Abstract. Cytosol and nuclear androgen receptor concentrations were measured in freshly prepared and cultured Leydig cells of immature pig testis with exchange assays using [3H]methyltrienolone as labelled ligand. Androgen receptors in Leydig cells had high affinity for [3H]methyltrienolone and steroid binding specificity typical of an androgen receptor. The mean receptor concentrations were 76 fmol/mg protein and 210 fmol/mg DNA for cytosol and nuclei, respectively. In sucrose gradients, cytosol androgen receptors sedimented in the 4 S region. The cells maintained androgen receptors under culture conditions. Exposure of cultured cells to [3H]methyltrienolone (10 nmol/l) resulted in accumulation of androgen receptors in the nuclei with maximal uptake by 1 h. We conclude that methyltrienolone binding sites with characteristics of androgen receptors were identified in both cytosol and nuclei of porcine Leydig cells.

Androgen receptors have been detected in the rat testis and also in human testicular tissue. Using various biochemical techniques, androgen receptors have been found in whole testis (Hansson et al. 1974; Mulder et al. 1975), seminiferous tubules (Hansson et al. 1974; Mulder et al. 1975; Sanborn & Steinberger 1975), germ cells (Sanborn et al. 1975), in interstitial tissue (Frederik et al. 1976; Verhoeven 1980), and in other cell types (Verhoeven 1980) of rat testis, but the exact location has remained unclear. The development of techniques for cell separation has provided a means to specify more clearly what cell types are targets for androgen action. In purified cell fractions of rat testis, androgen receptors have been detected in both Sertoli cells (Sanborn et al. 1981) and Leydig cells (Gulizia et al. 1983). Furthermore, in a recent study, androgen receptors were identified in several rat and mouse testicular cell lines (Nakhla et al. 1984).

We have used immature pig Leydig cells in primary culture as a model in studies of testicular steroidogenesis (Orava et al. 1985a,b). To further characterize this model we investigated the presence of cytosol and nuclear androgen receptors in freshly prepared and cultured porcine Leydig cells. The data reported here suggest that a binding protein with characteristics of an androgen receptor is located in these cells.

Materials and Methods

Reagents

[17-methyl-3H]-methyltrienolone (SA 86 Ci/mmol) and non-radioactive methyltrienolone were purchased from New England Nuclear Corp (Boston, MA) and [14C]-methylated bovine serum albumin from Amersham International (Amersham, UK). Non-radioactive steroids were from Steraloids Inc (Wilton, NH). Hexylene glycol was supplied by Fluka AG (Buchs, Switzerland), hydroxylapatite by Bio-Rad Laboratories (Richmond, VA) and collagenase/dispase by Boehringer (Mannheim,
FRG). RPMI 1640 medium, foetal calf serum and Fungizone® were from Gibco Europe Ltd (Paisley, Scotland). DME/F12-medium contained Ham’s F12 Nutrient Mixture and Dulbecco’s Modified Eagle’s Medium (1:1 v/v) (KC Biological Inc, Lenexa, KS) with the addition of 1.2 g/l sodium bicarbonate, 15 mmol/l Hepes, 20 mg/l gentamycin, and 2.5 mg/l Fungizone. Other chemicals, of the highest purity available, were from Sigma Chemical Co (St. Louis, MO) or Merck AG (Darmstadt, FRG).

**Preparation of Leydig cells**

Testes were from 3–4 week old piglets. Testicular pieces were enzymatically dispersed at 35°C for 1.5 h with gentle shaking in DME/F12-medium containing collagenase/dispase (0.3 g/l) and 0.05 g/l trypsin inhibitor. Interstitial cells were further purified on a continuous Percoll gradient (0–90%) in RPMI 1640 medium containing 15 mmol/l Hepes. About 1 x 10⁷ cells were layered on the top of a 35-ml gradient and after centrifugation at 800 x g for 20 min, Leydig cells were obtained in a band corresponding to densities between 1.055–1.072 as determined by histochemical 3β-hydroxysteroid dehydrogenase staining (Mendelson et al. 1975) and by hCG stimulated testosterone production in short-time (2 h) incubation of the cells from various density fractions. The purity of Leydig cells was 90–95%.

