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UP-REGULATION OF ANDROGEN RECEPTOR BINDING IN MALE RAT FAT PAD ADIPOSE PRECURSOR CELLS EXPOSED TO TESTOSTERONE: STUDY IN A WHOLE CELL ASSAY SYSTEM

GIOVANNI DE PERGOLA,* XUEFAN XU,[1] SHUMIN YANG,[1]
RICCARDO GIORGINO and PER BJORNTORP[1]

Istituto di Clinica Medica, Endocrinologia e Malattie del Ricambio, Università di Bari, Piazza G. Cesare 10, 70124 Bari, Italy and [1]Wallenberg Laboratory and Department of Medicine I, Sahlgren's Hospital, University of Goteborg, 413 45 Goteborg, Sweden

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Summary—Binding of androgens to adipocytes has previously been evaluated using cytosol fractions without taking into account nuclear binding, although the latter is suggested to be close to the physiological site of action. In the present study, performed in differentiated fat pad adipose precursor cells, we describe a simple, reliable and reproducible androgen binding assay in a system with intact cells. Tritiated and unlabeled methyltrienolone (R1881) were used to define specific and unspecific androgen binding. Triamcinolone acetonide was added to prevent the binding of R1881 to other types of receptors. Differentiated adipose precursor cells contain a homogeneous class of high affinity androgen binding sites, and binding is saturable and reversible. Binding apparently occurs at one site, with a K_d in the range of physiological androgen concentration (about 4 nM). Competition studies indicate that the receptor is specific for R1881, testosterone and dihydrotestosterone, which have approximately the same affinity, while progesterone, estradiol and dexametasone show much lower affinity. Androgen binding was markedly enhanced after cellular exposure to R1881 and testosterone but not dihydrotestosterone, and this increase was dependent on protein synthesis, suggesting the formation of new receptors by these androgens.

In conclusion, fully differentiated adipocytes contain a specific, high affinity receptor, the density of which is dependent on androgens.

INTRODUCTION

Sex hormones appear to play a role in the distribution of body fat, with males having a greater proportion of upper body fat and females a greater proportion of lower body fat. Adipose tissue represents an important target for androgens. Recent studies have shown that administration of testosterone (T) to male hamsters [1, 2], middle-aged men [3], male or female rats [4–6] induces profound changes in adipose tissue metabolism. In culture conditions, we have demonstrated that testosterone effect is mediated by a direct action on adipose precursor cells [7]. All these effects require the binding to specific intracellular receptor proteins, since testosterone effects are shown even in the presence of aromatase inhibitors [7]. The interaction of androgen receptor–steroid complexes with specific chromatin acceptor sites in the nucleus leads to gene transcription and subsequent translation of mRNAs into biologically active proteins. To our knowledge, only cytosol receptors have been described in isolated adipocytes [8], or in cultured adipose precursor cells [7], but the cytosol assay has the inconvenience of understimating the receptor density (B_{max}) [9, 10]. Many studies in the rat suggest that the androgen receptor is located predominantly in the nucleus [11]. Androgen binding has been recently shown in a whole cell assay system in culture canine prostatic epithelial cells [12] or in genital skin fibroblasts [13, 14], and a specific binding for glucocorticoids has been described in adipose precursor cells [15]. The whole cell binding assay presents the advantage to ensure cellular and functional homogeneity and avoids any loss of receptors during the cell disruption. For these reasons, and in order to better understand the effects of androgens, it seemed useful to define androgen receptor (AR) binding

*To whom correspondence should be addressed: Dr G. De Pergola, via Putignani 236, 70122 Bari, Italy.

in a whole adipocyte assay, which gives the possibility of measuring the total number of sites (Cytosolic and nuclear). For this purpose, we performed this study in differentiated male rat adipose precursor cells, which constitute an *in vitro* model of adipocyte differentiation [7, 16, 17], and where specific binding for other steroid hormones has been shown [7, 18]. Moreover, when these cells are differentiated to mature adipocytes, they enable the study of the regulation of adipocyte function [7, 19–22]. Since little is known about the mechanisms controlling the androgen receptor concentration in adipocytes, the aim of the present study was first to define the binding parameters of AR in whole adipose precursor cells, and second, to evaluate the effect of androgens on the receptor number.

