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BINDING OF NORGESTREL TO RECEPTOR PROTEINS IN THE HUMAN ENDOMETRIUM AND MYOMETRIUM

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SUMMARY

The interaction of [^{3}H]-norgestrel with the endometrial and myometrial receptor proteins has been investigated. Norgestrel binding to 4.9 *S* and 7.8 *S* receptor proteins in the endometrial cytosol and 4.3 *S* receptor protein in the myometrial cytosol was observed. The binding protein in the endometrial nucleus sedimented at 5 *S*. The endometrial and myometrial norgestrel binding receptors were protein in nature and thermolabile. The dissociation constants of the endometrial and myometrial norgestrel binding receptor proteins were 0.7×10^{-9} M and 0.96×10^{-9} M respectively. The number of binding sites calculated was 0.34×10^{-12} mol/mg of endometrial cytosol protein and 0.3×10^{-12} mol/mg of myometrial cytosol protein.

Progesterone and norethisterone showed maximum competition for norgestrel binding sites while chlormadinone acetate, oestradiol, testosterone and corticosterone competed poorly.

INTRODUCTION

Specific retention of progesterone in the oestrogen primed rat uterus was shown [1–2] after an injection of [1,2-^{3}H]-progesterone. Subsequently the binding of progesterone to rat uterine cytosol was reported [3–4]. The binding of progesterone to the human endometrium and myometrium was reported by several workers [5–8]. Verma and Laumas[6] characterised the human endometrial and myometrial receptor proteins for progesterone. Although some information on the competitive displacement of progesterone by progestogens from uterine progesterone receptors is available [8–9], as yet little is known about the binding characteristics of progesterone receptors to synthetic progestational compounds.

It is known that norethynodrel, chlormadinone acetate and norgestrel are selectively taken up and retained by the human endometrium and myometrium [10–12] and rat uterus [13–14]. It has been found [15] that norethynodrel is localized mainly in the 105,000 *g* cytosol and nuclear fractions in the uterus of rat and human. In the human endometrium, myometrium and rat uterus, norethynodrel was shown to bind to the receptor proteins [15]. Norgestrel, a synthetic progestogen is a mixture of D and L-sterioisomers, the former being the biologically active form. D,L-Norgestrel in combination with ethinyl oestradiol (Ovaral, Wyeth Lab) is used as an oral contraceptive [16]. It has also been extensively tested as a continuous low dose oral progestin for fertility control [17]. It has been proposed to produce its antifertility effect by its actions on the cervical mucus and at the uterine level. However, its precise mechanism of action at the uterine level is not understood. Since [^{3}H]-norgestrel after an i.v. injection to women has been found to be selectively taken up by and retained in the endometrium and myometrium [12], the study of the possibility of its binding to the receptor proteins in human uterus was undertaken, which should help in an understanding of its mode of action at the uterine level.

MATERIALS AND METHODS

Steroids. (14,15-^{3}H)-D,L-norgestrel (S.A. 293 mci/mmol) was obtained from Schering A.G., Berlin. The purity of the radioactive steroid was checked by paper and thin layer chromatography before use. Norgestrel* and its 3αOH, 5βH; 3βOH, 5βH metabolites (Wyeth Lab. U.S.A.). Chlormadinone acetate (E. Merck, Germany), Norethindrone, Norethindrone acetate (Schering A.G., Berlin), Oestradiol-17β, progesterone, corticosterone, testosterone (Sigma Chemical Co., St. Louis, U.S.A.) were used in this study.

The uterine tissue was obtained from patients who underwent hysterectomy for 3rd degree prolapse of the uterus. The subjects were in reproductive age and were in general good health having a regular menstrual cycle.

* Trivial names of steroids used: 13β-Ethyl-17α-ethynyl-17β-hydroxygon-4-en-3-one; 6-Chloro-17α-acetoxy-4, 6-pregnadiene-3,20-dione acetate; 17α-Ethynyl-17β-hydroxy-4-estren-3-one; 17α-Ethynyl-17β-hydroxy-4-estren-3-one, 17β-acetate.

