order to analyze if catecholestrogen stimulation of proliferation was mediated by α_2-adrenoceptors a stimulating concentration of each catecholestrogen was incubated with increasing concentrations of the specific antagonist yohimbine. The same 30 nM concentration was chosen because all of the catecholestrogen were stimulatory at that concentration. As a parallel assay, a curve with the same concentrations of this adrenergic antagonist was performed and no effect of this compound alone was observed. In the case of 4-OH-E_2, cell proliferation was not affected by the presence of increasing concentrations of yohimbine, suggesting that the effect of 4-OH-E_2 is not mediated by α_2-adrenoceptors. This result is concordant with the absence of displacement of the tritiated rauwolscine. All the rest of the hydroxylated catecholestrogens showed a partial reversion by this antagonist. A very high yohimbine concentration was necessary in order to inhibit the stimulatory action of 2-OH-E_1, coincidently with its high affinity in displacement assays. This reversion, although partial, suggest that at least part of the mitogenic effect of catecholestrogens (with the exception of 4-OH-E_2) could be mediated by the α_2-adrenoceptors expressed in human breast cancer cells. Clonidine has shown a stimulatory effect on cell proliferation in previous work from our laboratory [13,14].

In order to evaluate the possibility of proliferation stimulation via one of the ERs, the estrogen receptor downregulator ICI 182780 was used. This compound causes ubiquitination and destruction of the ER–ICI 182780 complex, resulting in downregulation of the ER [32,33]. In every case, a partial reversion of the mitogenic action of catecholestrogen was observed by this compound, suggesting that part of this action is mediated either directly or indirectly by ERs.

4-OH-E_2 is similar to E_2 in its ability to bind to and activate the classical ER-α. Interestingly, the interaction of this estrogen metabolite with the ER appears to occur with a reduced dissociation rate compared with E_2, suggesting that the association of 4-OH-E_2 with the ER may last longer than that for its parent hormone, E_2 [10]. 2-OH-catecholestrogens also showed mitogenic activity in both MCF-7 and T47D and this activity was inhibited by the antiestrogens tamoxifen and ICI-182780 [30].

Breast cancer cell lines, such as MCF-7, undergo a robust proliferative response to estrogens [34]. MCF-7 cells were used for the experiments as cells that express both ER-α and -β [20]. MDA-MB-231 cells were chosen in order to confirm the proliferation effect of catecholestrogens in a cell line which express only ER-β and not ER-α [20]. In the ERα-negative cell line MDA-MB-231, again, 2-OH-E_1, 2-OH-E_2 and 4-OH-E_1 significantly stimulated cell proliferation, and the α_2-adrenergic antagonist rauwolscine reversed this effect to control levels. The simultaneous incubation of the catecholestrogen and the estrogen receptor downregulator did not reverse this effect. 4-OH-E_2 on the other hand showed no effect on cell proliferation in these cells. These results are expected, as ER-β has been mainly associated with inhibitory effects on cancer cell proliferation [35].

As a conclusion, these results clearly show that 2-OH-E_1, 2-OH-E_2 and 4-OH-E_1 exert at least part of the mitogenic action by binding to α_2-adrenoceptors. ERs are also implied in their action in ER-α positive MCF-7 cells. 4-OH-E_2 on the other hand, only binds to ERs and does not interact with α_2-adrenoceptors. The possibility of reversing both the estrogenic and the adrenergic effect of these compounds would perhaps encourage the validation of these drugs in clinical trials, considering their low toxicity and well-known pharmacokinetics.

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