EXPERIMENTAL

Animals

Male Sprague–Dawley rats weighing 100–200 g (Alab Stockolm, Sweden) were used as cell donors. They were housed under controlled conditions at constant temperature (24–26°C), 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, Sodertalje, Sweden).

Cell culture

Rats were decapitated, and epididymal fat pads were removed asceptically. Cells for primary culture were then liberated by collagenase and separated from adipocytes and remaining tissue fragments by flotation, filtration, and sedimentation procedures, as described in detail previously [16]. Cells were then cultured in multi-well dishes (1.6 cm in diameter) at an inoculation density of 40,000 cells/cm^2. Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.1 mg/ml cephalotin and 100μU/ml insulin, 33 mM biotin, 200 pM triiodothyronine (T_3) and 10% charcoal treated fetal calf serum was used as culture medium. Charcoal-treated serum (steroid-free serum) was prepared as previously reported [7]. Media were changed every other day.

Androgen receptor binding assay

General protocol. Experiments were performed after 7 days culture, with confluent differentiated cells [7, 16]. A synthetic androgen methyltrienolone (R1881), was chosen as ligand, because of its high affinity to cytosolic androgen receptors in adipose precursor cells [7] and stability to metabolic conversion [23]. After 7 days culture, medium was removed with a suction pump and the monolayer washed 3 times with medium 199. Cells were then incubated (in duplicate) at 37°C in medium 199, in the presence of labeled and/or unlabeled R1881. 10 μM triamcinolone acetonide was added to all incubation tubes, since R1881 has some affinity for progesterone and glucocorticoid receptors [24], and is highly specific for androgen receptors when triamcinolone acetonide is included in the assay [25]. The non-specific binding was determined by dilution of the isotope in the presence of an excess (20 μM) of non-radioactive R1881. After 60 min incubation, the binding medium was removed and the monolayers were washed 5 times in order to remove all free steroids. The cells were then solubilized in 1.0 ml of 0.20 M NaOH. The solution was transferred into scintillation vials and 10 ml of toluene-based scintillation fluid were added. Radioactivity was counted in a LKB 1211 Rack-beta liquid scintillation counter (LKB Beckman, Stockholm, Sweden). Specific binding was calculated as the difference between total binding and non-specific binding, and expressed as femtomoles of steroid bound per milligram protein. Parallel wells of the same culture dish were used for determination of total cellular proteins, measured according to the method of Lowry *et al.* [26], following their solubilization with 0.2 NaOH from the monolayers.

Saturation curves and determination of the binding parameters. Binding sites were saturated in the presence of increasing concentrations (0.5–30 nM) of [^{3}H]R1881. The results were analyzed according to the method of Scatchard [27], permitting the calculation of affinity (K_d) and total number of sites (B_{max}) by linear regression.

Competition studies. Cells were incubated at 37°C for 1 h with 10 nM [^{3}H]R1881 in the absence or presence of different concentrations of radioinert competitors. In addition, all incubations contained radioinert 10μM triamcinolone acetonide.

Time-course for up-regulation of androgen receptor. Confluent monolayers were incubated for 24 h with 10 nM radioinert androgens: R1881 or testosterone or dihydrotestosterone (DHT). In dose–response curve experiments,

cells were incubated for 24 h with increasing concentrations (from 10 nM to 1 μM of testosterone. At the end of every incubation, wells were washed with medium 199 and androgen binding was assayed. In time-course experiments, monolayers were incubated for different time periods (1–48 h) with 10 nM testosterone. The further procedure was as described above.