Tissue preparation. Uterine tissue was collected from the operation theatre immediately after hysterectomy and processed further [6]. Sub-cellular fractionation was carried out by ultracentrifugation of the homogenate into nuclear (800 *g* pellet) and cytosol (105,000 *g* supernatant) fraction in a Beckman Ultracentrifuge Model L. The purification of the nuclear pellet was done as described [18].

Sucrose density gradient. Linear gradients (4.8 ml) of 5–20% sucrose concentration containing 10% glycerol in 0.01 M Tris–HCl buffer pH 7.4 were prepared [19]. Cytosol (0.2 ml) was incubated with 0.2 uCi of (14,15-^{3}H)-D,L-norgestrel at 0°C for 30 min, layered on the gradient and spun at 165,000 *g* for 16 h in a Beckman Ultracentrifuge Model L using SW-50 Rotor. Human serum albumin (Sigma Chemical Co. U.S.A.) was run as a marker and the approximate sedimentation coefficient ($S^{\circ}_{20}W$) for the receptor was calculated according to Martin and Ames[20].

Determination of the dissociation constant of the binding proteins. The hormone binding kinetics were carried out by equilibrium dialysis as described earlier [19]. A Scatchard plot of bound vs unbound (B/U) norgestrel was plotted on the *Y*-axis against the molar concentration of bound (B) on the *X*-axis. An intrinsic dissociation constant (K_D) and number of binding sites was calculated from the Scatchard plot [21].

Immuno-absorption of the plasma proteins from the cytosol. Rabbit anti-normal human serum was polymarized using gluteraldehyde [22] Plasma proteins if any, in the cytosol were absorbed by mixing the cytosol with the immunoabsorbent gel. The binding capacity of the cytosol was then studied by equilibrium dialysed method.

Determination of the molecular weight, molecular (Stokes) radius and frictional ratio (f/f_0). The molecular weight of the binding proteins by gel filtration was calculated according to Andrews [23] using 2 mg of non-enzymic protein markers (Mann Res. Lab.). Dextran blue was used to measure the void vol. of the column. Molecular weights of the binding proteins from the sucrose gradient sedimentation values were calculated in relation to the human serum albumin [20]. The molecular (Stokes) radii and frictional ratios of the binding proteins were determined [19].

Analysis of the bound steroid. The fractions containing radioactive steroids associated with protein peaks obtained from the endometrial and myometrial cytosol after Sephadex chromatography were pooled and extracted with diethyl ether. The ether extract was dried and chromatographed on thin layer alumina plates using benzene:ethanol (99:1, v/v) solvent system and processed [12].

Protein estimation. Protein concentration in the endometrial and myometrial cytosol was estimated by the method of Lowry *et al.*[24].

RESULTS

The sedimentation profile of the norgestrel binding endometrial cytosol proteins (Fig. 1) showed norgestrel binding to a 4.9 *S* and 7.8 *S* receptor proteins. The 4.9 *S* receptor contained higher amounts of norgestrel than the 7.8 *S* receptor.

The sedimentation gradient analysis of the norgestrel binding myometrial cytosol (Fig. 2) showed norgestrel binding to a 4.3 *S* receptor protein. No 8 *S* norgestrel binding protein was found in the myometrial cytosol.

