Testicular Steroid Hormones

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Testicular Steroid Hormones

Castration of Male Rats (Orchiectomy)

Procedure

Castration of young male rats is performed with minimal bleeding in animals weighing less than 60 g. The animal is anesthetized. A small transversal incision is made in the skin on the ventral site over the symphysis. The testis lying in the scrotum is gently pushed into the abdominal cavity. With a pair of fine forceps, the abdominal wall is opened. The epididymal fat pad, easily seen, is grasped with the forceps, and the testis with the epididymis is pulled out from the wound. The ductus deferens with the testicular vessels is crushed with artery forceps and the testis together with the epididymal fad pad cut off with a pair of fine scissors. There is almost no bleeding in young animals. In older animals, ligation of the testicular vessels together with the ductus deferens may be necessary. The same procedure is performed on the other side. The skin wound is closed with one or two wound clips. The animal recovers immediately. With some skill, the operation can be performed very rapidly (Bomskov 1939).

Modifications of the Methods

Dorfman (1969) recommended removing the testes through an incision in the tip of the scrotum. In our hands, the procedure described above was preferable.

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Dorfman RI (1969) Androgens and anabolic agents. In: Dorfman RI (ed) Methods in hormone research, vol IIA. Academic, New York, pp 151–220

Caponizing of Cockerels (Orchiectomy)

Procedure

This is a classical bioassay for androgens. White Leghorn cockerels are used for surgery at approximately 6 weeks of age. The animals, fasted 24 h prior to surgery, were anesthetized with ether and placed on their sides. An incision is made between the last two ribs, the muscle layer is divided, and the incision is pulled apart with small retractors. The testis is found close to the midline of the posterior abdominal wall, alongside the vena cava. The capsule enclosing the testis is cut and the gonad is removed. It is imperative to remove the testis intact, as fragments left behind are usually vascularized and persist, giving rise to incompletely caponized animals. The incision is closed by a suture. The contralateral testis is removed in a similar fashion (Bomskov 1939).

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Androgenic and Anabolic Activity

In Vitro Methods

Androgen Receptor Binding

Purpose and Rationale

Rat ventral prostate (Bonne and Raynaud 1974; Liao et al. 1974; Grover and Odell 1975; Ojasoo and Raynaud 1978; Raynaud et al. 1979; Winneker et al. 1989; Duc et al. 1995) and mouse kidney (Isomaa et al. 1982) serve as sources for androgen receptors. Human androgen receptors have been prepared from transfected COS-1 cells (Teutsch et al. 1994). Labeled androstanolone, 5α-dihydrotestosterone, testosterone, and, more recently, methyltrienolone (R 1881) have been used as radioligands.

Procedure

Androgen Receptor Assay

Cytosol is prepared from ventral prostate glands of adult male rats castrated approximately 24 h before use. The tissue is homogenized in TMDG buffer (10 mM Tris, 20 mM sodium molybdate, 2 mM dithiothreitol, 10 % glycerol, pH = 7.4) at room temperature using a motor-driven glass homogenizer and centrifuged at 135,000 g for 1 h. Aliquots of the supernatant (cytosol) are diluted to contain 40 mg tissue/ml and incubated for 1 h or overnight with [17α-methyl-^{3}H]R 1881 (methyltrienolone, 5 nM final concentration, 87 Ci/mmol, New England Nuclear) in either the absence or presence of increasing concentrations (1 nM to 10 μM) of R 1881 or test compounds. Because R 1881 binds weakly to progesterone and glucocorticoid receptors, cytosols are pretreated with 1 μM triamcinolone acetonide to block these interactions. After a 1- or 18-h incubation period, a suspension of dextran-coated charcoal (1 % charcoal, 0.05 % dextran T-70, 0.05 % BSA) is added to the ligand–cytosol mixture and incubated for 5 min. The charcoal is removed by centrifugation at 1500 g for 10 min and the

supernatant (protein-bound [^{3}H]R 1881) counted using 10 ml of scintillation fluid (New England Nuclear) in a liquid scintillation spectrometer.

