Glucocorticoid Receptor in the Rat Uterus

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Abstract

³H-Dexamethasone binding sites with a K_d of ~ 0.7 nM and a maximum number of binding sites of ~ 0.3 pmoles/mg protein were demonstrated in the uterine cytosol of adrenalectomized rats only if dithiothreitol was present in the incubation mixture and the simultaneous presence of molybdate further enhanced the binding in the cytosol. The binding sites exhibited a high specificity for glucocorticoids and were depleted in a dose-dependent manner from cytosol after administration of dexamethasone to animals. The depletion was not due to the occupation of the binding sites by the dexamethasone administered and the rate of depletion was correlated with the inhibition of uterine growth induced by estrogen administration. The cytosol labeled with ³H-dexamethasone in the presence of dithiothreitol bound to DNA-cellulose efficiently after heating at 25°C for 30 min and the binding was inhibited by pyridoxal 5'-phosphate added to the reaction mixture. The effect of heating on the DNA-cellulose binding was abolished by molybdate in the incubation mixture.

From these observations, it was concluded that ³H-dexamethasone binding sites in the rat uterus were physiologically active glucocorticoid receptors.

The presence of cytoplasmic receptors has been considered to be essential in expressing most glucocorticoid responses in target tissues. However, in the rat uterus, inhibition by glucocorticoids of a number of estrogen-induced responses has been well documented (Szego and Roberts, 1953, Szego and Daws, 1969, Campbell, 1978, Markaverich *et al.*, 1981, Stewart *et al.*, 1983), while the lack of glucocorticoid receptors has also been reported (Giannopoulos, 1973, Higgins *et al.*, 1973, Ballard *et al.*, 1974, Booth and Colas, 1975). Recently, Panko *et al.* (1981) observed ³H-glucocorticoid

binding sites in the rat uterine cytosol with the buffer containing monothioglycerol and examined the physicochemical nature of those binding sites. However, it did not seem to be sufficiently clear whether the binding sites observed by these authors were physiologically active glucocorticoid receptors or not.

In a previous work in this laboratory (Noguchi *et al.*, 1983), it was observed that binding of ³H-dexamethasone to the uterine cytosol of rats was markedly enhanced by simultaneous addition of molybdate and dithiothreitol. In the present study, to further elucidate the physiological significance of the ³H-dexamethasone binding sites in the rat uterus, some binding characteristics and

dynamics after administration of glucocorticoid were examined.

Materials and Methods

Chemicals

[1, 2, 4- ^3H] Dexamethasone (^3H -Dex, specific radioactivity, 42 Ci/mmol) was purchased from Amersham Internatl. Ltd. (Amersham, UK). Unlabeled steroids were obtained from Sigma Chemical Co. (St. Louis, MO, USA). DNA-cellulose was prepared according to the method of Alberts and Herrick (1971) using calf thymus DNA (Sigma, Type I) and cellulose powder (Whatmann, CF-11, Kent, UK). Other chemicals used were of analytical grade from Nakarai Chemicals Co. (Kyoto, Japan).

Animals and tissue preparations

Female rats of Wistar strain weighing 180–200 g were mostly used. In experiments where the effect of hormone administration on the cytosolic ^3H -Dex binding sites was examined, animals were adrenalectomized or adrenalectomized and ovariectomized 12–16 days prior to using. Hormones were administered subcutaneously as 30% ethanol-saline solutions. To prepare cytosols, tissues were homogenized in 5 (liver and thymus) or 10 (uterus) volumes of ice-cold TEM buffer solution (10 mM Tris-HCl, pH 7.4, 0.5 mM EDTA and 2 mM 2-mercaptoethanol) supplemented with or without 10 mM sodium molybdate (Mo) and 10 mM dithiothreitol (DTT). Homogenates were centrifuged at $10,500\times g$ for 1 h and the resultant supernatant was used as cytosol after treatment with 1/3 volume of dextran-coated charcoal (DCC, 2.5% charcoal-0.25% dextran in TEM buffer) to remove free steroids.