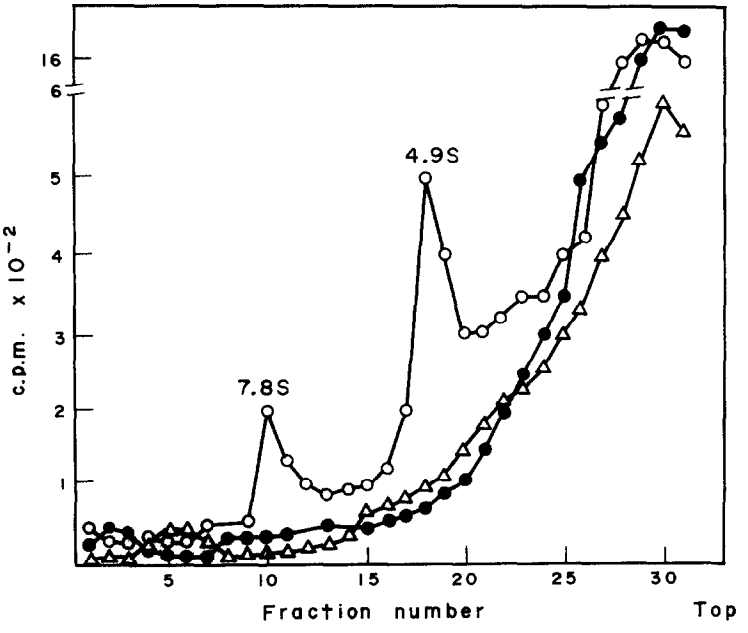

Fig. 1. Sucrose gradient sedimentation pattern of endometrial cytosol norgestrel binding proteins. ○——○ Cytosol + [^{3}H]-norgestrel. ●——● Cytosol heated at 60°C for 20 min + [^{3}H]-norgestrel. △——△ Cytosol + [^{3}H]-norgestrel + 2 μg unlabeled norgestrel.

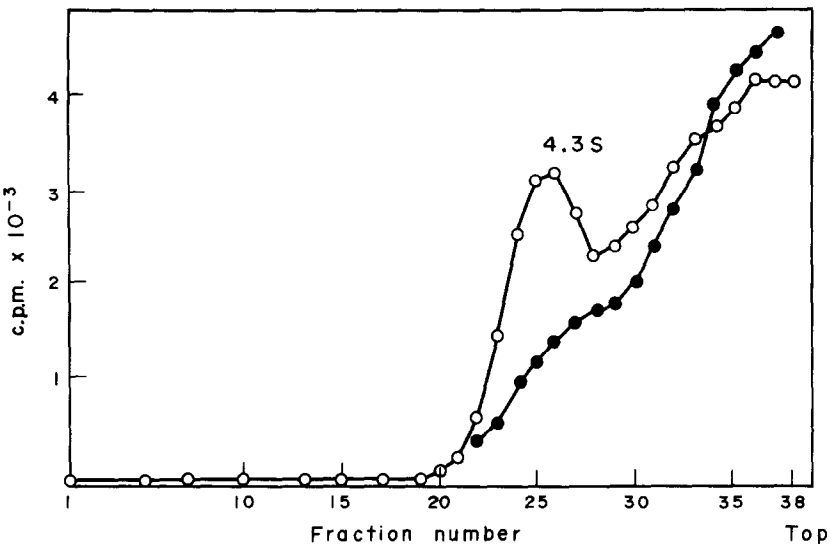

Fig. 2. Sucrose gradient sedimentation pattern of myometrial cytosol norgestrel binding protein. O——O Cytosol + [^{3}H]-norgestrel. ●——● Cytosol heated for 20 min at 60°C + [^{3}H]-norgestrel.

Sucrose density gradient sedimentation profile of norgestrel binding receptor protein in the nucleus (Fig. 3) showed a sedimentation coefficient value of 5 *S*.

Analysis of bound steroid. About 93% of the steroid was present as norgestrel in the endometrial cytosol eluted from Sephadex colum, nearly 4.8% was found to be 3α*OH*, 5β*H* compound and about 2% were the polar metabolites. In the myometrial cytosol 91% was present as norgestrel, about 5% was the 3α*OH*, 5β*H* compound and the rest 4% were the polar metabolites.

Effect of sulphydryl reacting agents on the binding. Cytosol treated with 2-mercaptoethanol showed 40% more binding compared to the cytosol without mercaptoethanol treatment.

