Journal of Steroid Biochemistry. Vol. 10, pp. 529 to 534
Pergamon Press Ltd 1979. Printed in Great Britain

HIGH AFFINITY BINDING OF OESTRADIOL-17β IN THE NUCLEI OF HUMAN ENDOMETRIAL CELLS

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SUMMARY

The development of a specific assay for high affinity nuclear receptors for use in human endometrium is described. A nuclear suspension and 700 *g* supernatant were prepared from 150 mg of fresh tissue and aliquots incubated at 4°C for 18 h in the presence of 0.1–1.0 nM [^{3}H]-oestradiol-17β. Loosely bound [^{3}H]-oestradiol-17β was removed from the nuclear preparation by washing in a millipore filter unit and from the 700 g supernatant by incubation with dextran-charcoal at 2°C for 15 min. Preliminary results obtained in normal human endometrium and in endometrial carcinoma are presented.

INTRODUCTION

Anderson and his colleagues [1] have amply demonstrated the central role that specific nuclear receptors play in modulating oestrogen stimulated growth in the rat uterus. Therefore, in order to be able to use human endometrium for studies of hormone-regulated cellular growth, an assay to measure these receptors in human tissue is required. Oestrogen receptors of physiological importance must not only be specific for biological oestrogens but must have both high affinity (K_D approximately 10^{-10} M), and limited capacity. A suitable assay, which satisfies these criteria, has been developed for human mammary carcinoma [2]. None of the assays currently used in human endometrium measures affinity or capacity of nuclear receptors routinely. This paper describes a similar assay for human endometrium and presents some results obtained in normal and neoplastic endometrium.

EXPERIMENTAL

Tissue. Fresh tissue was obtained either by curettage or from hysterectomy specimens. One portion was placed in formol saline for histological examination, and the remainder into ice-cold Hanks solution for transport to the laboratory for oestrogen receptor analysis. All procedures were carried out at 2°C unless otherwise stated.

Materials. [6,7-^{3}H]-oestradiol-17β (85 Ci/mmol) was obtained from the Radiochemical Centre, Amersham. The purity was confirmed by thin layer chromatography. Unlabelled steroids were obtained from Sigma Co. and, unless otherwise stated, all other chemicals were obtained from B.D.H. Chemicals.

The buffers used were: HED: 20 mM Hepes, 1.5 mM EDTA, 0.25 mM dithiothreitol pH 7.4; Dextran-charcoal: 0.25 M sucrose, 0.15% Norit A charcoal 0.0015% dextran in HED pH 7.4; HM: 0.15 M KCl; 0.002 M $MgCl_2$; 0.05 M Tris–HCl; 0.5 M sucrose; pH 7.5.

Tissue fractionation. Fresh tissue was homogenized gently in HED (50 mg/ml) using a Teflon-glass homogenizer on ice. The homogenate was centrifuged at 700 *g* for 10 min, and the supernatant decanted. The crude nuclear pellet obtained was washed and resuspended to the original volume in 0.15 M sodium chloride, and an aliquot taken for DNA estimation. In some experiments, a glass-glass homogenizer was used and a 5000 *g* centrifugation employed to prepare the nuclei. In some other experiments the nuclei were resuspended and incubated in HED instead of 0.15 M sodium chloride.

Measurement of [^{3}H]-oestradiol binding. Aliquots of 150 μl of nuclear suspension or 700 *g* supernatant were each incubated with 50 μl of 7 to 9 different concentrations of [^{3}H]-oestradiol-17β (0.1-1.0 nm) with and without 100-fold excess of diethylstilboestrol, at varying temperatures, and for varying durations.

After incubation, 100 μl aliquots of the nuclear suspension were washed with 15 ml 0.15 M sodium chloride on Whatman GF/C filters using a Millipore filter unit. The filters were dried at 60°C overnight and then counted in Xylene-PPO 5 g/l in a Packard Tricarb 3330 at an average efficiency of 43%. Total radioactivity was determined for a 50 μl aliquot of nuclear suspension.