**Measurement of cytosol androgen receptor concentration**

Freshly-isolated or cultured Leydig cells were sedimented by centrifugation, washed once with homogenization buffer (50 mmol/l Tris-HCl, 0.1 mmol/l EGTA, 12 mmol/l monothioglycerol, 10 mmol/l Na₂MoO₄, and 10% (v/v) glycerol) and homogenized in a Dounce homogenizer (Contes Co Vineland, NJ) using a tight pestle. One ml of buffer was used for 10–20 x 10⁶ cells. The homogenate was centrifuged at 105 000 x g for 60 min at 2–5°C to yield a soluble fraction. Cytosol samples were prepared by dextran-charcoal pellets, which were prepared by centrifuging 2 ml dextran-charcoal suspension (2.5 g Norit A, 150 mg Dextran T-70, and 1 g gelatin per 1 of a buffer composed of 0.1 mol/l phosphate, 15 mmol/l NaN₃, and 0.15 mol/l NaCl, pH 7.4) at 1500 x g for 10 min and decanting the supernatants. The samples were incubated for 10 min with the charcoal, which was then removed by centrifugation, as described above. Aliquots of the cytosol were incubated with [³H]methyltrienolone (0.1–8 nmol/l) with and without a 500-fold molar excess of testosterone at 4°C for 18 h. Bound and free steroids were separated using hydroxylapatite, as described previously (Isomaa et al. 1985). In this paper, cytosol receptors refer to those found in the cytosol fraction following homogenization and ultracentrifugation of the tissue in buffer. Correspondingly, nuclear receptors refer to those found in the nuclear fraction. At present there seems to be no information available on whether the distribution of androgen receptors found in vitro accurately reflects that prevailing in vivo.

**Determination of nuclear androgen receptor concentration**

Nuclei were isolated by using a hexylene glycol technique and nuclear androgen receptors were extracted with a buffer containing pyridoxal 5'-phosphate, as previously described for androgen receptors in mouse kidney (Isomaa et al. 1982). Briefly, the cells were washed with homogenization buffer (1 mol/l hexylene glycol, 0.1 mmol/l MgCl₂, 2 mmol/l dithioretil, 5 mmol/l EGTA, and 1 mmol/l Pipes, pH 7.5) and homogenized using two 10-sec bursts of an Ultra Turrax homogenizer (Janke & Kunkel KG, Staufen, FRG). The homogenate was filtered through nylon net and centrifuged at 1500 x g for 10 min. The crude nuclei were suspended in 1.8 mol/l sucrose made in a buffer containing 50 mmol/l Tris-HCl, pH 7.5, 2.5 mmol/l KCl, 2 mmol/l MgCl₂, and 1 mmol/l dithioretil, and were centrifuged at 110 000 x g for 1 h in a swing-out rotor. Nuclear receptors were extracted with pyridoxal 5'-phosphate-containing buffer (20 mmol/l sodium barbital, 1.5 mmol/l EDTA, 0.15 mol/l KCl, 5 mmol/l dithioretil, 5 mmol/l pyridoxal 5'-phosphate, 20% (v/v) glycerol, pH 8.0). The nuclei were gently suspended in extraction buffer using a Dounce homogenizer, incubated for 30 min at 4°C and sedimented at 80 000 x g for 30 min. The supernatant was used for receptor assay and bound and free steroids were separated using hydroxylapatite.

**Competition studies**

The steroid specificity of the androgen receptors was studied separately for cytosol and nuclear extracts. One hundred-fold molar excesses of various non-radioactive steroids were added to the receptor assay reaction containing 10 nmol/l (cytosol) or 15 nmol/l (nuclei) [³H]methyltrienolone.