Determination of ^3H -Dex binding sites in cytosols

Aliquots of cytosol were incubated with a series concentration of ^3H -Dex (1–10 nM) in the presence or absence of 500-fold molar excess of unlabeled dexamethasone (Dex) in a final volume of 0.4 ml at 0°C for 15 h. At the end of incubation, unbound steroids were removed with 0.2 ml of DCC at 0°C for 15 min and the radioactivity in an aliquot of the supernatant was determined after brief centrifugation. The radioactivity which was not displaced by the addition of 500-fold molar excess of radioinert Dex was referred to as the

non-specific binding and subtracted in all instances. The dissociation constant (K_d) and number of maximum binding sites (B_{max}) were calculated by the method of Scatchard (1949). Rates of inhibition by radioinert steroids and diethylstilbestrol were estimated as described previously (Izawa and Ichii, 1978). Exchange assay of glucocorticoid-bound cytosol was performed by the method of Banerji and Kalimi (1981) using p-hydroxymercuribenzoate and DTT.

Binding to DNA-cellulose of uterine cytosol labeled with ^3H -Dex

The cytosol was incubated with 20 nM ^3H -Dex in the TEM buffer supplemented with 20 mM DTT at 0°C for 4 h and treated with DCC to remove unbound ligands. The ^3H -Dex labeled cytosol was incubated at 25°C for 30 min, in the presence or absence of 10 mM Mo, and cooled in ice for 30 min. An aliquot of the labeled cytosol was incubated with the DNA-cellulose (40 mg powder containing 130 μg DNA) in a total volume of 1 ml at 0°C for 30 min with occasional vortexing. At the end of the incubation period, after a brief centrifugation at $3000\times g$ the supernatant was discarded. The pelleted DNA-cellulose was washed twice with 1.5 ml of the TEM buffer, the bound steroid was extracted with 1.5 ml of ethanol and the radioactivity was determined. In a parallel incubation, the radioactivity associated with plain cellulose was determined and subtracted from that associated with DNA-cellulose as the non-specific binding.

Analytical methods

Protein and DNA were determined by the methods of Lowry *et al.* (1951) and Burton (1968) using bovine serum albumin and calf thymus DNA as standards, respectively. The radioactivity was determined in a liquid scintillation spectrometer (Aloka, LSC-700) with automatic standardization for quenching correction.

Results

Binding parameters and specificity of ^3H -Dex binding to uterine cytosol

As reported previously (Noguchi *et al.*, 1983), ^3H -Dex binding to uterine cytosols was synergistically enhanced when both Mo and DTT were included in the incubation

Fig. 1. Scatchard plot of ^3H -Dex binding in the uterine cytosol of adrenalectomized rats.

Binding of ^3H -Dex in the cytosol from uterus of adrenalectomized rats was assayed in the TEM buffer (Fig. 1, $\blacktriangle$), in the buffer containing DTT (10 mM) (Fig. 1, $\triangle$) and in the buffer containing DTT (10 mM) and Mo (10 mM) (Fig. 1, $\circ$). K_d and the number of maximum binding sites are indicated in the figure as nM and pmoles per mg protein, respectively.

mixture of the binding experiments (Fig. 1); in the presence of Mo and DTT, K_d and number of maximum binding sites were approximately 0.7 nM and 0.3 pmoles per mg protein, respectively. Steroid specificity of the binding observed in the presence of Mo and DTT was also examined (Fig. 2). Steroids which possess the biological potency of glucocorticoid showed high rates of inhibition of ^3H -Dex binding, and progesterone inhibited the binding moderately, while estro-

Table 1. Binding to DNA-cellulose of the uterine cytosol labeled with ^3H -Dex.

Pretreatment of ^3H -Dex labeled cytosol*	Conditions for DNA-cellulose binding**	Bound radio-activity***
0°C for 30 min	0°C for 30 min	520 dpm
25°C for 30 min	0°C for 30 min	2675
25°C for 30 min	0°C for 30 min in the presence of 10 mM pyridoxal 5'-phosphate	710
25°C for 30 min in the presence of 10 mM Na_2MoO_4	0°C for 30 min	335

Uterine cytosol from adrenalectomized rats was incubated with 20 nM of ^3H -Dex $\pm$ 500-fold molar excess of unlabeled Dex in the presence of DTT (20 mM) at 0°C for 4 h and the labeled cytosol was prepared by the DCC method described in the "Materials and Methods".