After incubation, 900 μl of HED were added to the 700 *g* supernatant; 200 μl of the diluted sample taken to measure total radioactivity, and loosely bound [^{3}H]-oestradiol-17β was removed from the remainder using 0.15% dextran-charcoal (0.5 ml) at 2°C for 15 min. The supernatant obtained after a short centrifugation was counted in Triton–Xylene (1:2) – PPO 5 g/l in a Packard Tricarb 3330 at an average efficiency of 27%. A series of blanks containing [^{3}H]-oestradiol-17β and buffer were also treated

with dextran-charcoal to measure the background [3]. The data were plotted according to Scatchard [4] and the binding affinity and total number of receptors calculated. In routine analysis, because of low levels of non-specific binding measured, a duplicate sample containing 100-fold excess diethylstilboestrol was incubated only at the highest concentration of [^{3}H]-oestradiol-17β. The Scatchard plot was drawn using the data obtained for total binding without subtraction of non-specific binding. This conserved tissue and produced very similar results.

It has been assumed in standard steroid receptor theory that no unfilled receptor are ever found in the nucleus. However, as the data presented in this study show, no difference is found in maximal "exchange" levels whether determined at 4° or 30°C. For this reason we cannot rule out the possibility that some of the specific binding of [^{3}H]-oestradiol-17β represents filling of empty nuclear receptor sites. For the purposes of this paper, therefore, exchange is taken to mean a combination of exchange of endogenous bound nuclear hormone and binding to any empty nuclear sites present. The kinetics of nuclear exchange were investigated at different temperatures by preparing three series of tubes with aliquots from the same nuclear suspension and the same concentration (1 nM) of [^{3}H]-oestradiol-17β. Each series was incubated at a different temperature and, at intervals, duplicate samples from each series were removed and 100 μl aliquots treated as described previously to determine the total amount of tightly bound [^{3}H]oestradiol-17β and, therefore, the amount of exchange.

Protein assays were by the method of Lowry [5], and DNA assays by the method of Burton as modified by Katzenellenbogen and Leake [6].

RESULTS

Preliminary experiments showed that EDTA was an essential requirement in the homogenizing buffer in order to measure nuclear receptors and that there was no advantage in using sucrose containing buffers (Fig. 1). The loss of DNA from the nuclear suspension during the washing process on millipore filters was measured and found to be similar to that occurring when nuclei were washed by repeated centrifugation and resuspension (mean recovery 95.4%). Because low and variable levels of nuclear binding were found after exchange at 37°C, the temperature used by Anderson and his co-workers [7] with rat uterus, a series of experiments at different temperatures was performed. Figure 2 shows the rate of nuclear exchange at 37°, 30° and 20°C as the mean of two experiments performed in duplicate. All values are expressed as a percentage of the 30°C plateau value (at 3 h). It is clear that the exchange at 30°C far exceeds that at 37°C suggesting that receptor degradation may be occurring at the higher temperature. Although the rate of exchange at 20°C is slower, by 10 h of incubation it has reached a higher value than that obtained at 30°C but some degradation is detectable by 20 h. Figure 3 shows the rate of nuclear exchange at 20°, 10° and 4°C as the mean of five experiments performed in duplicate with all values