**Sucrose density gradient centrifugation of androgen receptors**

Cytosol was prepared from freshly isolated Leydig cells as described above. The only modification was that 0.5 mmol/l phenylmethylsulfonylfluoride (PMSF) was included in the homogenization buffer. Cytosol samples (200 µl) were layered onto 5–20% sucrose gradients made in cytosol buffer containing PMSF and centrifuged at 220 000 x g for 18 h at 0–2°C in a swing-out rotor. After centrifugation, fractions of about 200 µl were collected and were labelled with 10 nmol/l [³H]methyltrienolone with and without 5 µmol/l testosterone. After 3 h of incubation at 4°C, free steroids were removed with dextran-coated charcoal and the radioactivity in the supernatants was counted. Bovine serum albumin was used as a sedimentation marker.
Accumulation of androgen receptors to the nuclei

Leydig cells were cultured in DME/F12-medium supplemented with 5 mg/l porcine insulin, 5 mg/l human transferrin, 1 mg/l vitamin E, and 0.1% foetal calf serum. Before translocation experiments, cultures were pre-incubated in a humidified atmosphere of 95% air–5% CO₂ at 37°C for 3 days with a medium change every 24 h. The accumulation of nuclear receptors was examined by incubating Leydig cells with 10 nmol/l \(^{3}H\)methyltrienolone in the presence or absence of a 500-fold molar excess of testosterone and measuring the accumulation of radioactivity in the nuclei at various time intervals. After incubation, the cells were washed with medium several times and the nuclei were isolated as described above. Radioactivity was extracted from purified nuclei with ethanol and counted.

Other methods

Protein measurements were made with the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA) and DNA was measured by the method of Burton (1956).

Results

Binding of \(^{3}H\)methyltrienolone to cytosol and nuclear extracts of freshly-prepared Leydig cells is shown in Figs. 1 and 2. In both cases, a single class of binding sites was observed, with apparent \(K_d\) values of 0.7 nmol/l (cytosol) and 4 nmol/l (nuclei) for methyltrienolone. The mean concentration of cytosol receptors in separate cell preparations was 76 ± 24 fmoles/mg protein (mean ± SEM, N = 5), and that of nuclear receptors was 210 ± 59 fmoles/mg DNA (mean ± SEM, N = 3). If we...
Competition of various steroids with [3H]methyltrienolone binding to cytosol (AR<sub>C</sub>) and nuclear (AR<sub>N</sub>) androgen receptor from porcine Leydig cells. A 100-fold molar excess of competing steroids was used in duplicate incubations. Values are means from 3–5 separate experiments and are expressed in relation to testosterone. The inhibition of methyltrienolone binding by testosterone was set as 1.00.

<table>
<thead>
<tr>
<th>Steroid</th>
<th>AR&lt;sub&gt;C&lt;/sub&gt;</th>
<th>AR&lt;sub&gt;N&lt;/sub&gt;</th>
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</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>5α-Dihydrotestosterone</td>
<td>0.93</td>
<td>1.06</td>
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<tr>
<td>Methyltrienolone</td>
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<td>1.30</td>
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<tr>
<td>Progesterone</td>
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<td>ORG 2058</td>
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<td>NM</td>
</tr>
<tr>
<td>Oestradiol</td>
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<tr>
<td>Oestrone</td>
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<td>NM</td>
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<tr>
<td>Diethylstilboestrol</td>
<td>0.01</td>
<td>NM</td>
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<tr>
<td>Dexamethasone</td>
<td>0.00</td>
<td>NM</td>
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</tbody>
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NM: not measured.

We assume that each Leydig cell contains 5.1 pg of DNA and calculate cytosol receptor levels on the basis of DNA in tissue homogenate, the receptor concentrations are 1870 and 650 receptors per cell in the cytosol and nucleus, respectively.

We next studied the steroid binding specificity of Leydig cell androgen receptors. In these experiments, freshly-prepared Leydig cells were used. Owing to the limited amount of cells available, competition studies were performed using a single concentration of each competing steroid. Cytosol or nuclear extract was incubated with [3H]methyltrienolone alone and in the presence of a 100-fold molar excess of competing compounds (Table 1). As expected, methyltrienolone, 5α-dihydrotestosterone, and testosterone were the most potent competitors of [3H]methyltrienolone binding. Oestradiol, oestrone, and progesterone competed to some extent, whereas synthetic nonsteroidal oestrogen (DES) and a synthetic progestin (ORG 2058) had little effect on the binding of methyltrienolone to the androgen receptor. In addition, dexamethasone was completely inactive. To further characterize the macromolecular nature of the androgen receptor, sucrose gradient centrifugation was used. Since [3H]methyltrienolone seemed to partially dissociate during the long centrifugation time, the receptor was labelled after centrifugation. In sucrose gradients, the cytosolic androgen receptor sedimented in the 4 S region (Fig. 3).