* The ^3H -Dex labeled cytosol was pretreated as indicated in the Table and an aliquot of each (19758 dpm, 0.63 mg as protein) was used for the DNA-cellulose binding experiments.

** 1 ml of the assay mixture contained 40 mg of DNA-cellulose (130 μg as DNA) or plain cellulose and the labeled cytosol described above.

*** Bound radioactivity was obtained by subtracting the radioactivity bound to plain cellulose from that bound to DNA-cellulose. Results obtained in the additional two experiments were almost identical. For other experimental conditions, see the "Materials and Methods".

gen and androgen did not show any significant inhibitory effect. The binding specificity observed was a typical one which has been reported in glucocorticoid-receptor systems of target tissues (Izawa and Ichii, 1978, Noguchi *et al.*, 1983).

Binding to DNA-cellulose of uterine cytosol labeled with ^3H -Dex in the presence of DTT

The uterine cytosol was prepared from adrenalectomized rats in the TEM buffer and incubated with 20 nM of ^3H -Dex in the presence of 10 mM DTT at 0°C for 4 h. At the end of the incubation, unbound ligands

Fig. 2. Steroid specificity of the ^3H -Dex binding in the uterine cytosol. An aliquot of the uterine cytosol (1.1 mg as protein) prepared in the TEM buffer supplemented with DTT (10 mM) and Mo (10 mM) was incubated with ^3H -Dex (4.7 nM) at 0°C for 15 h in a final volume of 0.4 ml. To estimate the inhibitory effect of unlabeled competitors, various concentrations of competitors were added to the incubation mixtures as ethanol solutions at concentrations of ethanol less than 0.2% in all instances. The binding in the absence of competitor was 16700 dpm/tube.

were removed by the DCC method. The labeled cytosol was divided into two parts and further incubated at 25°C for 30 min in the presence or absence of 10 mM Mo. An aliquot of the heated cytosol was incubated with DNA-cellulose at 0°C for 30 min and the ^3H -Dex bound to DNA-cellulose was determined. The labeled cytosol heated at 25°C in the absence of Mo bound to DNA-cellulose, but the cytosol heated in the presence of Mo did not show any sign of binding to DNA-cellulose (Table 1). The addition of pyridoxal 5'-phosphate to the reaction mixture markedly reduced the binding of the cytosol heated in the absence of Mo to DNA-cellulose.

Depletion of ^3H -Dex binding sites in uterine cytosol after administration of Dex

Depletion of the cytoplasmic receptor after steroid hormone administration has been attributed to translocation of receptors to nuclei following binding with steroid hormones. Therefore, the depletion of the cytoplasmic receptor seems to be one of the indices to be used in evaluating the physiological roles of the binding sites for steroid hormones. For this reason, changes in the binding sites for ^3H -Dex 30 min after Dex-administration were examined (Fig. 3). K_d 's were not changed with the dose of Dex-administered (approximately 0.7 nM), while B_{max} 's of ^3H -Dex binding sites were de-

Fig. 3. Changes in the ^3H -Dex binding sites of uterine cytosol from adrenalectomized rats after administration of Dex.

Adrenalectomized rats were injected with 3, 6, 20 and 200 μg per head of Dex 30 min before sacrifice. Cytosols were prepared in the TEM buffer supplemented with Mo (10 mM) and DTT (10 mM) and aliquots (approximately 0.6 mg as protein per tube) were incubated with series concentrations of ^3H -Dex (1–10 nM) in a final volume of 0.4 ml at 0°C for 15 h. Specific bindings were analyzed by the method of Scatchard (1949). For other experimental conditions, see the "Materials and Methods".

creased in a dose-dependent manner (no injected control, 0.31, Dex 3 μg , 0.14, Dex 6 μg , 0.08, Dex 20 μg , 0.03 pmoles/mg protein, respectively, Dex 200 μg , not detectable). To ascertain whether the depletion is merely due to the occupation of the binding sites by Dex administration, the

Table 2. Effect of adrenalectomy, ovariectomy and estradiol-17 β administration on ^3H -Dex binding in the uterine cytosol.