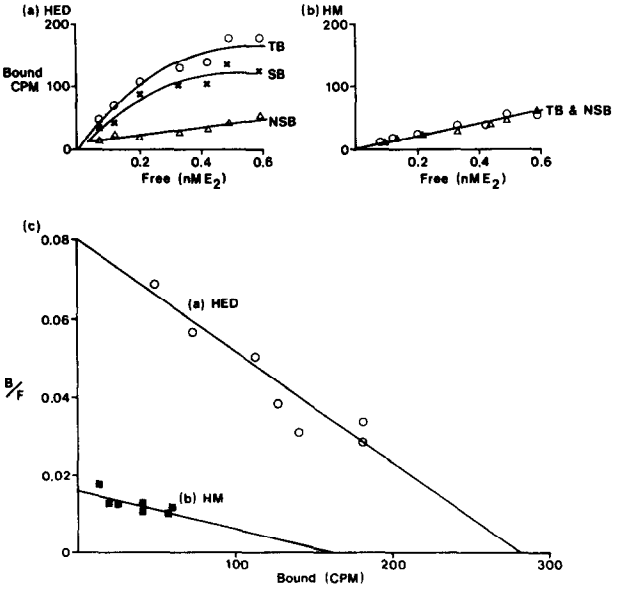

Fig. 1. (a) Human endometrium homogenized in HED and incubated at 30°C for 3 h in [^{3}H]-E_2 ± 100 fold excess DES. Nuclear binding shown at increasing concentrations of free ligand. Specific binding easily measured. (b) As above except that tissue was homogenized in HM. No specific binding seen. (c) Scatchard plots of the nuclear binding: O = homogenized in HED—shows high affinity binding ($K_D = 3.3 \times 10^{-10}$ M); ■ = homogenized in HM no high affinity binding measurable ($K_D = 9 \times 10^{-10}$ M). The DNA concentrations of both nuclear suspensions were very similar (O = 233 μg/ml; ■ = 228 μg/ml).

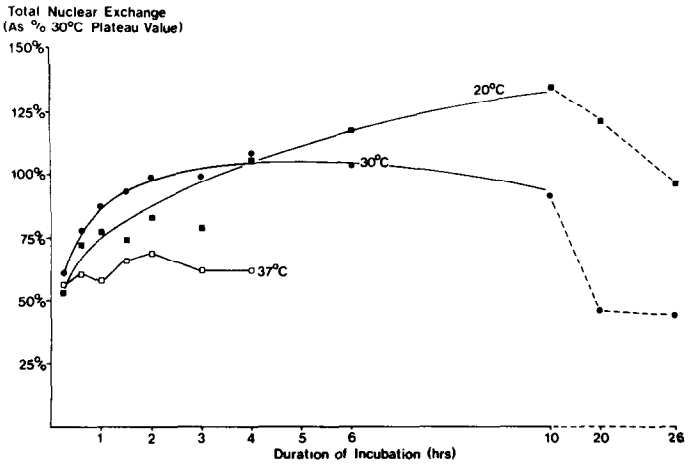

Fig. 2. Total nuclear "exchange" at 37°C (□), 30°C (●), and 20°C (■), expressed as a percentage of the 30°C plateau value.

expressed as a percentage of the 20°C plateau value. Incubation at 20°C gives the highest levels of exchange, the peak being reached between 8 and 12 h. Unfortunately, degradation of the receptor seems to occur thereafter, and after 18 h incubation exchange is similar at all three temperatures (about 85% of the 20°C maximum). In view of the similar levels of binding seen after 2 h at 30°C and after 18 h at 10° or 4°C, complete assays (i.e. using 9 different concentrations of [^{3}H]-oestradiol-17β) were performed on the 700 *g* supernatant and nuclei using a 2 h incubation at 30°C and an 18 h incubation at 10°C. This comparison was made on four separate occasions. Overall, there was little to choose between the different incubation conditions except that more low affinity binding was observed at 30°C, particularly in the 700 *g* supernatant. In all subsequent experiments the exchange was performed at 10° or 4°C for 18–24 h. A representative Scatchard plot of nuclear and 700 *g* supernatant receptors is shown in Fig. 4.

The nuclear (Fig. 5a) and 700 *g* supernatant (Fig. 5b) receptor concentrations found in histologically normal endometrium throughout the menstrual cycle are shown. The level of receptors in the nucleus seems to rise around day 9 to about 2800 fmol/mg DNA, but falls thereafter to a baseline of about 200 fmol/mg DNA at the end of the cycle and is maintained at this low level through the beginning of the next cycle. In contrast, the receptors in the 700 *g* supernatant remain at a fairly constant level throughout the cycle (mean (±S.D.) = 100 (±44) fmol/mg protein, n = 13; 1857 (±776) fmol/mg DNA, n = 12) save for a large increase at mid-cycle (mean (±S.D.) = 526 (±295) fmol/mg protein, n = 3; 5941 (±3015) fmol/mg DNA, n = 2).

