

EFFECTS OF OESTRIOL: PRELIMINARY RESULTS ON RECEPTOR KINETICS IN TARGET TISSUES OF POSTMENOPAUSAL WOMEN

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Summary—This *in vivo* investigation was done to study the effects of intravaginal oestriol (E_3) administration on endometrial, myometrial and vaginal tissue of normal postmenopausal women.

All women received intravaginal E_3 -suppositories (containing 0.5 mg E_3) once a day for 3 weeks prior to hysterectomy. The medication was continued until the day of operation. At the time of operation both uterine and vaginal tissue was obtained.

The receptor content in the *cytosol* was measured by a multiple point-dextran-coated-charcoal assay using [3H] E_2 and [3H]ORG-2058 as ligands. The receptor content in the *nucleus* was measured by incubating purified whole nuclei in 10 nM [3H] E_2 for 18 h at 0°C. We have shown that under these conditions there is a total exchange of all occupied receptors.

Preliminary data on 4 patients are available. Vaginal cytology clearly showed an increase of the maturation value. Oestrogen receptor concentrations in the cytosol of all three tissues studied were lower than those obtained in untreated women, suggesting nuclear transformation of the receptor as a consequence of treatment. The nuclear E_2 receptor levels cannot be compared with normal women yet.

Progesterone receptors in endometrial and myometrial cytosol seemed to be higher than those in untreated women, indicating effects of the treatment. In the human, vaginal progesterone receptor cannot be used as a marker for oestrogenic stimulation because only exceptionally could their presence be detected in either treated or untreated women.

INTRODUCTION

Oestriol (E_3) is used in the treatment of postmenopausal women with climacteric complaints because it is considered to be mainly vaginotropic and not uterotrophic [1, 2]. This dissociation in the effects on different target tissues is important as this may implicate that E_3 is a safe oestrogen with respect to overstimulation of the endometrial tissue. This dissociation depends on the mode of administration. When given in a way leading to prolonged elevation of blood levels, E_3 leads to the same uterotrophic effect as oestradiol (E_2) [3]. These findings are consistent with results of biochemical and pharmacological investigations: (1) Pharmacokinetic research has shown a peak concentration of E_3 in plasma in the first hours after oral or vaginal administration. Thereafter a rapid decline in E_3 -levels in plasma is seen, possibly due to a rapid conjugation in the liver, a rapid clearance because of its weak binding to serum proteins and excretion in the gall and urine [4]. (2) Receptor studies have shown that E_3 binds to the same receptor as E_2 , but at a lower affinity [5]. Kinetic experiments by Clark *et al.* [6] have demonstrated that the E_3 -receptor complex has a much shorter nuclear retention time (<6 h) than the E_2 -receptor complex (>12 h) in animals. Competition of E_3 for the E_2 binding sites has also been found in human endometrial cytosol [7].

However, these results do not explain the differences in the vaginotropic and uterotrophic effects of E_3 . Several explanations for this discrepancy can be considered: (1) Differences in the uptake and retention of E_2 and E_3 between different target tissues. Recently we have described that there was no difference between these two oestrogens in uptake and retention in endometrial, myometrial and vaginal tissue of postmenopausal women [8]. (2) Differences in the specificity of oestrogen receptors. There are some data indicating the existence of a specific binding protein for E_3 besides the specific oestrogen receptor in the human vagina [5], but its function is completely unknown. (3) Differences in the concentration of oestrogen receptors in the three target tissues. Our data [9] showed a lower receptor content in vaginal cytosol as compared to myometrial and endometrial cytosol of the same patient. (4) Differences in the subcellular processing leading to different responses after oestrogen stimulation. It is generally accepted that the induction of the progesterone receptor is a specific response of an oestrogen target tissue to an oestrogenic stimulation. Recently we have shown [10] that the specific progesterone receptor is nonmeasurable in the human vagina, even after oestrogenic stimulation and therefore cannot be used as a marker for stimulation in this tissue.

In view of these considerations we have initiated a study on the effects of E_3 in normal postmenopausal women. Oestriol is administered per os or per vaginam in different amounts and with variable intervals between two administrations. In this paper preliminary results will be given on the effects of E_3 on oestrogen and progesterone receptors measured in subcellular compartments.