Treatment of animals	No. of Exp.	^3H -Dex binding*	
		Kd (nM)	B _{max} (pmoles/mg protein)
None	8	0.57 $\pm$ 0.02	0.29 $\pm$ 0.01
Adrex, 8 days	7	0.65 $\pm$ 0.05	0.31 $\pm$ 0.01
Adrex & Ovex, 8 days	3	0.67 $\pm$ 0.03	0.30 $\pm$ 0.03
Adrex & Ovex, 12 days	3	0.75 $\pm$ 0.06	0.32 $\pm$ 0.02
Adrex & Ovex, 12 days plus E ₂ -17 β (20 μg /day), 2 days	4	0.62 $\pm$ 0.02	0.29 $\pm$ 0.01

* Values are expressed as means $\pm$ SEM.

Uterine cytosols were prepared from animals which had been adrenaectomized, ovariectomized and treated with estradiol-17 β as indicated in the Table. Binding parameters for ^3H -Dex of uterine cytosol were determined by the method of Scatchard (1949) as described in the "Materials and Methods".

exchange assay developed by Banerji and Kalimi (1981) was applied to the uterine cytosol prepared from animals which received 200 μg per head of Dex 30 min before. In this cytosol, no significant binding of ^3H -Dex to the cytosol was detected even by the exchange assay (data not shown). These results indicated that the depletion of ^3H -Dex binding sites in the uterine cytosol after Dex administration seemed to be the consequence of nuclear translocation of the receptor-complexes which were commonly observed in steroid-receptor systems of various target tissues (Anderson *et al.*, 1975, Bloom *et al.*, 1980, Freifeld *et al.*, 1974, Ichii, 1980, 1981, Isomaa *et al.*, 1979, Isotalo *et al.*, 1981, Kassis and Gorski, 1981, Mester and Baulieu, 1975, Milgrom *et al.*, 1973, Walters and Clark, 1978).

Effects of adrenalectomy, ovariectomy and estradiol administration on ^3H -Dex binding sites in the uterine cytosol

The binding capacity to ^3H -Dex in the

Fig. 4. Depletion and replenishment of ^3H -Dex binding sites in cytosols from the liver, thymus and uterus after administration of Dex. Adrenalectomized rats were sacrificed at appropriate time intervals after administration of Dex (doses are indicated in Fig. 4-C as $\mu\text{g}/\text{head}$) and cytosols of three tissues were prepared in the TEM buffer supplemented with DTT (10 mM) and Mo (10 mM). Binding of ^3H -Dex in cytosols of the uterus (Fig. 1), thymus (Fig. 4-A) and liver (Fig. 4-B) was assayed in the TEM buffer containing Mo (10 mM) and DTT (10 mM). Aliquots of cytosols (liver, 0.9, thymus, 0.7 and uterus, 0.6 mg as protein per tube, respectively) were incubated with series concentrations of ^3H -Dex (1–10 nM) in a final volume of 0.4 ml at 0°C for 15 h. Binding parameters were determined by the method of Scatchard (1949). Binding sites for ^3H -Dex in cytosols from uninjected adrenalectomized rats were taken as 100 (liver, 0.53, thymus, 1.23 and uterus, 0.31 pmoles per mg protein, respectively) and values were expressed as the relative bindings of the controls (mean $\pm$ SEM) in at least 3 experiments performed independently. For other experimental conditions, see the "Materials and Methods".

cytosol was not significantly altered after adrenalectomy, ovariectomy or estrogen administration during the experimental periods (Table 2). K_d 's for ^3H -Dex were not altered significantly by any of the treatments described above.

