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PROGESTIN BINDS TO THE GLUCOCORTICOID RECEPTOR AND MEDIATES ANTIGLUCOCORTICOID EFFECT IN RAT ADIPOSE PRECURSOR CELLS

XUEFAN XU,* JOHAN HOEBEKE[1] and PER BJÖRNTORP

Wallenberg Laboratory and Department of Medicine I, Sahlgren's Hospital, University of Göteborg, 413 45 Göteborg, Sweden and [1]Laboratoire de Biologie moléculaire des Récepteurs, Département de Biotechnologie, Institut Pasteur, 25 rue du Dr Roux, F-75724 Paris Cédex 15, France

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Summary—The binding of progestin and glucocorticoid hormones was examined in the cytosol of rat adipose precursor cells. Progestin binding sites of high affinity and limited capacity were present in the cytosol of adipose precursor cells from female rats, but not from male rats, by using [^{3}H]R5020 as radioligand. Glucocorticoid binding sites of high affinity and limited capacity were present in the cytosol of these cells from both male and female rats by using [^{3}H]dexamethasone and [^{3}H]triamcinolone acetonide as radioligands. The dissociation constants were in the physiological concentration range. Studies of competitive binding showed that progestin could compete with glucocorticoids at glucocorticoid binding sites. In a serum free medium glucocorticoid effect on cellular differentiation, monitored by glycerophosphate dehydrogenase (GPDH), was effectively counteracted by progesterone which by itself had no effect.

These results demonstrate that progestin receptor exists only in rat adipose precursor cells from female rats, while glucocorticoid receptor exists in rat adipose precursor cells of both sexes. Glucocorticoid effects on cellular differentiation in these cells are mediated by the glucocorticoid receptor. Progestin binds to the glucocorticoid receptor and antagonizes glucocorticoid effect on cellular differentiation in these cells.

INTRODUCTION

Recent studies have shown profound effects of steroid hormones on the regulation of lipolysis and differentiation in adipose precursor cells. For example, androgens enhance the lipolytic capacity of rat adipose precursor cells by increasing the apparent number of β-adrenoceptors [1]. Glucocorticoids are involved in the regulation of both lipolytic pathway [2, 3] and differentiation [4–12] in adipose precursor cells. These effects are mediated by specific intracellular receptors in these cells [1, 13]. Progesterone has been shown to increase activities of differentiation markers in rat adipose precursor cells in media containing serum [14]. It is, however, not clear from that study if the observed effects of progesterone are progesterone receptor mediated or via interactions on the glucocorticoid receptor by glucocorticoids present in the serum. The purpose of this study was to describe the molecular mechanisms responsible for cellular effects of progestin and glucocorticoid interactions in rat adipose precursor cells.

*To whom correspondence should be addressed.

EXPERIMENTAL

Animals

Male and female Sprague–Dawley rats weighing 100–200 g (Alab, Stockholm, Sweden) were used as cell donors. They were housed under controlled conditions at constant temperature (24–26°C) and humidity (50–60%), and with a 12 h light–darkness cycle. The animals were given tap water and pellet food containing 22.5% protein, 72.5% carbohydrate, 5% fat and sufficient vitamins and minerals (Ewos, Södertälje, Sweden). For [^{3}H]R5020 binding, female rats were ovariectomized (OVX) via midventral incision under light anesthesia with pentobarbital (Mebumal, ACO, Sweden). Seven to nine days after the operation they were killed by decapitation, and adipose precursor cells were prepared for culture.