Nuclear Androgen Receptor Exchange Assay

Ventral prostates are homogenized at 100 mg/ml in hexylene glycol buffer (1 M hexylene glycol, 1 mM $MgCl_2$, 2.0 mM dithiothreitol, 5.0 mM EGTA, 1.0 mM PIPES, pH = 7.4) using a motor-driven ground glass homogenizer. Homogenates are centrifuged at 1500 g for 10 min. The nuclear pellet is washed three times in homogenization buffer by gently resuspending the pellet in a Dounce homogenizer and centrifugation at 1500 g for 10 min. The washed nuclear pellet is resuspended in pyridoxal-5′-phosphate extraction buffer (20 mM sodium barbital, 5 mM pyridoxal-5′-phosphate, 5.0 mM dithiothreitol,1.5 mM EDTA, 150 mM KCl, 20 % glycerol, pH = 7.4) for 60 min at a final concentration of 60 mg tissue/ml. The extracted nuclei are centrifuged at 25,000 g for 30 min with the resulting supernatant being used in the same single saturating dose assay as described for prostate cytosol.

Evaluation

Binding of test substances to the androgen receptor (receptor affinity) is quantified by calculating the relative binding affinity (ratio of the molar concentration of unlabeled R 1881 to test substance required to inhibit the binding of [^{3}H]R 1881 by 50 % after correction for nonspecific binding) and equilibrium inhibitory binding constant ($K_i = IC_{50}/(1 + C)/K_d$, where C = the concentration of [^{3}H]R 1881 and the K_d for R 1881 is 1.3 nM).

In the nuclear androgen receptor exchange assay, treatment group means are compared to control means, using ANOVA and Dunnett's multiple comparison tests.

Modifications of the Method

Cell assays and animal assays are described for evaluation of androgens and antiandrogens (Raynaud et al. 1975; Liang et al. 1977; Sivelle et al. 1982; Liao et al. 1984; Traish et al. 1986; Stobaugh and Blickenstaff 1990; Christiansen et al. 1990; Humm and Schneider 1990; Neubauer et al. 1993).

Brown et al. (1981) studied antiandrogen effects on androgen receptor binding in cultured human newborn foreskin fibroblasts.

Tezón et al. (1982) studied the intracellular distribution of the androgen receptor in the rat epididymis under the influence of androgens and antiandrogens.

The use of tritiated 7α,17α-dimethyl-19-nortestosterone for the assay of androgen receptors was recommended by Schilling and Liao (1984).

Characterization and expression of a cDNA encoding the human androgen receptor was described by Tilley et al. (1989).

Hoyte et al. (1993) recommended 7α-methyl-17α-(E-2′-[^{125}I]iodovinyl)-19-nortestosterone as radioligand for the detection of the androgen receptor.

Structure–affinity relationships of various steroids structurally related to nomegestrol and progesterone for [^{3}H]testosterone binding to rat ventral prostate cytosol were reported by Botella et al. (1987).

Molecular cloning of human androgen receptor complementary cDNA has been reported by Chang et al. (1988) and Lubahn et al. (1988).

DNA binding of androgen receptor overexpressed in COS-1 cells has been reported by Von Krempelhuber et al. (1994).

Thoth et al. (1995) studied in vitro binding of 16-methylated C-18 and C-19 steroid derivates to the androgen receptor using cytosol of castrated rat prostate and [^{3}H]R 1881 as radioligand.

Chang et al. (1995) reviewed the structure and function of the androgen receptor and its role in the function of the mammalian system.

The interaction of androgen receptors with the androgen-response element in intact cells was investigated by Karvonen et al. (1997).

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Transactivation Assay for Androgens

Purpose and Rationale

Steroid agonistic and antagonistic properties can be evaluated by transactivation assays (Muhn et al. 1995; Fuhrmann et al. 1995, 1996). The transactivation assay assumes that steroid receptor proteins act as ligand-regulated transcriptional activators. After binding of hormone, the steroid receptor interacts with hormone-responsive elements of hormone-regulated genes, inducing a cascade of transcriptional events (Green and Chambon 1988). The hormone-dependent transcriptional activation can be determined in tissue culture by transfection of the steroid receptor under investigation and a reporter gene linked to a hormonally responsive promoter into cells. The transactivation assay allows determination of the agonistic and also the antagonistic potency of a given compound, by either induction or inhibition of reporter gene activity (Fuhrmann et al. 1992).