MATERIAL AND METHODS

Patients

Tissue samples were obtained from 4 postmenopausal women who underwent a hysterectomy for non-oncological reasons (age between 51 and 66 y, height 1.59–1.64 m, weight 54–88 kg). Patients received E_3 in a dosage of 0.5 mg per day as a vaginal suppository for a 3 week period prior to the operation. Beside the indication for the hysterectomy, the patients had no other signs of illness. The maturation value (M.V.) was determined on the vaginal smear before and after the period of treatment.

Tissues

The operations were performed under general anaesthesia using standard procedures. Immediately after extirpation the uterine and vaginal tissue adjacent to the cervix were placed on ice and transferred to the pathologist. Endometrial tissue and myometrium was obtained by excision; vaginal tissue was freed from connective tissue by excision after stretching. The remaining parts of the tissues were subjected to histological examination. The amount of endometrial tissue ranged from 0.07 to 1.0 g. About 1 g of myometrial and vaginal tissue was obtained from each patient. After investigation by the pathologist tissues were stored at -70°C .

Steroids

1,2,6,7- ^3H Oestradiol (sp.act. 99 Ci/mmol) was purchased from the Radiochemical Centre, Amersham, U.K. Diethylstilbestrol was obtained from Sigma Chemicals, Missouri, U.S.A. ^3H Org-2058 (sp.act. 42 Ci/mmol), a progestational compound with high affinity and specificity for the progesterone receptor, and unlabelled Org-2058 were obtained from the Radiochemical Centre, Amersham, U.K. Purity of the ligands ($>98\%$) was checked by thin layer chromatography at monthly intervals.

Buffer solutions

Buffer A: phosphate buffer of 0.01 M Na_2HPO_4 , 0.01 M NaH_2PO_4 , 0.001 M EDTA, 0.003 M NaN_3 , 0.1% thioglycerol and 10% glycerol, pH 7.5. Buffer B: buffer A with 500 U Trasylol (aprotinine) per ml. Buffer C: buffer A with 1 g bovine serum albumine (BSA, Povite, Oss, the Netherlands) per l. Buffer D: buffer C + 1% ethanol. Buffer E: buffer B with 10 g BSA per l.

Methods

The entire procedure described below was performed at $0-4^\circ\text{C}$. After weighing the tissue was minced with two scalpels in buffer B. The tissue was minced with a Buhler homogeniser (Braun, Melsungen, GFR) in 2–4 ml of buffer B dependant on the weight of tissue. After filtration through a cheese-cloth the suspension was homogenised in an automatic Pottertube (Braun, Melsungen, GFR) 3 times $5''$ at 500 rpm and centrifugated ($10'$ at 800 g). The pellet was used to prepare the nuclear fraction and the supernatant was used for a high speed cytosol. A purified nuclear fraction was prepared by washing the crude pellet with 10 ml of 5% Triton X-100 followed by centrifugation and 3 times with 10 ml buffer B and centrifugation, each time $10'$ at 800 g. Supernatants were discarded; the purified nuclear fraction was used for the estimation of the nuclear oestrogen receptors under exchange conditions.

Estimation of total nuclear oestrogen receptors

The purified nuclear fraction was resuspended in 1.5 ml buffer B. For exchange 250 μl of this suspension was added to tubes containing ^3H E_2 in a final concentration of 10^{-8} mol. To determine non-specific binding 10^{-5} mol DES was used in combination with 10^{-8} mol ^3H E_2 , dissolved in buffer E. Incubations lasted 20 h at 4°C . After incubation and centrifugation ($10'$ at 6000 g) the nuclear pellet was washed 2 times with 1 ml buffer A, supernatants were discarded. The resulting nuclear pellet was extracted with 0.5 ml 0.6 M KCl in buffer A, overnight at 4°C [11].