Cell culture

Rats were decapitated and fat pads were removed aseptically. Cells for primary culture were liberated by collagenase and separated from adipocytes and remaining tissue fragments by flotation, filtration and sedimentation procedures as described in detail previously [15]. Cells were cultured in multi-well dishes (1.6 cm in diameter, for enzyme assays) or petri

dishes (9 cm in diameter, for binding assay) at an inoculation density of 40,000 cells/cm^2. Dulbecco's Modified Eagle's Medium (Grand Island Biological Company, Paisley, Scotland) supplemented with 0.1 mg/ml cephalotin (Keflin, Eli Lilly, Fegersheim, France) and 100 μU/ml insulin (Actrapid^R, Novo Laboratories, Copenhagen, Denmark) was used as basal medium. For binding assay, cells were cultured with basal medium supplemented with 10% fetal calf serum (Lot No. 028134, Flow Laboratories, U.K.). Serum free medium [16] that is basal medium supplemented with biotin (33 μM, Sigma, St Louis, Mo., U.S.A.), pantothenic acid (17 μM, Sigma), transferrin (10 μg/ml, Sigma), triiodothyronine (200 pM, Sigma), and insulin-like growth factor (IGF-1, 100 ng/ml, Kabivitrum, Stockholm, Sweden) was used for enzyme assay.

Binding assay

After several days of culture (as stated in figure legends) in basal medium supplemented with 10% fetal calf serum and 100 μU/ml insulin, culture dishes were placed on ice. Culture medium was then removed with a suction pump, and the monolayer washed with ice-cold TEMG-buffer (10 mM Tris–HCl, 1.5 mM EDTA, 12 mM monothioglycerol, and 10% (v/v) glycerol, pH 7.4). Cells were then removed by a rubber policeman with TEMG-buffer in the dish. The cell suspensions were then sonified for 30 s at 40 W with a microtip of a sonifier (Soniprep 150, MSE, Sussex, England) and centrifuged at 48,000 g for 60 min at 0°C.

Hormones dissolved in 99.5% ethanol were pipetted to incubation tubes and evaporated by N_2. To each tube 50 μl TEMG was added. To determine the saturation of the binding, aliquots (200 μl) of high speed supernatant between the top lipid cake and sediment were incubated with increasing concentrations of [^{3}H]R5020 (promegestone, SA 85 Ci/mmol, New England Nuclear, Dreieich, West Germany), [^{3}H]dexamethasone (SA 49.9 Ci/mmol, New England Nuclear) or [^{3}H]triamcinolone acetonide (SA 46.6, New England Nuclear) in the absence or presence of a 1000-fold excess of radioinert R5020 (New England Nuclear), dexamethasone (Sigma) or triamcinolone (Sigma) for 3 h at 0°C [17]. Bound and free radioligand were then separated by gel filtration chromatography on 5 × 60 mm Sephadex LH-20-100 (Sigma) columns equilibrated with TEMG [18]. Two hundred microliters of incubate were pipetted into the column, and washed in with 200 μl TEMG. After 30 min, the protein peak was eluted into a scintillation vial with 800 μl TEMG, whereafter 10 ml toluene-based scintillation fluid were added. Radioactivity was then counted in a LKB 1211 Rackbeta liquid scintillation counter (LKB Beckman, Stockholm, Sweden). The amount of specifically bound hormone was considered to be total binding (without radioinert hormones) minus nonspecific binding (with radioinert hormones).