Effect of cycloheximide on androgen receptor up-regulation. In three series of experiments, cells were exposed for 24 h to 10 nM testosterone, both in the presence or in the absence of 10 μg/ml cycloheximide. Androgen binding was then performed.

Chemicals and reagents. Collagenase (type 1), charcoal (Norit-A), triiodothyronine, biotin, testosterone, dihydrotestosterone, triamcinolone acetonide, estradiol, progesterone, dexamethasone, cycloheximide were all purchased from Sigma Chemical Co. 17-α[methyl-^{3}H]methyltrienolone (R1881; SA 81.8 Ci/mmol) an methyltrienolone were from New England Nuclear (Dreieich, West Germany). Insulin (Actrapid) was supplied by Novo Laboratories (Copenhagen, Denmark). Medium 199 was obtained from Statens Bakteriologiska Laboratorium (Stockolm, Sweden). Dulbecco's modified Eagle's medium was purchased from Gibco (Paisley, Scotland) and cephalotin from Eli Lilly (Fergersheim, France). Fetal calf serum was purchased from Flow Laboratories (England).

Statistical method. The significance of differences were calculated with analysis of variance by using the Scheffe T-test. Results are expressed as the mean $\pm$ SEM.

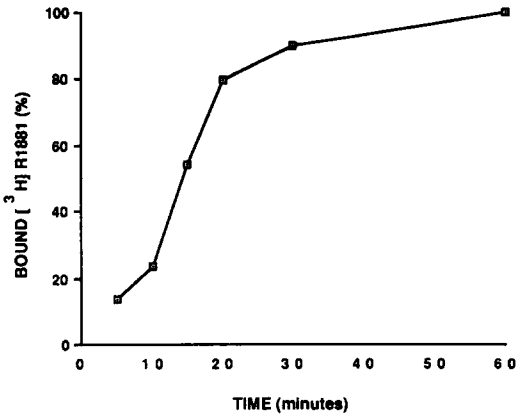

Fig. 1. Determination of time-dependence of [^{3}H]R1881 binding to differentiated adipose precursor cells. Cells were incubated at 37°C with 10 nM [^{3}H]R1881. After different times, incubation was stopped and binding analysed as described in Materials and Methods. All values represent the percent of specific binding at equilibrium in 1 out of 3 representative experiments.

RESULTS

Determination of equilibrium conditions: stability and reversibility of specific binding

Figure 1 shows an association experiment performed at 37°C. The plateau of specific binding was achieved rapidly, within 30 min and maintained at least 60 min. A monophasic kinetic curve was also observed in dissociation experiments, with total deplacement within 30 min (not shown).

Specificity of binding

Figure 2 shows the competition of various non-radioactive steroids for [^{3}H]R1881 binding. R1881 was replaced by other androgens (testosterone and dihydrotestosterone), but only

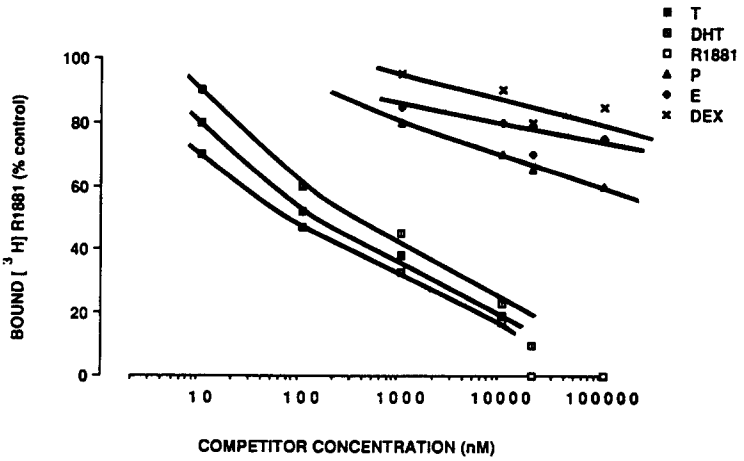

Fig. 2. Specificity of binding. Differentiated adipose precursor cells were incubated 60 min at 37°C in the presence of 10 nM [^{3}H]R1881 and increasing concentrations of non-radioactive steroids. Specific binding in the presence of these steroids is expressed relative to the specific binding of [^{3}H]R1881 alone. Means of 3 experiments are represented and the SEM of each point was not over 5%.

slightly affected by other types of steroids (progesterone, estradiol and dexamethasone). The order of affinity was: R1881 > testosterone > dihydrotestosterone > progesterone > estradiol > dexamethasone.