After dilution with 2.5 ml buffer A, to decrease the KCl-molarity to 0.1 M, the incubation mixture was centrifugated for $10'$ at 6000 g. The supernatant will contain the KCl-extractable fraction and the pellet will contain the non-KCl-extractable fraction. The KCl-extractable fraction contains the free, unbound radioactivity and the receptor-bound radioactivity. In order to separate these two fractions a protamine-sulfate (PS) precipitation was performed by adding 1 vol of 1.5 mg PS per ml in buffer A. After $10'$ at 4°C the solution was centrifugated ($10'$ at 6000 g), the supernatant, containing the free, unbound activity, was discarded. The PS-pellet was washed three times with 1.0 ml buffer A and the remaining pellet was extracted three times with 0.5 ml of ethanol. The combined ethanol-supernatant were used to measure the radioactivity by liquid scintillation counting. The non-KCl-extractable fraction was washed three times with 1.0 ml of buffer A, the remaining pellet was transferred with ethanol to a liquid scintillation vial and the total radioactivity in this pellet was counted.

Estimation of cytosol oestrogen and progesterone receptors

The concentration of oestrogen receptors ($E_2\text{Rc}$) and progesterone receptors (PgRc) was determined in

the high speed cytosol after centrifugation during 30' at 100,000 *g* (Kontron ultracentrifuge). The cytosol of myometrium, endometrium and vagina was incubated with tritium labelled ligands with and without excess of unlabelled ligand to correct for nonspecific binding. Because of the limited amounts of cytosol, 8 point assays were done in micro-titer plates. To prevent adsorption of the tracers to the plates, E_2 was dissolved in buffer C and Org-2058 in buffer D. Aliquots of cytosol (50 μ l) were incubated with [3 H] E_2 in concentrations of 5.10^{-10} to 5.10^{-9} M with and without DES at 10^{-6} M or with [3 H]Org-2058 in concentrations of 1.10^{-9} to 5.10^{-9} M with and without unlabelled Org-2058 in a 100-fold excess. After incubation for 18 h at 4°C under continuous shaking, bound and unbound ligands were separated by dextran coated charcoal (for E_2 : 0.25% charcoal and 0.025% dextran in buffer A; for Org-2058: 1.0% charcoal and 0.01% dextran plus 0.1% BSA in buffer A). After centrifugation (20' at 6000 *g*) an aliquot of the supernatant was counted.

Total protein was determined by the BioRad protein assay according to Bradford[12], reagents from BioRad, Alphen a/d Rijn, The Netherlands.

Liquid scintillation counting was done in a Packard 2660 Tri-Carb spectrometer with optifluor

Table 1. The maturation value, calculated according to Meisels[14] in 4 patients before the start and after 3 weeks of 0.5 mg E_3 per day intra-vaginally

	Maturation value	
	Before	After
1	52.5	60
2	xx	90
3	2.5	80
4	50	70

xx = Unreliable because of colpitis.

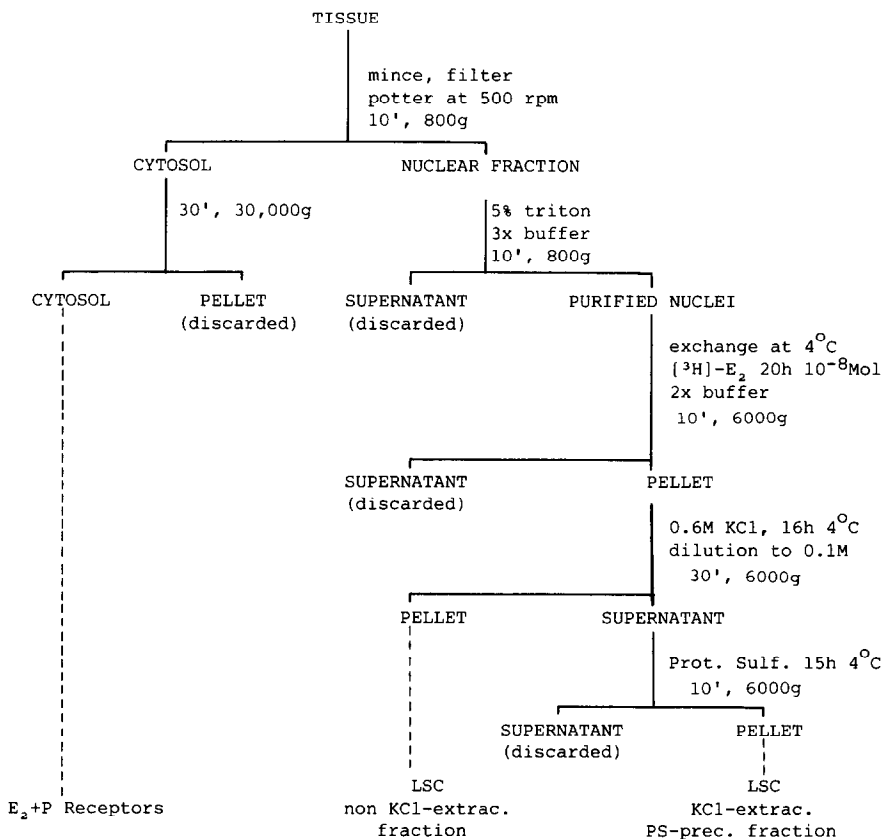
(Packard Instruments) as scintillator. Receptor data were analysed according to Scatchard [13]. The whole procedure is summarised in Scheme 1.