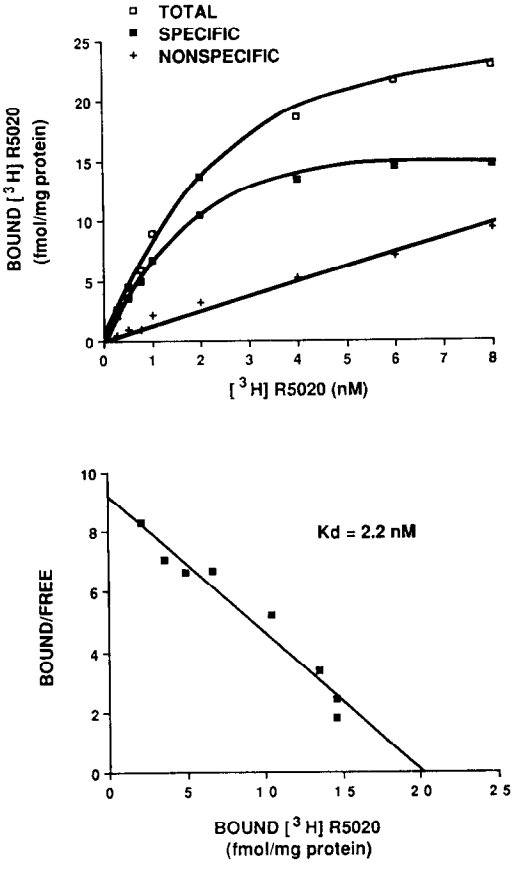

Fig. 1. Saturation (upper) and Scatchard (lower) plots of [^{3}H]R5020 binding to cytosol of adipose precursor cells from parametrial adipose tissues of OVX rats after 7 days culture in basal medium supplemented with 10% fetal calf serum. Each point represents the mean of 3 separate experiments with duplicate determinations.

Scatchard analysis [19] was performed on the specifically bound fraction.

To determine the specificity of binding, aliquots of high speed supernatant were incubated with 2 nM [^{3}H]R5020, 20 nM [^{3}H]dexamethasone or 6.5 nM [^{3}H]triamcinolone acetonide and increasing concentrations of nonlabelled R5020, dexamethasone, triamcinolone, progesterone (Sigma), 17β-estradiol (Sigma) or testosterone (Sigma).

To determine if progestins were competing with dexamethasone for the same binding site, aliquots of high speed supernatant were incubated with increasing concentrations of [^{3}H]dexamethasone in the presence of different concentrations of R5020. Scatchard analysis was performed on the specifically bound fraction.

To determine the concentration of glucocorticoid binding sites in cells from different adipose tissues and after varying duration of culture, aliquots of high speed supernatant were incubated with 10 nM [^{3}H]triamcinolone acetonide in the absence or presence of 200-fold excess of radioinert triamcinolone. The specifically bound fraction was used for calculation.

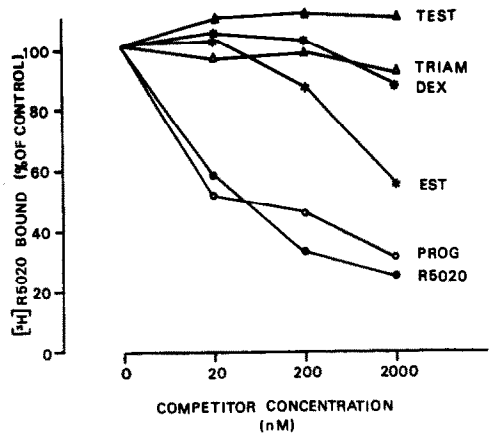

Fig. 2. Inhibition study of [^{3}H]R5020 binding by R5020, progesterone (PROG), 17β-estradiol (EST), dexamethasone (DEX), triamcinolone (TRIAM), testosterone (TEST) to cytosol of adipose precursor cells from parametrial adipose tissues of OVX rats after 7 days culture in basal medium supplemented with 10% fetal calf serum. The figure is representative of 3 experiments performed in duplicate.

Determination of protein and GPDH activity

Protein determination was performed according to Lowry *et al.*[20]. GPDH activity was measured according to the method of Wise and Green[21] by determining spectrophotometrically the oxidation of dihydronicotinamide adenine dinucleotide (NADH, Sigma) at 340 nm. Specific activity is expressed as nmol of product formed/min/mg protein.

Statistical method

Statistical significance was calculated with analysis of variance by using the Scheffe F-test.

RESULTS

Figure 1 shows the binding of R5020 in parametrial adipose precursor cells of OVX rats after 7 days in primary culture. The binding was saturable. Scatchard analysis indicated a K_d of 2.2 nM, and a maximal binding capacity (B_{max}) of 20.5 fmol/mg protein. This saturation curve could not however be demonstrated in adipose precursor cells from

