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UTERINE PROGESTERONE AFTER PROGESTERONE TREATMENT: ON THE DISAPPEARANCE OF CYTOSOLIC PROGESTERONE RECEPTORS

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SUMMARY

The effect of progesterone (P) treatment on P receptors and on the resultant P concentrations in the cytoplasm and other subcellular fractions was studied. Cytoplasmic P receptors in the uterus of oestrogenized rabbits were reduced by about 60% after 24 h of single injection of P. However, P concentration in the intact tissue as well as in the cytoplasm (supernatant), as determined by radioamminoassay, increased significantly. The increase in P concentrations in other fractions (nuclear, mitochondrial + microsomal) was not significant.

In order to recover cytoplasmic receptors from nuclei, the nuclear pellets were stirred with the buffer solution and the washings were collected. Although P concentration in the cytoplasm so extracted (nuclear-extract) from the nuclear fraction was significantly higher in the case of progesterone treated rabbits, the receptor concentration was lower as compared to those from the untreated animals. Neither stripping of cytoplasm of the endogenous P nor its loading with P *in vitro* led to any increase or decrease respectively in P receptors. Determination by radioamminoassay of progesterone concentrations in the loaded and the stripped cytoplasm showed that these procedures were highly effective.

These data indicate that the occupation of the receptors and its subsequent influence on the receptor determination *in vitro* cannot account for the reduction of P receptors unless one postulates that progesterone when bound endogenously to its receptor, after progesterone treatment, does not exchange as readily as that bound *in vitro*. It seems also unlikely that the reduction of cytoplasmic P receptors was simply due to their translocation in the nucleus.

INTRODUCTION

Considerable insight into the cellular mechanisms of hormone action has been gained during the past decade. Biologically active steroids seem to exert their effects in a similar manner which is characterized by their binding, as a first step, to specific proteins (receptors) present only in the cytoplasm of target cells. Following this primary event, the complex of steroid and receptor rapidly enters the nucleus and is thought to bind to acceptor sites on the nuclear chromatin. The binding initiates physiological changes by modifying gene expression. Although there are still difficulties in clearly demonstrating this for all steroids, there is a good reason to believe that this two step mechanism of action, originally postulated by Jensen *et al.*[1] and Gorski *et al.*[2], is common to oestrogens, progesterone, glucocorticoids and mineral-corticoids.

Until recently there has been some debate on the existence of specific progesterone receptors in the mammalian uterus (e.g. [3, 4]). However, this now seems resolved and evidence showing that the progesterone receptors are under both oestrogen and progesterone control has also been presented [5]. Milgrom *et al.*[6] reported an interesting finding that cytosolic progesterone receptors in the guinea-pig uterus decreased markedly within a short time after progesterone injection, which has subsequently been confirmed [7]. So far no clear explanation for this somewhat paradoxical observation has been given. The possibility for the nuclear translocation of the receptor has been recently investigated and the data show that this does not fully explain the disappearance of the receptor from the cytoplasm [8]. It has also been suggested that a certain number of the progesterone receptors, after progesterone administration, are already occupied and these are not measured by the usual receptor assay method.

It is not known whether the progesterone-induced disappearance of the cytosolic progesterone receptor observed in the guinea-pig holds true for other species. There is some circumstantial evidence that progesterone might have a similar effect in the human uterus [9, 10]. In the present study we have investigated this phenomenon in the rabbit uterus. Rabbit is particularly interesting in this regard because the reproductive activity of the rabbit is acyclic and progesterone withdrawal seems to be the stimulant for the induction of parturition in this species. Data are presented in this paper on the effect of progesterone treatment not only on progesterone receptors but also on the resultant progesterone concentrations in the cytosol and other subcellular fractions.