The cytosol (0.2 ml) when incubated with parachlormercuribenzoic acid (500 μg/ml) affected the

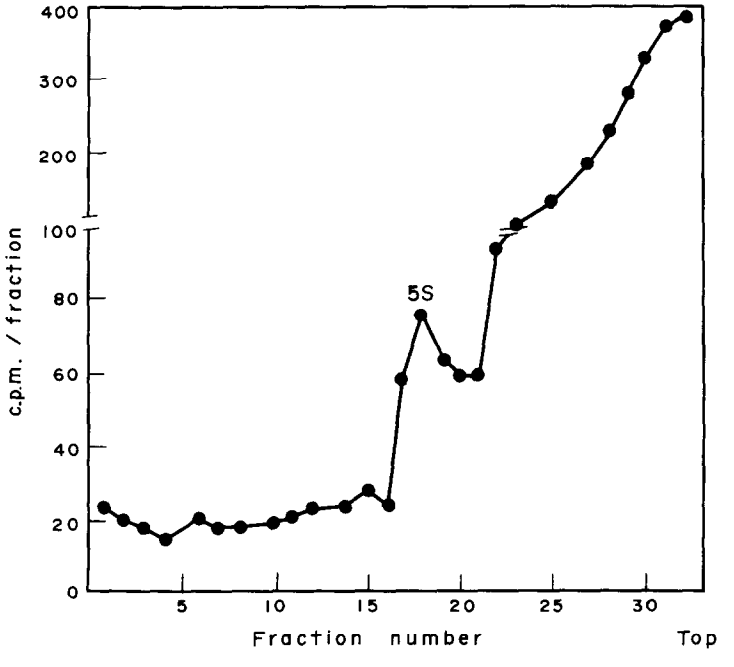

Fig. 3. Sucrose gradient sedimentation pattern of endometrial nuclear norgestrel binding protein. Endometrium was incubated with 1 μci of [14, 15-^{3}H]-D,L-norgestrel at 37°C for 1 h. The endometrial nuclear fraction was isolated as described in "Materials and Methods" and homogenized in 0.4 M KCl allowed to stand for 1 h and centrifuged at 105,000 *g* for 30 min. From the supernatant 0.2 ml nuclear steroid complex was layered on 10–30% sucrose gradient and spun at 165,000 *g* for 16 h.

Table 1. Relative binding affinities for various steroids to norgestrel binding protein in the human endometrial and myometrial cytosol

Steroids	Endometrial cytosol Mean*RA	Myometrial cytosol Mean RA*
Norgestrel	100	100
Progesterone	33	24
Norethindrone	14	10
Norethindrone acetate	<1	<1
Chlormadinone acetate	<1	<1
Testosterone	<1	<1
Oestradiol-17β	<1	<1
Corticosterone	<1	<1

Endometrium and myometrium was homogenized in 4 vol. of cold buffer (0.01 M Tris–HCl, 1.0 mM EDTA pH 7.4). The cytosol was diluted using equal vol. of 80% glycerol (v/v) in buffer.

To each assay tubes containing 15,000 c.p.m. of [^{3}H]-norgestrel (in 0.2 ml buffer) or [^{3}H]-norgestrel plus various concentration of unlabeled steroids, 0.3 ml cytosol was added. The contents of the tubes were mixed by shaking and incubated for 16 h at 4°C. The free steroid was separated from the bound using dextran coated charcoal [19]. The relative binding affinities of steroids were determined as described [9, 28]. Competition curves for each steroid tested were made by plotting per cent bound radioactivity against the log of the amount of competing steroid added. The amount of the ligand needed to decrease the binding of [^{3}H]-norgestrel by 50% was calculated from the plots. The competing ability was then estimated relative to non-labeled norgestrel. All estimations were made in duplicate using different cytosol preparations.

*RA represents the mean of the values from two different cytosol in case of endometrium and that of three different cytosol for myometrium.

RA = Relative binding affinity.

binding of [^{3}H]-norgestrel to the myometrial cytosol. The binding in the 4.3 *S* region was significantly reduced.