RESULTS AND DISCUSSION

The effects of E_3 were studied by (1) The cytological and histological examination of the vagina and the uterus (2) The concentration and subcellular distribution of oestrogen and progesterone receptors.

ad 1

The effect of E_3 on the vaginal cytology was clearly demonstrable. In 3 of the 4 patients an increase in the maturation value can be seen, as shown in Table 1.



Schematic outline of the procedure used to measure oestrogen and progesterone receptors in the cytosol and the oestrogen receptor in the nuclear fraction. Details are described in the text (see section Materials and Methods).

In patient 2 the MV before the start of the E₃-medication could not be calculated due to an infected vaginal smear, after treatment the MV is very high. In previous observations in untreated normal post-menopausal women no MV above 50 had been seen [9]. The histology of the endometrium showed in patients no. 1 and 4 only an atrophic endometrium without mitosis, the two other women had an endometrium with some signs of proliferation.

ad 2 Cytosol E₂-receptors

Results on the E₂Rc and PgRc determinations in cytosol of endometrium, myometrium and vagina are given in Table 2. The E₂Rc concentrations in endometrial, myometrial and vaginal tissues are lower than those described for untreated postmenopausal women. The low concentrations in the vagina of our patients with a high MV after treatment is in accordance with data from Wiegerinck *et al.*[9], who found a significant negative correlation between the vaginal E₂Rc level and the maturation value in postmenopausal women. The following explanations can be considered: (a) In parabasal cells the E₂Rc concentration is higher than in intermediate cells and much higher than in superficial cells [15]. Vaginal tissue with a high M.V. contains relatively few parabasal cells and many intermediate and superficial cells. (b) The low level of E₂Rc in the cytosol of the vagina may reflect a translocation of cytosol receptors to the nucleus caused by the E₃ treatment. Comparison of the nuclear receptor concentrations in our patients with those in nontreated postmenopausal women is not possible, no data are available. Comparison of the E₂Rc in the cytosol with those in the nucleus (Table 3, total specific binding) shows a different

pattern for the vagina compared with endometrium and myometrium, the vagina has the lowest ratio between cytosolic and nuclear oestrogen receptors. In our opinion this indeed indicates a high translocation of the cytosol receptor to the nucleus in the vagina after the treatment with E₃.

Cytosol progesterone receptors

The PgRc concentrations in the cytosol from myometrium of our patients were higher than those from untreated postmenopausal women (<100 fmol/mg, data from 16). Also the endometrial PgRc level in the cytosol seems to be higher than those reported by Martin *et al.*[17] In the vaginal cytosol the PgRc concentrations were not measurable, in accordance with earlier observations [10]. In one patient however, the PgRc amounted to 90 fmol/mg protein in vaginal cytosol. We have no explanation for this discrepancy.