We furthermore studied the maintenance of androgen receptors under cell culture conditions. After three days in culture, with a medium change every day, the concentration of cytosol receptors had decreased to 40 fmol/mg protein, and the nuclear receptor concentration had decreased to about 50 fmol/mg DNA, resulting in a reduction of the total cellular receptor concentration to about half of that in freshly-isolated cells. Using cultured cells, the translocation of cytosol androgen receptors was studied by incubating cells with 10 nmol/l [3H]methyltrienolone in the absence or presence of non-radioactive testosterone and measuring the accumulation of radioactivity in the nuclei at various time intervals. The results shown in Fig. 4 indicate that maximal accumulation of nuclear androgen receptors occurred by 1 h and it had subsided by 18 h. The presence of a
Translocation of androgen receptors to the nuclei of cultured porcine Leydig cells. Cells were incubated with [3H]methyltrienolone (10 nmol/l) alone (●) or in combination with 5 µmol/l testosterone (▲). After the indicated times, nuclei were isolated with the hexylene glycol method as described in Materials and Methods. Nuclear-bound radioactivity was extracted with ethanol and the pellet was used for DNA determination.

500-fold excess of non-radioactive testosterone in the medium completely prevented the accumulation of radioactivity in Leydig cell nuclei. About 60% of the maximal radioactivity could be extracted with pyridoxal 5’-phosphate and about 60% of that was macromolecule-bound as measured by the hydroxyl apatite assay. The concentration of nuclear receptors calculated from the amount of radioactivity accumulated in the nuclei, therefore, is an over-estimation of nuclear binding sites.

Discussion

The possible existence of androgen receptors in porcine Leydig cells was investigated. Previous studies with rats have shown that the interstitial cell fraction contains androgen receptors and recent studies using Leydig cells purified on a Percoll gradient (Gulizia et al. 1983) and an established Leydig cell line (Nakhla et al. 1984) have demonstrated that androgen receptor are present in this cell type. Our results with purified porcine Leydig cells are in line with these findings. The purity of our cell preparation was high (90–95%), which makes it unlikely that the receptors we measured were mostly located in a small fraction of other contaminating cells.

The cytosol and nuclear binding proteins we identified had several characteristics typical of an androgen receptor. They had a high affinity for methyltrienolone, they were specific for androgens and were translocated from the cytosol to the Leydig cell nucleus in vitro. Methyltrienolone was used as a ligand to measure androgen receptors, since it does not bind to extracellular androgen-binding proteins and is not metabolized during receptor assay (Bonne & Raynaud 1975). It does, however, bind to the progestin receptor and the glucocorticoid receptor. The fact that these did not interfere is supported by the results of the competition studies. Since neither ORG 2058, a synthetic steroid with high specificity for the progestin receptor, nor dexamethasone competed for [3H]methyltrienolone binding in Leydig cells, our assay apparently is specific for androgen receptors. Competition by progesterone probably does not represent inhibition of methyltrienolone binding to the progestin receptor. Similar data have been obtained in other studies (Wagle et al. 1983), which suggests that the testicular androgen receptor binds progesterone with an affinity which is clearly lower than that of active androgens. In addition, under our incubation conditions 20–25% of the progesterone was metabolized to other steroids which we have not identified, but which may contain androgenic steroids.

There are discrepancies in the literature concerning the binding of oestrogens to the androgen receptor. Several investigators have shown that physiological oestrogens bind to some extent to the androgen receptor from the testis (Sanborn et al. 1975; Brown et al. 1975; Monet-Kuntz & Terqui 1983), but not to that from the prostate, epididymis or seminal vesicle (Wilson & French 1976). Our data, showing some affinity of testicular androgen receptors for oestrone and oestradiol but not for DES, are in harmony with those findings.

The function of androgen receptors in Leydig cells is unknown. Histochemical studies in animals with testicular feminization have suggested that androgen receptors may be required for complete maturation of this cell type (Bardin et al. 1973). On the other hand, recent studies with rats have suggested that androgens may regulate testosterone production in Leydig cells via an ultra-short
loop feedback mechanism (Adashi & Hsueh 1981; Darney & Ewing 1981). Additional studies are needed to clarify this mechanism in porcine Leydig cells.

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References


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