Saturation curves

Figure 3a represents a typical saturation curve, analyzed according to the method of Scatchard (Fig. 3b). The level of non-specific bound hormone progressively increased with the increase of radiolabeled concentration, and it ranged between 15 and 40%.

Effect of androgens on R1881 binding

Androgen receptor binding progressively increased during 1 day exposure to androgens, reaching the plateau at 24 h (Fig. 4). The up-regulation was induced by R1881 and testosterone, but not by dihydrotestosterone (Fig. 5). The receptor sensitivity to testosterone was dose-dependent (Fig. 6). Cycloheximide totally abolished the androgen-induced receptor up-regulation (Fig. 7).

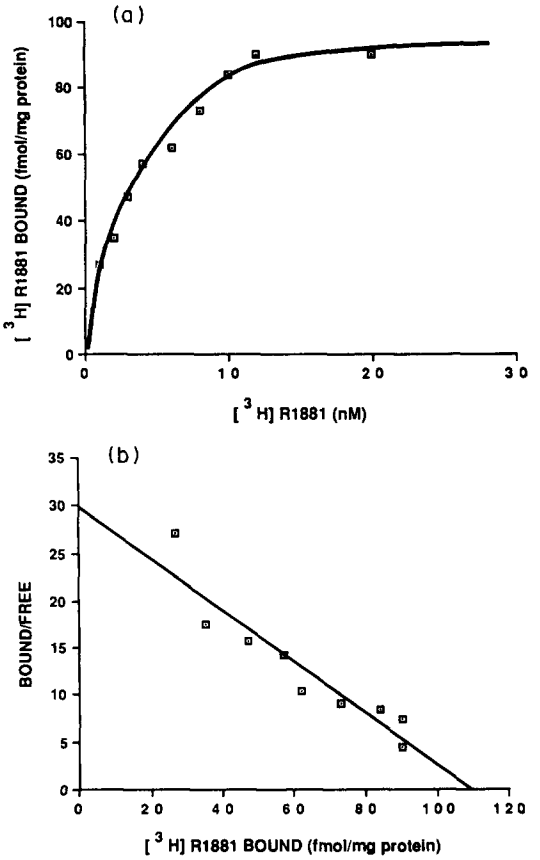

Fig. 3. Saturation (a) and Scatchard (b) plots of specific [^{3}H]R1881 binding to intact differentiated adipose precursor cells. One out of 3 representative experiments.

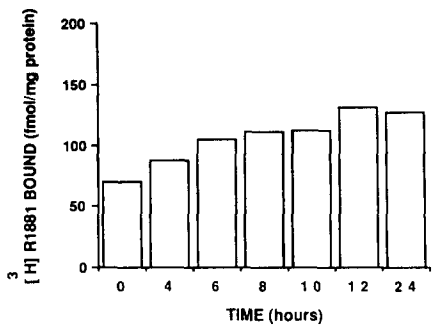

Fig. 4. Time-dependence of effect of testosterone on [^{3}H]R1881 to differentiated adipose precursor cells. Cells were incubated 60 min at 37°C in the presence of 10 nM [^{3}H]R1881 after incubations for the indicated time periods in the presence of 10 nM testosterone. One out of 3 representative experiments.