Comparison of the pattern of depletion and replenishment of ^3H -Dex binding sites in cytosols from the uterus after administration of Dex with those of the liver and thymus

The time-course of the depletion of the binding sites was compared in these tissues (Fig. 4-C). As reported by Sando *et al.* (1979), ^3H -Dex binding to the thymus cytosol was significantly enhanced by Mo and

DTT and it was observed that these reagents act to increase the binding affinity as well as the binding sites of the cytosol to ^3H -Dex (Fig. 4-A). The effect of Mo and DTT on ^3H -Dex binding to the liver cytosol was relatively small (Fig. 4-B). Therefore, Mo and DTT were included in the reaction mixtures of ^3H -Dex binding to cytosols in this study. Within 30 min after injections of 20 μg of Dex, the shortest period examined, maximum depletions of ^3H -Dex binding sites in cytosols from all tissues were observed but rates of depletion of the binding sites in these tissues were slightly different; in the liver, the binding sites completely disappeared, while 15% and 5% of the binding sites of the uninjected controls still remained in the thymus and uterus. The patterns of replenishment were also different significantly in these 3 tissues; in the thymus and uterus, the binding sites were replenished to approximately 50% of the untreated controls by 7 h after treatment with 20 μg of Dex, while in the liver, only 10% of the binding sites of the control were observed at this time. Replenishment of the binding sites in the uterine cytosol was much more rapid than that in the liver cytosol; in the uterine cytosol from animals which received 200 μg of Dex, the rate of replenishment was higher than that observed in the liver cytosol of animals which had received 20 μg of Dex. The physiological significance of the rapid replenishment of the binding sites observed in the uterine cytosol is not clear at the present moment.

Effect of addition of uterine cytosol on ^3H -Dex binding to liver cytosol

In our previous study (Izawa *et al.*, 1984), the presence of cytosolic inhibitor(s) which specifically inhibits the ^3H -Dex binding was demonstrated in cytosols of the male accessory sex organs and a similar inhibitor(s) in the uterine cytosol was also suggested. When the uterine cytosol prepared from animals injected with 200 μg of Dex 30 min

Fig. 5. Effect of the uterine cytosol on ^3H -Dex binding in the liver cytosol. Liver cytosol from female adrenalectomized rats (0.9 mg as protein) prepared in the TEM buffer supplemented with Mo (10 mM) was incubated with increasing concentrations of ^3H -Dex (1–10 nM) $\pm$ 500-fold molar excess of unlabeled Dex in the presence or absence of the DCC-treated uterine cytosol (0.6 mg as protein) or the cytosol heated at 60°C for 15 min, prepared from animals which received 200 μg per head of Dex 30 min prior to sacrifice, in a final volume of 0.4 ml at 0°C for 15 h. At the end of the incubation period, unbound steroids were removed with 0.2 ml of the DCC and the bound radioactivity in an aliquot of the supernatant was measured. K_d and number of maximum binding sites were determined by the method of Scatchard (1949). K_d 's are 1.3 (O, control), 2.8 (Δ , untreated uterine cytosol) and 3.0 nM ($\blacktriangle$, heated uterine cytosol), respectively. The maximum binding site was approximately 0.5 pmoles per mg protein in all instances. For other experimental conditions, see the "Materials and Methods".

before sacrifice was added to the liver cytosol from adrenalectomized rats, K_d was elevated from 1.3 to 3.0 nM without any

change in the number of maximum binding sites (Fig. 5, approximately 0.50 pmoles/mg liver protein). Pretreatment of the uterine cytosol at 60°C for 15 min did not alter the inhibitory activity significantly. These results are essentially consistent with the observation in experiments of cytosols from male accessory sex organs (Izawa *et al.*, 1984). From these observations, it seems that an inhibitor(s) of which activity is inactivated by the addition of Mo and DTT is also present in the uterus.