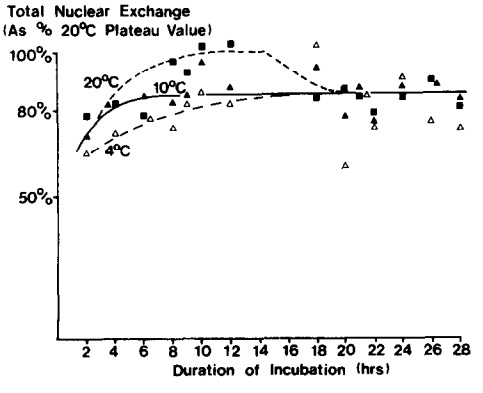

Fig. 3. Total nuclear "exchange" at 20°C(■), 10°C(▲), and 4°C(△), expressed as a percentage of the 20°C plateau value.

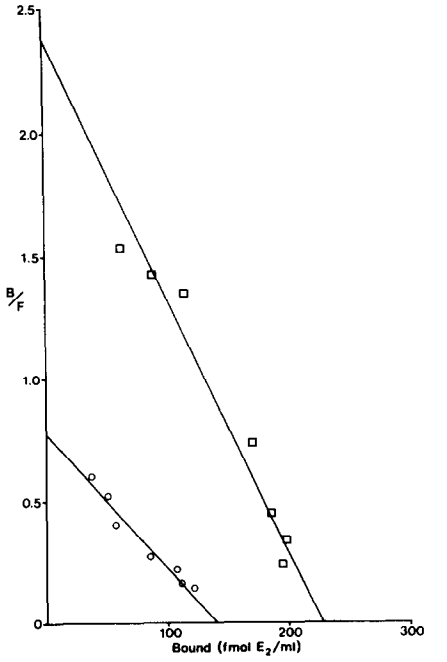

Fig. 4. A Scatchard plot of nuclear (○) and supernatant (□) binding in normal human endometrium. Nuclear K_D = 1.8 × 10^{-10} M, 459 fmolE_2/mg DNA; 700 *g* supernatant K_D = 0.97 × 10^{-10} M, 43 fmolE_2/mg protein.

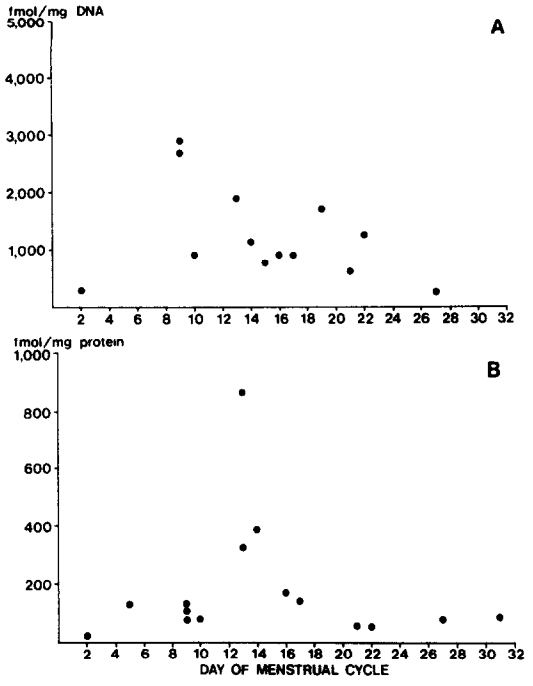

Fig. 5. (a) High affinity nuclear oestrogen receptors throughout the menstrual cycle in histologically normal endometrium. (b) High affinity oestrogen receptors in the 700 *g* supernatant throughout the menstrual cycle in histologically normal endometrium.

Eight specimens of endometrial carcinoma have been studied (Table 1). One showed no evidence of receptors in either the 700 *g* supernatant or the nuclei. This was the only adenoacanthoma (a tumour containing squamous elements). The remainder showed levels of high affinity binding in the nuclei which were similar to those seen in normal endometrium. The 700 *g* supernatant, in some cases, contained lower levels of high affinity binding than normal endometrium.

DISCUSSION

Measurement of physiological oestrogen receptor levels should include an estimate of binding affinity because, *in vitro*, oestrogens bind with low affinity to sites of no apparent physiological importance. Other work suggests that functional binding sites in human tissue have a $K_D \sim 10^{-10}$ M [8; see also Fig. 1]. In common with other workers using rat uterus [7] and a human breast tumour cell line [9], we find the dissociation constant of the oestradiol receptor is similar in both cytoplasmic and nuclear fractions.