DISCUSSION

This report describes the application of a simple, reliable and reproducible whole cell androgen receptor assay in differentiated adipose precursor cells. The finding of a specific cellular androgen binding in adipose precursor cells from male rats is in agreement with our previous study, where androgen binding was described in cytosol [7]. Compared to the earlier study, the receptor capacity and affinity in intact cells were very similar to that found in cytosol, thus suggesting that during the cellular preparation most of the nuclear receptors might leave the nuclei and appear in the cytosol preparation, as previously suggested [28]. The K_d here found is in the range of physiological androgen concentration [7].

Radioinert androgens were very effective as inhibitors of radiolabeled androgen binding, in contrast to other steroids (progesterone,

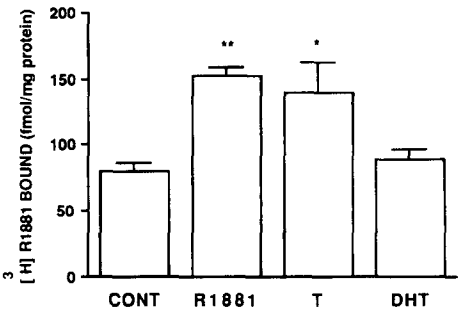

Fig. 5. Effects of androgens on [^{3}H]R1881 binding to differentiated adipose precursor cells. Cells were incubated for 60 min at 37°C in the presence of 10 nM [^{3}H]R1881 after exposure for 24 h to 10 nM testosterone, 10 nM R1881 or 10 nM dihydrotestosterone (DHT). The values are the means ± SEM of three experiments. $^*P < 0.05$ and $^{**}P < 0.01$ represent the significance of the statistical difference with control.

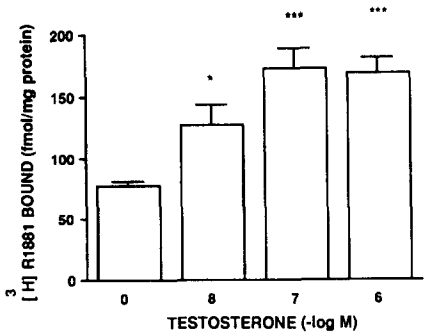

Fig. 6. Effects of different testosterone concentrations on [^{3}H]R1881 binding to differentiated adipose precursor cells. Cells were incubated 60 min at 37°C in the presence of 10 nM [^{3}H]R1881 after exposure for 24 h to the indicated concentrations of testosterone. The values represent the means ± SEM of five experiments. $*P < 0.05$ and $***P < 0.001$ represent the significance of the statistical difference with control.

estradiol and dexamethasone), demonstrating a high specificity of receptor.

Recent reports have shown that androgen target cells have the ability to autoregulate their androgen receptor concentrations by regulating both the stability of receptors and the induction of new receptor synthesis [13, 29–33]. In this study, testosterone and R1881, but not dihydrotestosterone produced a significant increase in receptor levels. This effect was already apparent after 4 h and reached a maximal effect within 24 h. Moreover, it required synthesis of proteins, suggesting that the effect was due to the formation of new receptors, rather than receptor availability for binding. Dihydrotestosterone also showed less capacity than testosterone in competing with specific binding of R1881. These results, combined with our previous results showing that an increase in isoproterenol-stimulated lipolysis and in β-adrenergic receptor number is induced by cell exposure to testosterone but not to dihydrotestosterone [7], strongly suggest that testosterone effects do not require conversion to dihydrotestosterone and are mediated by an increase in androgen receptor binding.

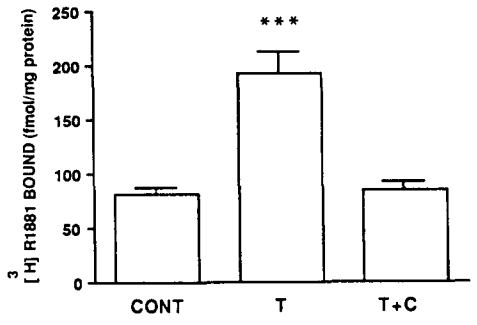

Fig. 7. Effects of testosterone (T) and cycloheximide (C) on [^{3}H]R1881 binding to differentiated adipose precursor cells. Cells were incubated 60 min at 37°C in the presence of 10 nM [^{3}H]R1881 after exposure for 24 h to 10 nM testosterone, in the presence or in the absence of 10 μg/ml cycloheximide. The values represent the means ± SEM of three experiments. $***P < 0.001$ represents the significance of the statistical difference with control.