EXPERIMENTAL

Animals. All rabbits, weighing in average 3 kg, were oestrogenized by a single injection of 4 mg Estradurin i.m. (LEO, Sweden). With this treatment high levels of oestradiol not only in plasma but also in tissue

are maintained for at least 2 weeks [11]. The control group received no further treatment and the animals were used after 4–5 days of Estradurin injection. The progesterone group received an injection of 2 mg progesterone in oil s.c. 4 days after the oestrogen injection and the animals were killed after 24 h.

Preparation of tissue and subcellular fractions. Rabbits were killed by cervical dislocation, the uterus was removed and placed in ice-cold saline. After quickly scraping off the endometrium, the tissue was washed twice with TEDG buffer solution containing 10 mM Tris–HCl (pH 8), 1 mM EDTA, 1 mM dithioerythritol and 10% glycerol. Tissues were then blotted dry, weighed and a piece saved for total estradiol-17β and progesterone determinations. The remaining tissue was homogenized in the above buffer with a Polytron homogenizer. The homogenate was centrifuged at 1000 *g* for 10 min to obtain the crude nuclear pellet. The supernatant was centrifuged at 110,000 *g* for 1 h to obtain the mitochondrial + microsomal pellet, and the cytosolic supernatant. The crude nuclear pellet obtained above was suspended in TEDG buffer and stirred for 2 h at 4°C to obtain the so-called nuclear-extract which is essentially a wash of the nuclear fraction containing the cytoplasm that was trapped in or loosely bound to the nuclear fraction. The residual pellet after centrifuging the nuclear-extract was called the nuclear fraction. Endogenous steroid concentrations in the particulate fractions and the supernatant were determined by the method described previously [12, 13].

Receptors. Progesterone receptor concentrations were determined in the cytosolic supernatant and also in the nuclear-extract by the usual radioisotopic procedure. Excess cortisol was present in the progesterone receptor assays to eliminate progesterone binding to any CBG present. Binding to receptor was measured after 24 h of incubation at 4°C. In some cases oestrogen receptors were also determined. Preliminary experiments were done to validate the assay method. The concentration of receptor sites and the apparent dissociation constant (K_D) were determined by Scatchard analysis.

RESULTS

Data obtained in support of the validation of the receptor assay method are shown in Table 1 and Fig. 1. The effect of receptor protein concentration both on the binding sites concentration and apparent dissociation constant (K_D) is shown in Fig. 1. The binding sites increased whereas the affinity decreased with increasing protein concentration. There was, however, only a small difference in these parameters at a protein concentration between 0.48 and 0.60 mg/ml of the incubation medium. In all further experiments, therefore the protein concentration was kept around 0.5 mg/ml. Although receptor protein was diluted with the buffer containing glycerol, a part of the reduction in receptor sites may be due to a greater instability of the receptor in diluted solutions.

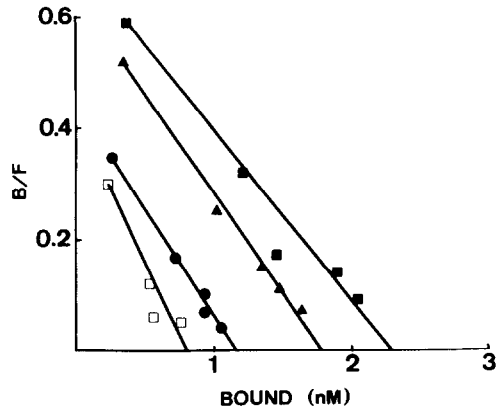

Fig. 1. Effect of protein concentration on cytosolic progesterone receptor determinations. Various protein concentration used were (□) 0.24, (●) 0.36, (▲) 0.48 and (■) 0.60 mg/ml.

Table 1 shows that between 5 and 24 h of incubation the concentration of binding sites did not change, nor did the K_D values. Between 1 and 5 h the binding site concentration increased considerably (not shown). After 40 h of incubation, however, there was some decrease in the concentration of binding sites and K_D values. The absence of glycerol from the incubation and the isolation medium resulted in significant reduction of the receptor sites (not shown).