Procedure

Vector Construct

CV-1 cells are stably transfected with the rat androgen receptor and pMMTV-CAT7. The pMMTV-CAT plasmid containing the mouse mammary tumor virus promoter linked to a chloramphenicol acetyltransferase gene is prepared according to Cato et al. (1986).

Cell Culture and Transfections

The culture medium of CV-1 cells stably transfected with the rat androgen receptor and pMMTV-CAT7 is supplemented with 400 μg/ml G418 (Gibco BRL) and 5 μg/ml puromycin.

Stable and transient transfections are performed using Lipofectin Reagent (Gibco BRL) according to a procedure recommended by Felgner and Holm (1989). Stable transfections are carried out according to Fuhrmann et al. (1992). For transient transfection, 1×10^6 COS-1 or CV-1 cells are plated onto 100-mm dishes 1 day prior to transfection. Cells are typically about 80 % confluent after 24 h. Before transfection, cells are washed twice with 1 ml Opti-MEM (Gibco BRL) per dish. For each dish, 5 μg hAR expression plasmid and 5 μg pMMTV-CAT are diluted with 1 ml Opti-MEM; in addition, 50 μg Lipofectin Reagent is diluted with 1 ml Opti-MEM. Next, DNA and Lipofectin Reagent dilutions are combined in a polystyrene snap-cup tube to obtain 2 ml of transfection solution per dish,

gently mixed, incubated at room temperature for 5 min, and added to the washed cells. After 5 h the transfection solution is replaced by 6 ml DMEM supplemented with 10 % fetal calf serum.

To study the effect of the test hormones, transiently transfected cells are trypsinized, pooled, and replated onto 60-mm dishes at a density of 4.5×10^5 per dish 24 h after transfection. Stably transfected cells are seeded onto 6-well dishes (1×10^5 cells per dish). Cells are cultured in medium supplemented with 3 % charcoal-stripped fetal calf serum and the appropriate hormones for 48 h. Cells are cultured with 1 % ethanol as a negative control for the reporter gene induction. Transactivation assays with transiently or stably transfected cells are carried out at least three times.

CAT Assay

Transiently transfected cells and stably transfected cells are disrupted by freezing (ethanol/dry ice bath) and thawing (37 °C water bath) three times. Protein concentrations of the cell extract are determined according to Bradford (1976). The CAT assay is performed according to Gorman et al. (1982).

Evaluation

CAT activity is calculated as percentage conversion from chloramphenicol to acetylated chloramphenicol. Concentration–response curves for CAT induction are established to demonstrate the potency of the test hormone. The synthetic androgen R 1881 (10^{-10} to 10^{-6} mol/l) is used as the standard.

For antiandrogenic activity, CAT activity in the presence of 0.5 nmol/l R 1881 is set as 100 %, and relative CAT activity is calculated as a percentage of this value. Concentration–response curves for CAT inhibition are established with increasing concentrations of the antihormone.

Critical Assessment of the Method

See chapter "▶ Anterior Pituitary Hormones"

Modifications of the Method

Warriar et al. (1993) examined the ability of dihydrotestosterone (DHT) and various antiandrogens to stimulate or to inhibit the transcription activation of mouse mammary tumor virus-bacterial chloramphenicol acetyltransferase (MMTV-CAT) in CV-1 cells.

White et al. (1994) described a simple and sensitive high-throughput assay which can be adapted for several classes of steroid agonists and antagonists. A DNA cassette, containing a synthetic steroid-inducible promoter controlling the expression of a bacterial chloramphenicol acetyltransferase gene (GRE5-CAT), was inserted into an Epstein–Barr virus episomal vector which replicates autonomously in primate and human cells. This promoter/reporter system was used to generate two stably transfected human cell lines. In the cervical carcinoma cell line HeLa, which expresses a high level of glucocorticoid receptor, the GRE5 promoter is inducible over 100-fold by the synthetic glucocorticoid dexamethasone. In the breast carcinoma cell line T47D, which expresses progesterone and androgen receptors, the GRE5 promoter is inducible over 100-fold by either progesterone or dihydrotestosterone (DHT). These cell lines can be used to screen large numbers of natural and synthetic steroid agonists and antagonists in microtiter wells directly using a colorimetric chloramphenicol acetyltransferase (CAT) assay.