In summary, the present study demonstrates high affinity, specific, saturable and reversible androgen binding in intact adipose cells. Moreover, it shows for the first time that the androgen receptor number in adipocytes is increased by the exposure to androgens. The quantitative binding assay described here seems to allow future investigations on the relationship between androgen binding and metabolism in adipocytes.

REFERENCES

1. Pecquery R., Leneveu M. C. and Giudicelli Y.: Influence of androgenic status on the alpha$_2$/β-adrenergic control of lipolysis in white fat cells, predominant alpha$_2$-antilipolytic response in testosterone treated castrated hamsters. *Endocrinology* **122** (1988) 2590–2595.
2. Pecquery R., Dieudonne M. N., Leneveu M. C. and Giudicelli Y.: Evidence that testosterone modulates *in vivo* the adenylate cyclase activity in fat cells. *Endocrinology* **126** (1990) 241–245.
3. Rebuffé-Scrive M., Mårin P., and Björntorp P.: Male adipose tissue. *Int. J. Obesity* **13** (Suppl. 1) (1989) 181.
4. Gray J. M., Nunez A. A., Siegel L. I. and Wade G. N.: Effects of testosterone on body weight and adipose tissue: role of aromatization. *Physiol. Behav.* **23,** (1979) 465–469.
5. Krotkiewski M., Kral J. G. and Karlsson J.: Effects of castration and testosterone substitution on body composition and muscle metabolism in rats. *Acta Physiol. Scand.* **109** (1980) 233–237.
6. De Pergola G., Holmäng A., Svedberg J., Giorgino R. and Björntorp P.: Testosterone treatment of ovariectomized rats: effects on lipolysis regulation in adipocytes. *Acta Endocr.* **123,** (1990) 61–66.
7. Xu. X., De Pergola G. and Björntorp P.: The effects of androgens on the regulation of lipolysis in adipose precursor cells. *Endocrinology* **126** (1990) 1229–1234.
8. Miller L. K., Kral J. G., Strain G. W. and Zumoff B.: Androgen binding to human adipose tissue. *6th Annual Meeting of North American Association for the Study of Obesity*, Bethesda, Md (1989) (Abstr).
9. Gadson P. F., Russel J. D. nd Russel S. B.: Glucocorticoid receptors in human fibroblasts derived from normal dermis and keloid tissue. *J. Biol. Chem.* **259** (1984) 11236–11241.
10. Steiner A. E. and Wittliff J. L.: A whole-cell assay for glucocorticoid binding sites in normal human lymphocytes. *Clin. Chem.* **31** (1985) 1855–1860.
11. Tilley W. D., Marcelli M. and McPhaul M. J.: Recent studies of the androgen receptor: new insights into old questions. *Molec. Cell. Endocr.* **68** (1990) C7–C10.
12. Turcotte G., Chapdelaine A., Roberts K. D. and Chevalier S.: Androgen binding as evidenced by a whole cell assay system using cultured canine prostatic epithelial cells. *J. Steroid Biochem.* **1** (1988) 69–76.
13. Evans B. A. J., Jones T. R. and Hughes I. A.: Studies of the androgen receptor in dispersed fibroblasts: investigation of patients with androgen insensitivity. *Clin. Endocr.* **20** (1984) 93–105.