Effect of Dex administration on the uterine growth induced by estradiol-17 β administration

As reported by Markaverich *et al.* (1981), estrogen administration evoked significant uterine growth, and simultaneous injection of Dex reduced the rate of uterine growth induced by estradiol. To examine the role of ^3H -Dex binding sites of the uterine cytosol in glucocorticoid antagonism of the uterotrophic responses to estrogens, various doses of Dex and 20 μg of estradiol-17 β were administered simultaneously to adrenalectomized and ovariectomized rats and the uterine wet weight was estimated 24 h

after hormone injection (Table 3). In the present study, complete inhibition was not observed even after treatment of animals with 500 μg of Dex. On the other hand, estrogen administration did not alter the pattern of depletion and replenishment of the ^3H -Dex binding sites in the uterine cytosol (data not shown).

discussion

The addition of Mo and DTT enabled us to examine the nature of ^3H -Dex binding sites in the uterine cytosol. Similar ^3H -Dex binding systems have already been recognized in the male accessory sex organs; the binding sites for ^3H -Dex are detected only if DTT is present in the incubation mixture and the simultaneous presence of Mo enhances the binding in cytosols from those tissues (Noguchi *et al.*, 1983). The presence of at least two different inhibiting factors for ^3H -Dex binding, one related to DTT and the other sensitive to Mo, is suggested by these results. Inhibiting factors for ^3H -Dex binding have also been detected in the uterine cytosol (Fig. 5, Izawa *et al.*, 1984). Therefore, it seems probable that the inhibiting factors mask the binding sites for ^3H -Dex during the period of tissue processing *in vitro*. The addition of Mo and DTT abolished the action of the inhibiting factors and the demasked binding sites become detectable.

Characteristics of the ^3H -Dex binding sites in the uterine cytosol examined in the presence of Mo and DTT are well fitted to the definition proposed for the steroid hormone receptors; the binding sites are high affinity ($K_d \sim 0.7$ nM for Dex) and limited number ($B_{\text{max}} \sim 0.3$ pmoles/mg protein) and exhibit a high specificity for glucocorticoids. Upon binding to the ligand, the binder in the uterine cytosol acquires the ability to bind to DNA-cellulose after brief heating. Furthermore, the binding sites in the cytosol

Table 3. Effect of Dex administration on uterine growth induced by estradiol-17 β administration

Treatment of animals*	No. of Exp.	Uterine wet weight (mg)**
None	13	80.5 $\pm$ 4.1
E ₂ (20 μg)	4	181.5 $\pm$ 10.0
E ₂ (20 μg) & Dex (20 μg)	4	145.0 $\pm$ 8.0
E ₂ (20 μg) & Dex (200 μg)	4	124.5 $\pm$ 6.5
E ₂ (20 μg) & Dex (500 μg)	4	104.7 $\pm$ 8.1

* Rats (weighing 190–210 g) which had been adrenalectomized and ovariectomized for 12–16 days received subcutaneous injections of estradiol-17 β (E₂) and Dex at doses indicated in the Table (per head) in 30% ethanol-saline solution.

** Uterine wet weight was estimated 24 h after administration of hormones. Values are expressed as means $\pm$ SEM.

are depleted after administration of Dex. Depletion of the cytoplasmic receptor after steroid hormone administration has been attributed to the translocation of steroid hormone-receptor complexes to nuclei. Therefore, dose-dependent depletion of the ^3H -Dex binding sites observed after administration of Dex in the uterine cytosol seems to be a line of evidence showing that the binder may have some physiological activity in that tissue.

Another unique feature of the present study is in finding that replenishment of the binding sites in the uterine cytosol takes place rapidly following the depletion induced by Dex administration. The period of depletion is dependent on the dose of hormone administered and varies from tissue to tissue (Figs. 3 and 4, Ichii, 1981). The replenishment in the uterine cytosol of animals which received 200 μg per head of Dex is faster than that in the liver cytosol of animals treated with 20 μg of Dex. The length of the receptor depletion is well correlated with the amount of tyrosine aminotransferase induction in the liver (Izawa *et al.*, 1982). It has been considered that the biological action of glucocorticoid as well as that of androgen (Ichii, 1980) is exerted during the period of receptor depletion. The administration of Dex blocks the uterine growth induced by estradiol but complete inhibition is not attained even with the highest dose examined (500 μg /head, Table 3). This may be explained, at least in part, by the fact that the ^3H -Dex binding sites in the uterine cytosol replenish rapidly. Therefore, it may require higher dose of or more frequent injections of Dex to maintain the level of the binding sites in a depleted state. Factors which determine the length of receptor depletion in tissues, except the dose of hormone administered, have not been examined and may provide subjects to be explored.

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