Table 1. Effect of incubation time on cytosolic progesterone receptor determinations

Incubation time (h)	Receptor sites ($\times 10^{-12}$ mol/mg prot)	K_D ($\times 10^{-9}$ M)
5	2.56	3.6
24	2.58	3.2
40	2.39	2.6

The effect of progesterone treatment of animals on the cytosolic progesterone receptors was typical of that shown in Fig. 2. After 24 h of progesterone administration the concentration of the receptor sites

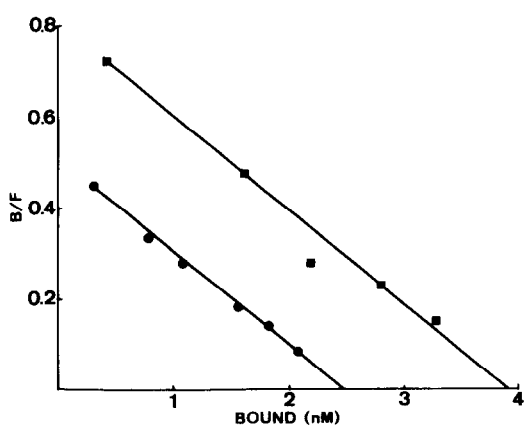

Fig. 2. Scatchard analysis of the data for the determination of cytosolic progesterone receptors in control (■) and progesterone treated (●) rabbits.

Table 2. Progesterone concentration in the uterus tissue and in isolated subcellular fractions obtained from control progesterone-treated rabbits†

Preparation	Progesterone (ng/mg protein) Control ($n = 6$)	P-treated ($n = 6$)
Intact tissue	0.10 ± 0.01	0.15 ± 0.01*
Nuclear	0.18 ± 0.02	0.21 ± 0.01
Nuclear-extract	0.07 ± 0.01	0.19 ± 0.03**
Mitochondrial + microsomal	0.28 ± 0.05	0.37 ± 0.05
Supernatant	0.05 ± 0.01	0.21 ± 0.03**

† Glycerol was present in the isolation medium.
* $P < 0.05$. ** $P < 0.01$

decreased considerably, from a mean value of 3.5 to 1.05×10^{-12} mol/mg protein, whereas the receptor affinity (K_D values) remained unchanged. This is consistent with the data reported on the guinea-pig cytosolic progesterone receptors [6, 7].

In order to elucidate the mechanism of progesterone-induced reduction in progesterone receptors we, as a first step, studied the distribution of progesterone in the control and the progesterone treated animals. The results are shown in Table 2. After progesterone treatment the progesterone concentration in the uterine tissue, as expected, increased significantly [11]. Among the subcellular fractions this increase was significant for nuclear-extract and the supernatant, whereas the slight increase seen in the mitochondrial + microsomal fraction was not significant. When these fractions were prepared using a medium not containing glycerol no significant increase in progesterone concentration in any of the subcellular fractions was found (Table 3). The increase in tissue concentration was, however, significant. This might indicate that progesterone bound to cytosolic receptor had already dissociated and subsequently was distributed among the other fractions which resulted in some increase in progesterone concentration in each fraction but no specific increase in the cytosolic fractions as shown in data of Table 2.

Although we found that the receptor concentration per mg protein was highest in the mitochondrial + microsomal fraction, the protein concentration in this fraction per unit weight of the tissue was lowest (Table 4). This distribution did not change after progesterone treatment. Since there was a specific increase in progesterone concentration of the cytosol, the distribution (per cent) after progesterone treatment of total progesterone had also changed markedly.