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In Vivo Methods

Chicken Comb Method for Androgen Activity

Purpose and Rationale

This is a historical bioassay based on the response of the comb of castrate cockerels (capons). The method has been used by many authors to assay androgenic activity and has been found to be extremely useful for the isolation and structural elucidation of natural androgens. Many modifications have been published (reviewed by Dorfman 1969).

Procedure

Prior to assay, the surface area (sum of the length plus height of each individual comb) is determined by a millimeter rule placed directly on the comb. The capons are injected q.d. intramuscularly for 5 days consecutively with a solution or suspension of the test compound, and reference animals are treated with the androgen standard in 1 ml olive oil. Then 24 h after the last injection, the combs are measured again, and the growth of the comb is expressed as the sum of length and height in millimeters. Groups of eight animals are used for at least two doses of the test compound and the standard. In this method, animals can be used repeatedly.

Evaluation

The experimental parameter is the surface area of the comb. The mean values of each group are calculated, dose–response curve for the test compound and the standard is plotted, and potency ratios are calculated where possible.

Similar studies were reported by Gallagher and Koch (1935), Greenwood et al. (1935), and Oesting and Webster (1938).

Modifications of the Method

The hormones, dissolved in oil, have been applied locally to the capon's comb instead of by injection. A greater sensitivity has been achieved with this modification (Fussgänger 1934; McCullagh and Cuyler 1939).

Newly hatched chicks of either sex have been used to study the growth of combs after systemic or local administration (Frank et al. 1942; Dorfman 1948). White Leghorn chicks are used at an age of 2–3 days. They are kept in a brooder with thermostatic control. An oily solution (0.05 ml) of the test compound or the standard is applied to the comb daily for a period of 7 days. Then 24 h after the last application, the animals are autopsied. Body weights are determined. The combs are removed by two longitudinal incisions along the base of the comb at its juncture with the scalp. The comb is freed from the scalp, touched lightly on a towel to remove blood, and weighed. Dose–response curves are established.

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Weight of Androgen-Dependent Organs in Rats

Purpose and Rationale

Androgens affect the development of secondary sex organs in the male rat (ventral prostate, seminal vesicles). The growth of the ventral prostate, the seminal vesicles, and the musculus levator ani is dependent on the presence of male sexual hormones. Weight development of the musculus levator ani was considered to indicate anabolic activity, and weight development of the ventral prostate and seminal vesicles was considered to indicate androgenic activity (Dorfman 1969).

Procedure

Immature male Sprague–Dawley rats weighing about 55 g are orchiectomized. Animal body weight is recorded at the beginning and at the end of the experiment.

The animals are treated with test compounds by gavage (orally) in 0.5 ml 0.5 % carboxymethylcellulose or by subcutaneous injection in 0.2 ml sesame oil suspension once per day over a period of 10 days. Testosterone is given in doses of 0.02, 0.1, and 0.5 mg per animal subcutaneously and methyltestosterone orally in doses of 0.25, 1.5, and 5 mg per animal. Controls receive the respective vehicle. Ten animals are used for each group. On day 10 at autopsy, the seminal vesicles, ventral prostate, and levator ani muscle are dissected out and weighed. The seminal vesicles are squeezed and dried to remove the fluid.

Dissection of the levator ani muscle is performed after removal of the skin in the scrotal area between the base of the penis and the anus. The posterior aspect of the perineal complex is cleared of fat and connective tissue with forceps, particular care being taken to expose the constrictions at either end of the levator ani where it joins the bulbocavernosus muscle. The rectum is transected just caudad to the point where the musculus levator ani loops around it dorsally. The body of the levator ani is then freed of the rectum and is removed by incisions at the points of attachment to the bulbocavernosus muscle. The levator ani is cleared of any connective tissue and weighed to the nearest 0.1 mg.