14. Gad Y. Z., Berkovitz G. D., Migeon C. J. and Brown T. R.: Studies of up-regulation of androgen receptors in genital skin fibroblasts. *Molec. Cell. Endocr.* **57** (1988) 205–213.
15. Hainique B., Moustaid N., Quignard-Boulange A., Ardouin B. and Lavau M.: Glucocorticoid binding during the differentiation of 3T3-F442A fibroblasts into adipocytes. A possible regulatory effect of insulin. *Biochem. Biophys. Acta* **931** (1987) 347–353.
16. Björntorp P., Karlssoln M., Pertoft H., Petterson P., Sjostrom L. and Smith U.: Isolation and characterization of cells from rat adipose tissue developing into adipocytes. *J. Lipid Res.* **19** (1978) 316–324.
17. Björntorp P., Karlsson M., Petterson P. and Sypniewska G.: Differentiation and function of rat adipose precursor cells in primary culture. *J. Lipid Res.* **21** (1980) 714–723.
18. Xu X., Hoebeke J. and Björntorp P.: Progestin binds to the glucocorticoid receptor and mediates antiglucocorticoid effect in rat adipose precursor cells. *J. Steroid Biochem.* **36** (1990) 465–471.
19. Xu X. and Björntorp P.: Effects of sex steroid hormones on differentiation of adipose precursor cells in primary culture. *Exp. Cell Res.* **173** (1987) 311–321.
20. Sypniewska G., Xu. X., Hager A., Lindhal A. and Björntorp P.: Effects of age, obesity and growth hormone on adipogenic activity in human plasma. *Int. J. Obesity* **11** (1987) 263–273.
21. Roncari D. A. K.: Hormonal influences on the replication and maturation of adipocyte precursors. *Int. J. Obesity* **5** (1981) 547–552.
22. Wiederer O. and Loffler G.: Hormonal regulation of the differentiation of rat adipocyte precursor cells in primary culture. *J. Lipid Res.* **28** (1987) 649–658.
23. Bonne C. and Raynaud J. P.: Assay of androgen binding sites by exchange with methyltrienolone (R1881). *Steroids* **27** (1975) 497–507.
24. Doré G. C., Gilbert J., Ojasoo T. and Raynaud J. P.: Correspondence analysis applied to steroid receptor binding. *J. Med. Chem.* **29** (1986) 54–60.
25. Zava D. T., Landrum B., Horwitz K. B. and McGuire W. L.: Androgen receptor assay with [^{3}H]methyltrienolone (R1881) in the presence of progesterone receptors. *Endocrinology* **104** (1979) 1007–1012.
26. Lowry O. H., Rosenbrough N. J., Farr A. L. and Randal R. J.: Protein measurements with the pholin phenol reagent. *J. Biol. Chem.* **193** (1951) 265–275.
27. Scatchard G.: The attractions of protein for small molecules and ions. *Ann. N.Y. Acad. Sci.* **51,** 660–672.
28. Gorski J.: The nature and development of steroid hormone receptors. *Experientia* **42** (1986) 744–750.
29. Blondeau J. P., Corpechot C., Legoascone C., Baulieu E. E. and Robel P.: Androgen receptors in the rat ventral prostate and their hormonal control. *Vitamins Horm.* **33** (1975) 319–344.
30. Kaufman M., Pinsky L. and Feder-Hollander R.: Defective up-regulation of the androgen receptor in human androgen insensitivity. *Nature* **293** (1981) 735–737.
31. Pinsky L., Kaufman M., Gil-Esterban C. and Sumbulian D.: Regulation of the androgen receptor in human genital skin fibroblasts, with a review of sex steroid receptor regulation by homologous and heterologous steroids. *Can. J. Biochem. Cell Biol.* **61** (1982) 770–778.
32. Syms A. J., Norris J. S., Panko W. B. and Smith R. G.: Mechanisms of androgen-receptor augmentation. Analysis of receptor synthesis and degradation by the density-shift technique. *J. Biol. Chem.* **260** (1985) 455–461.
33. Evans B. A. J. and Hughes I. A.: Augmentation of androgen-receptor binding *in vitro*: studies in normals and patients with androgen insensitivity. *Clin. Endocr.* **23** (1985) 567–577.