This study clearly demonstrates that nuclear oestrogen receptors in human endometrium are unstable at 37°C, the temperature used in rodent studies [7]. The same appears to be true in human breast carcinoma tissue—at least in the post-menopausal woman [10]. The most reproducible incubation conditions in the authors' hands were 4° or 10°C for 18 h. Such conditions may not reflect maximal uptake, but, on average, yielded 85% of the binding found after 12 h at 20°C. Incubation at 30°C for 2–6 h may provide a satisfactory alternative. The incubation conditions chosen appear to allow virtually complete exchange of labelled oestradiol with endogenous hormone in both 700 *g* supernatant and nucleus, enabling total receptor sites to be measured [11]. However, because 4° and 30°C give identical results, no distinction can be made between exchange of filled sites and filling of empty sites, therefore the proportions of exchange cannot be accurately assessed.

Inclusion of EDTA in the homogenizing buffer was found to be essential for reproducible estimation of high affinity nuclear binding. There seemed to be no real difference in nuclear or 700 *g* supernatant binding when a glass–glass tissue grinder was used instead of Teflon–glass, nor when the nuclear pellet was obtained by centrifugation at 5000 *g* instead of 700 *g*. This confirms the findings of Bayard and his colleagues [11] using cytosol alone.

Taking the concentrations of endogenous oestradiol found by Pollow and his colleagues [12] in human endometrium (approximately 300 pg/g except at mid-cycle when a maximum value of 1000 pg/g was recorded) and assuming that none is lost during homogenization or washing and that 75% of the endogenous non-specifically bound oestradiol will be associated with the supernatant fraction, the expected concentrations of endogenous oestradiol present would be 0.03×10^{-9} M (0.10×10^{-9} M at mid cycle) in the incubation of 700 *g* supernatant and 0.01×10^{-9} M (0.03×10^{-9} M) at mid cycle) in the nuclear incubation. Since these levels can be expected

Table 1. Oestradiol-17β receptor in human endometrial carcinoma

Histology	Nuclear receptors fmol/mg DNA	Nuclear receptors $K_D \times 10^{-10}$ M	700 *g* supernatant receptors fmol/mg protein	700 *g* supernatant receptors $K_D \times 10^{-10}$ M
Moderately well differentiated	100	2.2	58	2.1
Well differentiated	665	5.0	10	1.5
Moderately well differentiated	1273	2.3	8	0.8
Adenoacanthoma	NIL	—	NIL	—
Mesonephroid adenocarcinoma	7200	5.5	144	5.0
Moderately well differentiated	2106	2.9	36	3.2
Moderately well differentiated	6360	2.4	66	1.5
Moderately well differentiated	423	4.6	40	5.9

to be even further reduced by homogenization losses and washing of the nuclear pellet only minimal dilution of the specific activity of the added [^{3}H]-oestradiol-17β would be expected.

In other studies, nuclear oestrogen "receptors" have been measured in human endometrium and endometrial carcinoma, either by incubating fresh tissue with labelled oestradiol [13–16], or by pre-incubating the tissue with high concentrations (0.1 μg/ml) of non-radioactive oestradiol followed by incubation of a suspension of the crude nuclear pellet at 37°C in the presence of 6 nM [^{3}H]-oestradiol-17β [17]. None of these methods allowed the affinity of binding to be measured and, where high concentrations of oestradiol were used, carried the risk of including low-affinity sites. In this study the levels of nuclear high affinity oestrogen binding found are somewhat less than those reported by Gurpide and his colleagues [15]. This may be due to the high concentrations of labelled steroids used by these workers. The values found by Crocker and his co-workers [17] are similar to those reported here while the studies of Lunan and Green [14], and Soutter [16] gave much lower values. It is clear therefore that in the last two studies the majority of nuclear receptor sites were still occupied by endogenous oestradiol-17β at the end of the incubation. It is rather surprising that the levels of nuclear "receptors" in the study conducted by Crocker and his colleagues should be so similar to those reported here, since in that study the tissue was pre-incubated with 0.1 μg oestradiol-17β/ml for 1 h prior to a nuclear exchange with 6 nM [^{3}H]-oestradiol-17β for 1 h at 37°C. One would expect the pre-incubation to increase the nuclear oestradiol receptor levels, and indeed they showed that this was the case in samples of cystic hyperplasia. The similarity between their results and ours may be coincidence in that the increased translocation may have been balanced by receptor degradation in their 37°C assay. In common with all these other studies, the data presented here show depressed levels of nuclear "receptors" in the luteal and early follicular phases of the cycle.