Effect of hydrolytic enzyme. The treatment of myometrial cytosol with trypsin resulted in the displacement of radioactivity from 4.3 *S* region to the top of the gradient, whereas treatment with ribonuclease did not affect the binding in the 4.3 *S* region.

Influence of unlabeled norgestrel and temperature. The addition of 2 μg unlabeled norgestrel to the endometrial cytosol incubated with [^{3}H]-norgestrel abolished norgestrel binding in the 7.8 *S* and 4.9 *S* region (Fig. 1) when analysed on the sucrose density gradient, thus confirming that 7.8 *S* and 4.9 *S* proteins represented the binding receptors.

The binding protein in the endometrium (Fig. 1) and myometrium (Fig. 2) was denatured by heating.

Ligand specificity of the binding protein. The relative binding affinities for various steroids for norgestrel binding protein in endometrial and myometrial cytosol is shown in Table 1. Among the steroids tested, progesterone and norethindrone showed affinity for the norgestrel binding protein in the endometrial and myometrial cytosol whereas, testosterone, corticosterone, oestradiol-17β, norethindrone acetate and chlormadinone acetate did not show much affinity for norgestrel binding components in the cytosol.

Binding of norgestrel to cytosol after immunoabsorption. The binding of norgestrel to the cytosol after immunoabsorption was not abolished, but was decreased from 72% to 58% in the endometrium and 66% to 50% in the myometrium. Thus the specific norgestrel binding proteins from the endometrial and myometrial cytosol were not absorbed by immunoabsorption.

Dissociation constant of the binding proteins. The Scatchard plot (Fig. 4) gave an apparent dissociation constant of 0.7×10^{-9} M for the endometrial norgestrel binding protein and 0.96×10^{-9} M for myometrial norgestrel binding protein. The number of binding sites was 0.34×10^{-12} mol/mg of endometrial cytosol protein and 0.3×10^{-12} mol/mg of myometrial cytosol protein.

Molecular weights, molecular (Stokes) radii and frictional ratios of the binding proteins. The molecular weights, calculated from Sephadex chromatography and sucrose gradient were found to be 66,000 and 76,000 respectively for the lighter binding protein in the endometrial cytosol. The heavier binding protein in the endometrial cytosol had a molecular weight of 152,000. The molecular weight of the myometrial cytosol norgestrel binding protein by Sephadex chromatography and sucrose gradient analysis was found to be 62,000 and that of endometrial nuclear norgestrel binding protein 68,000 and 77,400 respectively.

The molecular radius of the endometrial and myometrial cytosol norgestrel binding protein was 35 Å and 34 Å respectively. The norgestrel endometrial nuclear receptor had a stokes radius of 36 Å. The frictional ratios of norgestrel binding proteins in the endometrial and myometrial cytosol were 1.31 and 1.34 respectively and that of nuclear norgestrel receptor was 1.33.

DISCUSSION

Studies on the interaction of norgestrel with endometrial cytosol showed that it binds with two specific receptor proteins with sedimentation coefficients of 4.9 *S* and 7.8 *S*. In the myometrium, norgestrel showed binding to only one protein which sedimented at 4.3 *S*. Norgestrel binding protein in the endometrial nuclei had a sedimentation coefficient of 5 *S*. No precise information about the binding of contraceptive steroids with receptor proteins in the human endometrium and myometrium is available. The available information on the binding of progesterone to human endometrium and myometrium shows that progesterone binds with 4.5 *S* and 4.1 *S* proteins respectively [6]. However, apart from 4 *S* binder a 7*S* binding component for progesterone in the human endometrium have been reported [7].