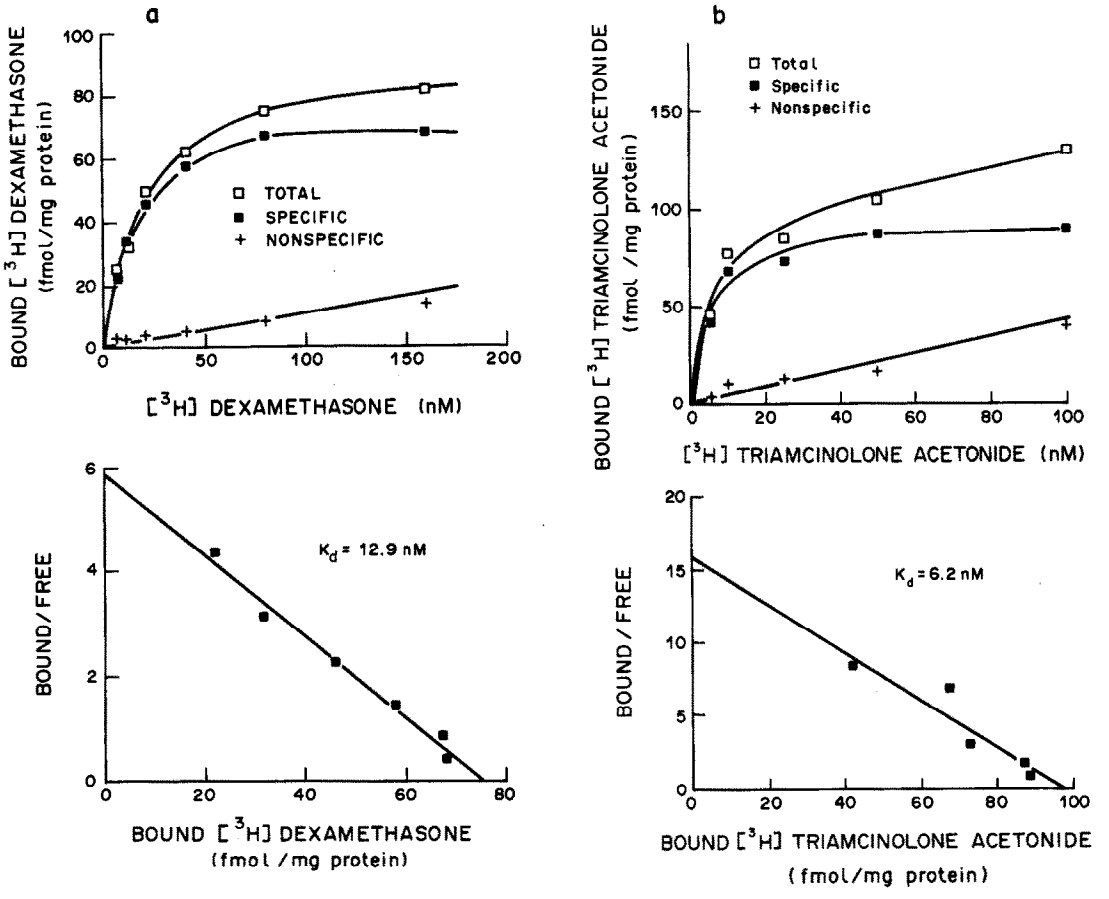

Fig. 3. Saturation (upper) and Scatchard (lower) plots of [^{3}H]dexamethasone (a) and [^{3}H]triamcinolone acetonide (b) binding to cytosol of adipose precursor cells from epididymal fat tissues after 4 days culture in basal medium supplemented with 10% fetal calf serum. Each point represents the mean of 2 separate experiments with duplicate determinations.

epididymal and perirenal adipose tissue of male rats.

The specificity of this binding was tested next (Fig. 2). Whereas the progestins, R5020 and progesterone, effectively inhibited binding of [^{3}H]R5020, high concentrations of 17β-estradiol, triamcinolone, dexamethasone and testosterone had little or no effect.

The binding of glucocorticoids to cytosol prepared from epididymal adipose precursor cells is shown in Fig. 3. The binding was saturable. Dexamethasone had a dissociation constant (K_d) of 10.3 ± 1.5 nM ($n = 4$) and a maximal binding capacity (B_{max}) of 81.9 ± 11.6 fmol/mg protein ($n = 4$) (Fig. 3a). Triamcinolone acetonide had a K_d of 6.7 ± 0.2 nM ($n = 3$), and B_{max} of 101.7 ± 15.9 fmol/mg protein ($n = 3$) (Fig. 3b). Similar saturation curves could also be demonstrated in adipose precursor cells from female rats (not shown).