In a very recent publication, Bayard and his colleagues [11] used an approach similar to that described in this paper except that the homogenizing buffer did not contain EDTA, the incubation was conducted at 30°C for 3 h and a single concentration of labelled ligand (20 nM) was used so that the affinity of binding could not be measured. The non-specific binding was measured using an incubation with a 100-fold excess of non-radioactive oestradiol-17β. The levels of nuclear binding they report are somewhat lower than those found in the present study and show a symmetrical distribution throughout the menstrual cycle. Their cytosol values are very much lower than those reported here and show a fall in the luteal phase of the cycle.

The levels of high affinity oestradiol-17β binding in the 700 *g* supernatant found in the present study are, in their turn, less than those found by Crocker and his colleagues [17], who used essentially the same technique on a 105,000 *g* supernatant. However, they too, found a large mid-cycle peak of cytosol receptors, but in their study it returned slowly to early follicular levels during the third week of the cycle. Difficulties in dating samples accurately probably account for this difference between these two sets of data. Both Limpaphayom and his group [18] and Robertson and his colleagues [19] used assays which were said to measure only the "available" cytosol receptor sites although the 20 min incubation at 30°C employed by the latter group may have included some exchange. Both these groups found levels less than those reported here but neither observed a clear difference between proliferative and secretory endometrium. In the latter study there were several much higher values obtained close to mid-cycle but reported as having been obtained either before or after mid-cycle. This reflects the difficulty of identifying the mid-cycle period from histological data alone. On the other hand, Pollow and his co-workers [12], using 16 h incubations at 4°C, a Tris–HCl, EDTA, 2-mercaptoethanol, 20% glycerol homogenizing buffer and dextran-charcoal removal of endogenous, unbound steroids before incubation, obtained levels of cytosol receptor which declined steadily from 6000 fmol/mg protein in the mid-follicular phase to about 1000 fmol/mg protein in the luteal phase. It is unlikely that the differences between that study and the data presented here are due to the presence of endogenous, unbound hormones. It has already been shown that these would not be expected to be present in significant concentrations and furthermore, since these levels are maximal at midcycle [12], any difference attributable to them would also be expected to be greatest then. In fact the reverse is found. Removal of endogenous, unbound hormones from the 700 *g* supernatant of human endometrium with dextran-charcoal prior to incubation does not make a significant difference to the level of receptors or to the K_D as determined by Scatchard analysis (unpublished observations). It is tempting to speculate that the rapid, transient rise of receptor levels in the 700 *g* supernatant reported here is due to a sudden reduction in the rate of nuclear translocation at a time when the rate of production of cytoplasmic receptors is still high. The return to lower levels would occur when, once again, the rate of production balanced the rate of translocation.

The one endometrial carcinoma which did not have measurable receptors was a rapidly growing adenoacanthoma which metastesised to the lungs 10 months after an extended total hysterectomy, deep x-ray therapy and radium treatment. All of the remainder had oestrogen receptors present in the 700 *g* supernatant and nucleus to a greater or lesser extent. The presence of high affinity bound, endogenous oestradiol in the nuclei of these tumours suggests that their growth may have been influenced, to some extent at least,

by the woman's own oestrogens. Whether these tumours would respond satisfactorily to hormonal therapy is not known. A large scale trial would be necessary to investigate that possibility. In the meantime, these assays will be useful in examining the molecular events that result in DNA synthesis and cell replication in human endometrium.

Acknowledgements—We thank the consultant staff of the Western Infirmary, Glasgow, for allowing us to study their patients and the theatre staff for their unfailing cooperation. We also thank Professor K. C. Calman, Department of Clinical Oncology, University of Glasgow, who generously provided facilities to allow this work to be performed, and Professors C. R. Whitfield and R. M. S. Smellie for their advice and encouragement.

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