Table 3. Progesterone concentration in the uterine tissue and in isolated subcellular fractions obtained from control and progesterone-treated rabbits†

Preparation	Progesterone (ng/mg protein) Control ($n = 4$)	P-treated ($n = 5$)
Intact tissue	0.07 ± 0.01	0.09 ± 0.01*
Nuclear	0.13 ± 0.03	0.13 ± 0.01
Nuclear-extract	0.03 ± 0.01	0.06 ± 0.01
Mitochondrial + microsomal	0.37 ± 0.01	0.42 ± 0.02
Supernatant	0.05 ± 0.01	0.06 ± 0.01

† Glycerol was absent from the isolation medium.
* $P < 0.05$

Table 4. Distribution (per cent) of protein in subcellular fractions from control and progesterone-treated rabbits

Preparation	Per cent total protein Control ($n = 4$)	P-treated ($n = 4$)
Nuclear	21.32 ± 1.56	18.01 ± 2.05
Nuclear-extract	17.12 ± 0.94	18.24 ± 0.91
Mitochondrial + microsomal	8.30 ± 0.28	10.93 ± 0.65
Supernatant	53.26 ± 2.20	52.82 ± 1.67

In order to rule out the possibility that reduction of the receptor was simply due to the occupation of the receptors after progesterone treatment, receptor concentrations were determined in the supernatant after loading it with progesterone. The results shown in Fig. 3 clearly indicate that the loading of the receptor *in vitro* had no influence on the outcome of the measured receptor concentration. A determination of the progesterone concentration in the cytosol after loading showed (Table 5) that the loading increased the amount of progesterone to levels greater than those found in the cytosol obtained from the progesterone treated rabbits (see Table 2).

To further check the above point on the occupation of the receptors the cytosol was first stripped of endogenous progesterone and receptors were determined thereafter. The data shown in Table 6 indicate that

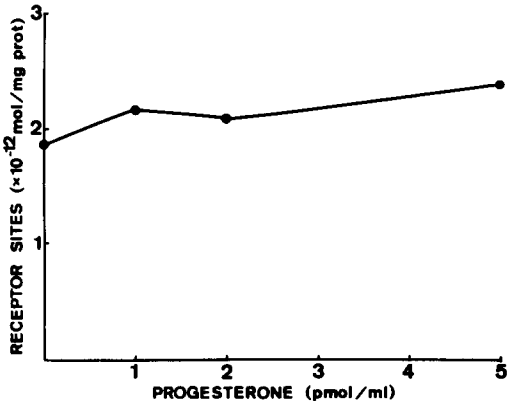

Fig. 3. Effect of loading of cytosol with various concentrations of progesterone on receptor concentration. Aliquots of supernatant were incubated with various concentrations of progesterone for 5 h at 4°C. Charcoal was added to remove the unbound progesterone. After spinning and decanting, the supernatant was filtered through GF/C (Whatman) filters to remove the remaining charcoal particles. The concentration of the receptors in the loaded cytosol was determined by the usual isotopic method.

Table 5. Progesterone concentration in cytosolic fraction after *in vitro* loading with progesterone

Experiment No.	Progesterone (ng/mg protein)	
	Unloaded	Loaded
1	0.17	0.59
2	0.08	0.50
3	0.08	0.63

See Fig. 3 for loading procedure.

there was no significant difference in the concentration of the receptor sites in the stripped and the unstripped cytosolic fractions obtained either from the control or the progesterone-treated rabbits. The determination of the progesterone concentration in the stripped and unstripped cytosol indicated (data not shown) that more than 80% of the endogenous progesterone could be stripped by the present stripping method (see footnote, Table 6).

Finally, to check whether the transfer of the cytosolic receptor to the nucleus could account for the diminished amount of progesterone receptors, we extracted the crude nuclear pellets with TEDG buffer and determined the receptor in the extract so obtained.