Evaluation

The ratio of organ weight to body weight is calculated for every animal and each organ (relative organ weights). Dose–response curves are constructed for each organ comparing the test compound with the standard in order to calculate potency ratios. An increase in the weight of seminal vesicles and ventral prostate indicates androgenic activity, whereas an increase in the weight of musculus levator ani is considered to indicate anabolic activity.

In evaluating steroids for possible use as anabolic agents, Hershberger et al. (1953) suggested the use of the levator ani to ventral prostate ratio, which is defined as the ratio of the increase in levator ani weight divided by the increase in ventral prostate weight.

The method has been used by several authors (Korenchevsky and Dennison 1935; Eisenberg and Gordon 1950; Junkmann 1957; Kincl 1965; Eisler 1964; Dorfman 1969; Kuhnz and Beier 1994).

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Nitrogen Retention

Purpose and Rationale

Anabolic agents induce positive nitrogen balance in the living organism (Polish 1964; Dorfman 1969). Many modifications of this assay principle have evolved. Stafford et al. (1954) suggested a method involving the measurement of nitrogen excretion in the castrated rat fed a liquid diet and in nitrogen balance.

Procedure

Twenty-five-day-old rats are castrated and kept untreated for 67 days, reaching about 300 g in body weight on normal laboratory diet. After 67 days, they are changed to a liquid diet, forced-feeding regime. The diet contains carbohydrates and fat, as well as casein and brewer's yeast as a nitrogen source. At the start, the rats receive 10 ml of feed per day, and this is increased to 26 ml per day. Feeding is continued for 30 days with simultaneous administration of the test drug once a day. Twenty-four-hour urine specimens are collected three times weekly and analyzed for total nitrogen.

Evaluation

Indices are calculated, such as greatest daily retention, which is defined as the difference between the lowest daily nitrogen value after the beginning of treatment and the preinjection mean; the total nitrogen retention, which is the sum of the differences between the preinjection excretion and the daily values during the retention period; and the number of days in the retention period.

Modifications of the Method

A method for the assay of anabolic steroids in the monkey (*Macaca mulatta*) has been suggested by Stucki et al. (1960). Nitrogen retention expressed as total nitrogen retained per day during the treatment period is chosen as the end point.

Critical Assessment of the Method

The assay is time consuming and labor intensive, and its use is no longer recommended.

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Antiandrogenic Activity

General Considerations

Antiandrogens may exhibit their activity both peripherally on androgen-dependent tissues and by feedback action at a central site (Neumann et al. 1970, 1977; Mainwaring 1977; Neri 1977; Raynaud et al. 1977; Neumann 1985; Moguilewski and Bouton 1988). They compete with the peripheral androgen receptors and thus inhibit the effect of endogenous or exogenous androgens. Centrally, they inhibit gonadotropin secretion and thereby diminish testosterone production by the gonads. In addition to their effects on reproduction and accessory sexual organs, antiandrogens inhibit sebum production (anti-acne drugs) and delay androgen-dependent hair loss (alopecia). The methods to detect and quantify gonadotropin inhibition are described in chapter "▶ Vitiligo Models".

Inhibition of 5α-reductase, an enzyme located in tissues such as the prostate, is one pharmacological approach to inhibit benign prostate hyperplasia in men. Such inhibitors reduced the conversion from testosterone to 5α-dihydrotestosterone (DHT).

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In Vitro Methods

Inhibition of 5α-Reductase

Purpose and Rationale

Testosterone is converted to 5α-dihydrotestosterone (DHT) by the enzyme 5α-reductase which is specifically localized in some androgen-responsive target tissues (e.g., prostate, seminal vesicle, epididymis, skin, and sebaceous glands), whereas in other androgen-sensitive tissues, such as the skeletal muscles and the central structures, the androgenic hormone is testosterone. Inhibition of 5α-reductase provides a selective approach to androgen deprivation in DHT-target tissues, such as the prostate. The 5α-reductase inhibitors are applied in the therapy of benign prostate hyperplasia.

Procedure

5α-Reductase preparations are obtainable from prostates of various species, such as human, dog, and rat.