Evidence presented showed that norgestrel binding macromolecule(s) were protein in nature. Norgestrel

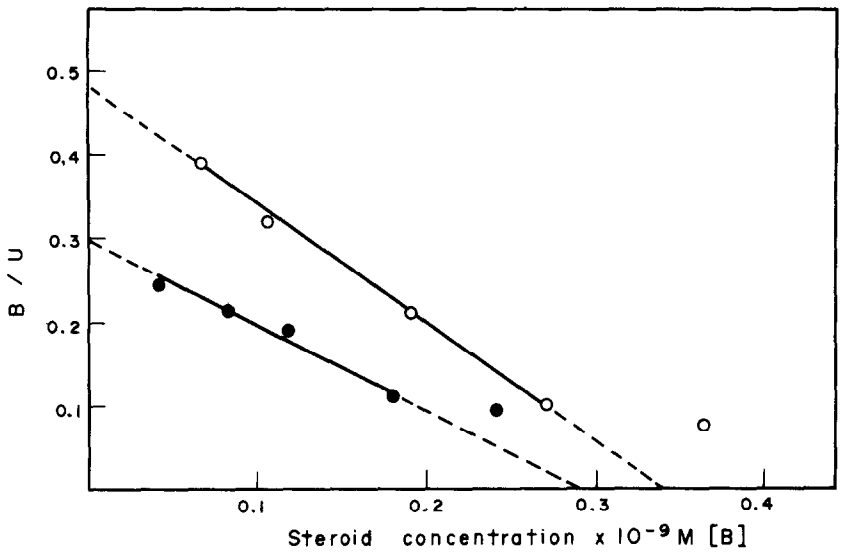

Fig. 4. Scatchard plot of uterine norgestrel binding proteins. The binding of [14,15-^{3}H]-D,L-norgestrel in the presence of increasing amounts of unlabeled norgestrel to endometrial and myometrial cytosol (1 ml) was carried out by equilibrium dialysis against 10 ml Tris–HCl buffer pH 7.4 at 4°C. The percent bound steroid was calculated [19] and Scatchard plot was drawn. The concentration of bound (B) against bound/unbound ratio (B/U). O——O Endometrial cytosol (1 mg protein/ml) ●——● Myometrial cytosol (0.94 mg protein/ml)

binding uterine receptor proteins may have -SH group or the involvement of -SH groups in maintaining the integrity of the receptor molecules was suggested by increased norgestrel binding to the uterine receptor proteins with mercaptoethanol treatment [25]. The -SH group involvement was further indicated with decreased norgestrel binding by PCMB treatment. The results showed that norgestrel binding proteins in the human endometrial and myometrial cytosol were thermolabile molecules. Similar observation has been reported earlier [6]. Thus norgestrel binding proteins in the human endometrium and myometrium showed similarities with progesterone binding proteins in these tissues.

Competitive displacement studies showed that progesterone and norethindrone competed for the norgestrel binding sites in the endometrial and myometrial cytosol. On the other hand, norethindrone acetate, chlormedinone acetate, corticosterone, testosterone and oestradiol did not compete to any appreciable extent. Moderate competition by norgestrel and progesterone for norethynodrel binding sites in the human myometrium has been reported [15]. In respect of ligand specificity, the norgestrel binding protein showed similarities to progesterone binding proteins in the human uterus [8]. High binding affinity for norgestrel to the uterine progesterone receptors under receptors stabilization condition has also been observed [26].

It is known that L-norgestrel does not bind to progestin receptors [27] and it also does not interfere with the binding of norgestrel to the receptors. Thus the binding affinities of norgestrel to uterine receptors would be due to the D-norgestrel component of the DL-norgestrel.

Norgestrel has little affinity for human CBG [8] and the binding of norgestrel to a specific plasma protein [19] with which corticosterone did not compete for norgestrel binding sites suggested that norgestrel binding protein in the uterus was different than corticosteroid binding globulin. The fact that norgestrel showed high binding to the immunoabsorbed endometrial and myometrial cytosol indicated that the binding proteins were of uterine origin.

The presence of 4.9 *S* and 7.8 *S* norgestrel binding proteins in the endometrial cytosol and 5 *S* receptor protein in the endometrial nucleus may point to the possibility of a transport mechanism of norgestrel in the human endometrium for expressing its action at the uterine level.

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