The results presented in Table 7 show that there was in fact a higher concentration of progesterone receptor in the nuclear-extract of the control than of the progesterone-treated rabbit. The receptor concentration in the extract was lower than in the native cytosol in the case of both control and progesterone-treated rabbits. The K_D for these receptors was similar to that found for the cytosolic receptors. These

Table 6. Effect of stripping cytosol of endogenous progesterone on receptor concentration

Group	Receptor sites ($\times 10^{-12}$ mol/mg protein)	
	Unstripped	Stripped
Control ($n = 4$)	3.61 ± 0.48	2.91 ± 0.47
P-treated ($n = 4$)	1.42 ± 0.12	1.48 ± 0.13

Stripping was done by mixing an aliquot of supernatant with charcoal for 30 min at 4°C. After centrifugation (1000 *g*) and decantation, the supernatant was filtered through GF/C (Whatman) filters, to remove any remaining charcoal particles, and used subsequently for receptor determination.

Table 7. Progesterone receptor determination in cytosol extracted from the nuclear fraction (nuclear-extract) of control and progesterone-treated rabbits

Group	Receptor sites ($\times 10^{-12}$ mol/mg protein)	K_D ($\times 10^{-9}$ M)
Control ($n = 7$)	0.76 ± 0.06	3.84 ± 0.53
($n = 6$)	0.52 ± 0.06*	4.31 ± 0.50

See Methods for the preparation of nuclear-extract.
* $P < 0.05$.

receptors therefore might simply reflect the cytosolic receptors trapped in or loosely bound to the nuclear fraction since they were extracted with the normal buffer (TEDG) rather than with the high salt solutions. In extracts of the nuclear fraction, made in high salt (0.4 M KCl) the concentration of the progesterone receptor (not shown) was similar in the control in the progesterone-treated rabbits.

DISCUSSION

The results of the present study show that there is a reduction in the cytosolic progesterone receptors in the rabbit myometrium after progesterone treatment of the rabbits as found earlier in the case of guinea-pigs. However, the reduction observed in the present study was not as marked as shown for the guinea-pig, since in that species the receptor level was reduced to less than 20% after progesterone treatment [6], whereas in the present study the level was reduced to only 40 to 50% of the control. Oestrogenization of the rabbits in the present study might have counteracted the effect of progesterone to some extent, but there is no available data in proof of this.

The present results further show that the occupation of the receptors and its subsequent influence on the receptor determination cannot simply account for the reduction of cytosolic progesterone receptors after progesterone treatment. It seems also unlikely that the reduction of cytoplasmic receptors was due to their translocation in the nucleus. This is consistent with the recent evidence that the increment in the nuclear progesterone receptor was too little to account for the reduction of the cytosolic progesterone receptors [8, 14].

The data on the progesterone distribution in the subcellular fraction indicated a specific increase in the cytosolic concentration of progesterone after progesterone treatment. This clearly reinforces the evidence for the presence of progesterone receptors in the cytoplasm. It appears that progesterone bound endogenously after progesterone administration to cytosolic receptors becomes inexchangeable or very slowly exchangeable compared to that bound *in vitro*. Further data are needed to test this possibility. Recent data show [15] that when filled *in vitro* with progesterone, the receptor is more stable than that not filled.

The reduction in the receptor level cannot be easily explained by a progesterone-induced degradation of the receptor since one would have in that case expected a reduced concentration of progesterone in the cytosolic fraction. This, however, was not the case as shown by the present data. One might argue, however, that the increased concentration of progesterone in the cytosolic fraction found after progesterone administration does not necessarily indicate that all of it was bound to its true receptor. On the other hand, if one postulates that progesterone in the cytosol after progesterone treatment was bound to two classes of binding sites (high and low affinity), one should see

an indication of this in the Scatchard plots used for the receptor measurements. This plot of the data from progesterone-treated animals was, except for its shift to the left (reduced binding sites), almost identical to that from the controls.

In conclusion the present results confirm in rabbit the reduction of cytosolic progesterone receptors after progesterone administration but this reduction was not as dramatic as reported for guinea-pig. An elucidation of the mechanism for this reduction, however, must await further information particularly on the nature of the receptor–progesterone complex formed endogenously.

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