The concentration of glucocorticoid binding sites in adipose precursor cells did not vary with duration of culture. Binding was found already after 2 days in culture in cells from epididymal adipose tissue (48 ± 10 fmol/mg protein, $n = 3$) and remained the same after 7 days in culture (51 ± 18 fmol/mg protein, $n = 3$). There was no difference between the number of binding sites in adipose precursor cells from epididymal, male perirenal (38 ± 7 fmol/mg protein, $n = 3$) or parametrial adipose tissue (52 ± 8 fmol/mg protein, $n = 3$) after 7 days in culture.

The specificity of glucocorticoid binding was assessed by inhibition experiment using different steroid hormones (Fig. 4). Dexamethasone, triamcinolone, progesterone and R5020 (in that order) inhibited [^{3}H]dexamethasone (Fig. 4a) and [^{3}H]triamcinolone binding (Fig. 4b), while testosterone and 17β-estradiol had no effect.

Analysis of the inhibition curve of [^{3}H]dexamethasone binding (Fig. 4a) by using a computer program [22] showed a monophasic pattern of dexamethasone and R5020 inhibition with a K_d of 16 and 207 nM respectively. The inhibition curve of triamcinolone showed a biphasic pattern with 15% high affinity sites ($K_d = 2.3$ nM) and 85% low affinity sites ($K_d = 54.6$ nM). The inhibition curve of progesterone also showed a biphasic pattern with 20% high affinity sites ($K_d = 7.2$ nM) and 80% low affinity sites ($K_d = 143$ nM).

In the presence of increasing concentrations of R5020 the maximal binding capacity of dexamethasone was not influenced while the apparent affinity decreased (Fig. 5).

In a serum free medium, glucocorticoids decreased GPDH activity in a dose-responsive manner. The concentration required for half-maximal effects was around 10 nM (Fig. 6).

In the serum free medium, progesterone alone had no effect on GPDH activity in a concentration range from 10^{-9} to 10^{-6} M, while at 10^{-5} M clear decreases were seen. In the presence of 10^{-7} M of glucocorticoids, which inhibited GPDH activity, progesterone