Frozen human prostates from benign prostatic hyperplasia patients are thawed and minced with a pair of scissors. The minced tissue is homogenized in three tissue volumes of medium A (20 mM potassium phosphate, pH 6.5, containing 0.32 M sucrose, 1 mM dithiothreitol, and 50 μM

NADPH) with a Brinkmann Polytron and a glass–glass homogenizer. The homogenate is centrifuged at 140,000 g for 60 min, and the pellets are washed with approximately three tissue volumes of medium A. The washed pellets are suspended at a concentration of 5–10 mg protein/ml in 20 mM potassium phosphate, pH 6.5, containing 20 % glycerol and 1 mM dithiothreitol.

Dog prostatic particulates are prepared from either fresh or frozen specimens of male mature dogs as described for human prostate. The washed pellets are suspended in medium A at a concentration of 30–60 mg protein/ml.

Ventral prostates from male Sprague–Dawley rats weighing 400 g are processed as described for human prostate, except that medium A without NADPH is used throughout the procedures. NADPH prevents inactivation of human and dog 5α-reductases during the preparation; the rat enzyme however is stable without the coenzyme.

For the 5α-reductase assay, reaction solutions are prepared in duplicate tubes containing 1 μM [^{14}C]testosterone, 1 mM dithiothreitol, 40 mM buffer (potassium phosphate, pH 6.5, for the rat and for the dog enzymes; Tris–citrate, pH 5.0, for the human enzyme), prostatic particulate (1 mg protein), and NADPH (50 μM for reaction with rat enzyme, 500 μM for reaction with human and dog enzyme) in a final volume of 0.5 ml. Test compounds or standard as inhibitors is added in 5 μl ethanol at concentrations between 10^{-9} and 10^{-5} M. The control tubes receive the same volume of ethanol. The reactions for the rat and dog enzymes are started by the addition of the prostatic particulates. The human prostatic particulate is premixed with NADPH before starting the reaction. The reactions are linear for at least 1 h at 37 °C. The reactions are carried out for 10–30 min and are stopped with 2 ml ethyl acetate containing testosterone, 5α-dihydrotestosterone, and androstenedione (10 μg each). After centrifugation at 1000 g for 5 min, the ethyl acetate phase is transferred to a tube and evaporated under nitrogen to dryness. The steroids are taken up in 50 μl ethyl acetate. The solutions are applied to Whatman LK5DF silica plates, and the plates are developed in either ethyl acetate to cyclohexane (1:1) at 25 °C or chloroform to methanol (96:4) at 4 °C. The plates are air-dried and the chromatography is repeated. Nonradioactive steroid standards are located by UV and by spraying with 1 % $CeSO_4$/10 % H_2SO_4 solution followed by heating. The radioactivity profiles are determined by scanning the plates or by scraping the silica in sections and counting in a scintillation counter. 5α-Dihydrotestosterone is the only radioactive product for the rat and human enzymes. With the dog enzyme 5α-dihydrotestosterone, 3α,17β-androstanediol, androstan-3,17-dione, and androstenedione are formed. The radioactivities of the first three products are combined for the calculation of the 5α-reductase activity.

Evaluation

IC_{50} values are calculated based on at least five dilutions of test preparations or standard.

Modifications of the Method

The method has been used by several authors (Bruchovsky and Wilson 1968; Corvol et al. 1975; Brooks et al. 1981; Liang et al. 1985; Rhodes et al. 1993; di Salle et al. 1993, 1994; Sudduth and Koronkowski 1993).

Using human genital skin fibroblasts and simian COS cells, specific inhibition of 5α-reductase type 1 has been observed (Hirsch et al. 1993).

Wennbo et al. (1997) reported that **transgenic mice** overexpressing the prolactin gene develop dramatic enlargement of the prostate gland.

Sigimura et al. (1994) described age-related changes of the prostate gland in the senescence-accelerated mouse and recommended this strain as a model of age-related changes in the prostate gland.

Neubauer et al. (1993) measured prostatic 5α-reductase in rats both in vitro and *ex vivo* and determined in vivo uptake of [^{3}H]testosterone by the prostate.