Nuclear oestrogen receptors

The nuclear oestrogen receptors were measured under exchange conditions. We have found that at 0°C during 18 h at 10⁻⁸ M [³H]E₂ a maximal exchange of the endogenously occupied receptor sites is obtained in nuclei from human oestrogen target tissues (data not shown). Results on nuclear receptors both for the KCl-extractable fraction and for the non-KCl-extractable fraction are given in Table 3 in fmol per g tissue. In both fractions specific and non-specific binding sites were observed.

The non-KCl-extractable fraction contained a larger amount of E₂-binding than the KCl-extractable fraction. In different patients the ratio between the specific and the nonspecific oestrogen binding is variable.

Table 2. The oestrogen receptor (E₂Rc) and progesterone receptor (PgRc) concentration, expressed as fmol/mg protein, in the cytosol of endometrial, myometrial and vaginal tissue of E₃-treated postmenopausal women

	E ₂ -Receptor			Pg-Receptor		
	Endo	Myo	Vag	Endo	Myo	Vag
P1	35	65	<5	<5	800	<5
2	60	83	<5	550	1800	<5
3	250	65	9	1630	700	90
4	85	140	<5	185	390	<5

Values in fmol/mg protein cytosol
The high speed cytosol was incubated with [³H]E₃ (3 × 10⁻¹⁰ – 2 × 10⁻⁹ M) with and without unlabelled DES (3 × 10⁻⁷ – 2 × 10⁻⁶ M) to distinguish between specific and a-specific binding (for details, see text Materials and Methods).

Table 3. The oestrogen receptor concentration, expressed as fmol/g of tissue, in the nuclear fraction of endometrial, myometrial and vaginal tissue of E₃-treated postmenopausal women

	KCl-extrac. PS-prec. fraction						KCl-resistant fraction					
	Endometrium		Myometrium		Vagina		Endometrium		Myometrium		Vagina	
	spec	aspec	spec	aspec	spec	aspec	spec	aspec	spec	aspec	spec	aspec
P1	11	8	12	4	2	1	38	180	110	31	34	70
2	15	4	5	2	2	2	77	50	56	42	26	51
3	5	2	1	1	1	1	63	65	27	18	16	35
4	33	5	25	6	10	3	790	130	400	170	510	74

Values in fmol/g tissue
The nuclear fraction was incubated with [³H]E₂ (10⁻⁸ M) with and without unlabelled DES (10⁻⁵ M) to distinguish between specific and a-specific binding. After incubation a 0.6 M KCl extraction was done to separate KCl-extractable from the non-KCl-extractable fraction (for details, see text Materials and Methods).

In the KCl-extractable fraction the free, unbound radioactivity has been separated from the receptor bound activity by protamine sulfate precipitation. In this fraction we found for all patients a higher amount of specific binding than of nonspecific.

Comparison of the amounts of specific binding sites in the KCl-extractable with those in the non-KCl-extractable fractions showed larger quantities of specific binding in the non-KCl-extractable fraction. In this respect it is remarkable that most investigators only measure the specific binding in KCl-extractable fractions and ignore the KCl-resistant fraction. In our hands even a second 0.6 M KCl extraction does extract only a very small portion of the KCl-resistant fraction. At the moment we do not know the physiological and biochemical differences between the two nuclear fractions.

In patient no. 3 we found a high E₂Rc in the cytosol of the endometrium and a high PgRc in endometrium and vagina, results suggesting response of these tissue to the E₃ treatment. In contradiction histological investigations of the endometrium did not show any evidence of oestrogen stimulation. The opposite was seen in patient no. 2; the endometrium showed signs of proliferation after treatment, E₂Rc and PgRc were comparable with those in untreated postmenopausal women. Regarding stimulation of the endometrium following low-dose vaginal E₃ administration, Englund *et al.* [18] observed some influence on the epithelium, studied by electron microscopy, after treatment of postmenopausal women with 0.5 mg ovula once a day. This is at variance with histological examinations of endometria of postmenopausal women treated with similar ovula by other investigators [19, 20], who did not find any sign of endometrial activity after treatment.

In conclusion. We have shown preliminary results on histologically and biochemically measurable effects of oestriol on human endometrial, myometrial and vaginal tissue in postmenopausal women. The results do raise different questions which hopefully can be answered in the future.

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