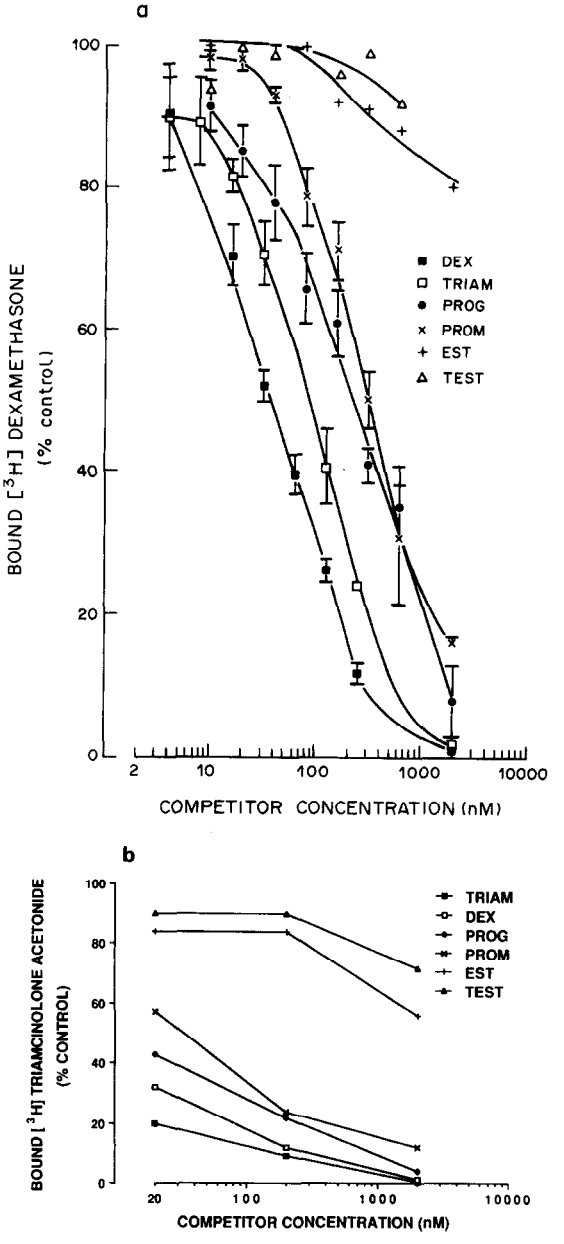

Fig. 4. Inhibition study of [^{3}H]dexamethasone (a) and [^{3}H]triamcinolone acetonide (b) binding by dexamethasone (DEX), triamcinolone (TRIAM), progesterone (PROG), R5020 (PROM), 17β-estradiol (EST), or testosterone (TEST) to cytosol of adipose precursor cells from epididymal adipose tissue after 7 days culture in basal medium supplemented with 10% fetal calf serum. For (a) the mean $\pm$ SEM of 3 experiments are shown. For (b) each point represents the mean of 2 separate experiments with duplicate determinations.

from 10^{-8} M to 10^{-6} M counteracted the glucocorticoid effect at least partly, while 10^{-5} M of progesterone had no counteracting effect (Fig. 7).

DISCUSSION

Specific cytosolic progestin binding sites were found in adipose precursor cells from OVX rats, but

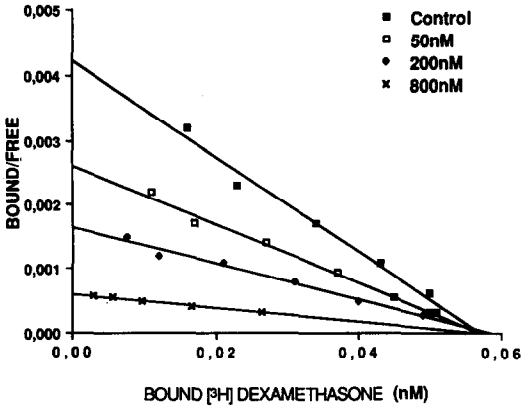

Fig. 5. Scatchard plot of specific [^{3}H]dexamethasone binding in the absence (Control) or presence of 50, 200, or 800 nM R5020. Cytosol was prepared from adipose precursor cells from epididymal fat tissues after 4 days in basal medium supplemented with 10% fetal calf serum. Each point represents the mean of two separate experiments with duplicate determinations.

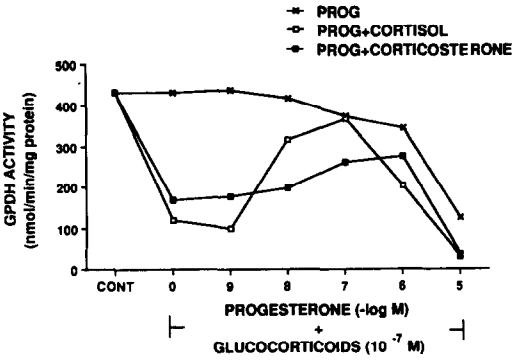

Fig. 7. Effect of progesterone on GPDH activity of adipose precursor cells from epididymal adipose tissue after 7 days culture in the absence or presence of glucocorticoids. Cells were cultured first in basal medium supplemented with 10% fetal calf serum for 2 days and then medium was changed to serum free medium supplemented with steroid hormones. Each point represents the mean of 2 separate experiments with duplicate determinations.

not from male rats. This is in agreement with early studies where progestin binding was found in female rat adipose tissue [23], but not in male rat adipose tissue [24]. The capacity and affinity of progestin binding for these cells was similar to that found for rat adipose tissue.

Glucocorticoid binding sites were found in adipose precursor cells from both male and female rats. The affinity of glucocorticoids for these cells was similar to that found for rat adipose tissue [25], human adipose tissue [17, 26], the 3T3-F442A cell line [13] and other tissues [27]. The concentration of cytosolic binding sites in these cells was also similar to that found in isolated adipocytes from rats [25]. The amount of cytosolic binding sites did not vary among cells from various adipose depots tested or after different duration of culture time.

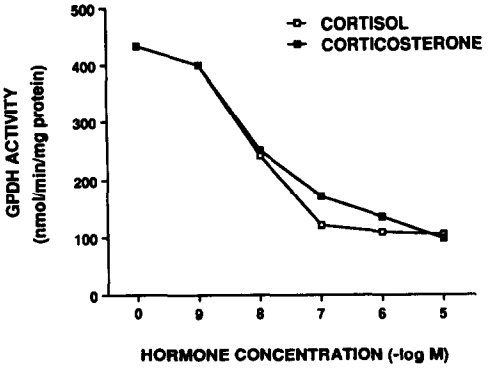

Fig. 6. Glucocorticoids effect on GPDH activity of adipose precursor cells from epididymal adipose tissue after 7 days culture. Cells were cultured first in basal medium supplemented with 10% fetal calf serum for 2 days and then medium was changed to serum free medium supplemented with cortisol or corticosterone. Each point represents the mean of two separate experiments with duplicate determinations.

The inhibition pattern of glucocorticoid binding by different steroids is in agreement with previous studies using other cell systems [13, 17, 25, 26, 28, 29]. The reason for biphasic patterns of the inhibition curves of triamcinolone and progesterone is not known. Progestins competed effectively with glucocorticoid binding here. However, the fact that an unlabeled steroid reduces glucocorticoid binding does not necessarily indicate true competition for a common binding site. For example, an unlabeled steroid might increase the nonspecific binding of the labeled ligand. Under such circumstances an apparent decrease in specific binding would be observed. Similarly, a non-competitive inactivation of the receptor by the unlabeled steroid would induce a similar effect. For these reasons, specific binding of increasing concentrations of [^{3}H]dexamethasone was measured in the presence of various concentrations of unlabeled R5020. Scatchard analysis of these experiments showed that the maximal binding capacity of dexamethasone was constant. Analysis of binding kinetics suggests that the competition of glucocorticoid binding by progestin in adipose precursor cells is indeed occurring over a common binding site. Although these competitive kinetics have been previously described in other cell systems [28, 29], this study demonstrates for the first time that the competitive kinetics also appear in adipose cells.

GPDH, a late marker of cellular differentiation [30] was inhibited by glucocorticoid hormones in a serum free medium. The glucocorticoid concentration required for half-maximal effect was found to be about 10 nM. This is in agreement with the K_d of glucocorticoids found here. This suggests that the effect of the glucocorticoids observed was mediated by the glucocorticoid receptor.

In culture under serum free conditions progesterone alone had no effect on cellular differentiation in physiological concentration range. Progesterone

could at least partially reverse the effect of glucocorticoids. The concentrations of progesterone needed for this effect corresponded to the concentrations needed to inhibit glucocorticoid binding to the glucocorticoid receptor.

In a previous study [14], progesterone was shown to be able to stimulate differentiation of adipose precursor cells from both male and female rats in a serum (fetal calf serum) supplemented medium. This fetal calf serum contained a high concentration of corticosterone. The effective progesterone concentrations were 10^{-7}–10^{-5} M. We could demonstrate in the present study that progestin binding was present only in adipose precursor cells from female rats with a K_d around 2 nM. It is therefore difficult to explain the observed effect of progesterone on male adipose precursor cells in serum supplemented medium. The effective progesterone concentrations were also much higher than the K_d of progestin binding, but near the K_d of progestin binding to the glucocorticoid receptor reported here ($K_d \approx 10^{-7}$ M). Taken together, it is suggested that when progesterone was added to serum supplemented medium (containing corticosterone), binding of progesterone to the glucocorticoid receptor blocked the receptor binding site without inducing the conformation changes necessary to induce the glucocorticoid receptor activity. The endogenous inhibitory effect of corticosterone on adipose differentiation in that condition was therefore counteracted by progesterone and a stimulating effect of progesterone on differentiation was therefore seen. This result again confirms that progesterone is an antagonist to glucocorticoids [3, 5, 31, 32].

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