At least two isoforms of 5α-reductase have been isolated (Andersson and Russell 1990; Jenkins et al. 1992). Recombinant human prostatic 5α-reductase types I and II were expressed using the baculovirus-directed insect cell expression system (Iehlè et al. 1993).

Iehlè et al. (1995) and di Salle et al. (1998) tested synthetic 5α-reductase inhibitors against both isoforms.

Tolman et al. (1995) identified a 4-azasteroid as a scalp isoenzyme selective inhibitor.

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In Vivo Methods

Chick Comb Method

Purpose and Rationale

This method is now obsolete for the evaluation of antiandrogens. Several modifications of the chick comb method have been described for antiandrogens applied either systemically or locally to the comb of intact cockerels (Dorfman and Dorfman 1960; Dorfman 1969).

Procedure

Male or female, 1–3-day-old White Leghorn chicks are housed at constant temperature in a heated incubator. Testosterone is incorporated into the finely ground chick starting mash at a concentration of 80 mg/kg of food to stimulate comb growth. The chicks are placed on this diet on day 1. The antiandrogen to be tested is dissolved in sesame oil and injected for several days. At 24 h after the last injection, the animals are sacrificed, the combs removed and blotted to remove blood, and weighed rapidly to the nearest 0.5 mg. Body weights are also determined.

Evaluation

The results may be expressed as absolute comb weights or as milligram of comb per gram of body weight. Dose–response curves for groups treated with increasing doses of antiandrogen are plotted.

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Antagonism of Androgen Action in Castrated Rats

Purpose and Rationale

In this modification, antiandrogens are administered to reduce or inhibit testosterone actions on androgen-dependent organs.

Procedure

Male Sprague–Dawley rats weighing 50–70 g are orchiectomized. Starting 1 day after castration, the rats are injected once daily for 7 days with 0.15 mg testosterone propionate in 0.1 ml sesame oil (agonist action). The test compounds (antiandrogens) are dissolved in sesame oil or suspended and injected subcutaneously daily at a separate site for the same test period of 7 days. Six to ten animals are used per group. At autopsy on day 8, weights of ventral prostate, seminal vesicles, and musculus levator ani as well as body weight are recorded.

Evaluation

The organ weight to body weight ratio is calculated for each order, preferably based on relative organ weight to 100 g of body weight. The inhibition by the antiandrogen is compared with agonist action in the groups of animals receiving testosterone propionate alone. Dose–response curves may be plotted for each organ and expressed as percentage inhibition of the agonist effect of testosterone by the antiandrogen.

Modifications of the Method

Dorfman (1962) described an antiandrogen assay using the castrated mouse. Weights of prostate and seminal vesicles were determined after injection of the antiandrogen test compounds and simultaneous injections of 2 mg testosterone over a period of 7 days.

Applications of the Method

The intrinsic antiandrogenic activity is an important parameter in the evaluation of the pharmacological activity of H_2-receptor antagonists (Winters et al. 1979; Broulik 1980; Baba et al. 1981; Sivelle et al. 1982;

Foldesy et al. 1985; Takeda et al. 1982; Neubauer et al. 1990). In general pharmacology studies, there is rarely any need for specific anti-hormonal tests. However, in toxicology studies, tests for antiandrogen activity may be warranted when the effects on the testes and androgen-dependent organs are found in repeated-dose studies.

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Antiandrogenic Activity in Female Rats

Purpose and Rationale

This is another historical bioassay of interest due to the design of exploring antiandrogenic activity. Neumann and Elger (1966) described a method for testing the antiandrogenic activity of compounds in immature female rats. The inhibition by the antiandrogen cyproterone of the trophic effect of testosterone on uterine and preputial growth was studied in intact as well as in castrated female rats (Neri et al. 1972; Snyder et al. 1989).

Procedure

Female Sprague–Dawley rats weighing 40–45 g are ovariectomized. One week later, the treatment is started for 12 days with daily subcutaneous injections of 0.3 mg testosterone propionate and several doses of the antagonist. Controls receive testosterone propionate only. At autopsy on day 13, the uteri and preputial glands are distracted out and weighed. The weight increase of female accessory sexual organs caused by the testosterone's action is dose-dependently reduced by the antiandrogen (in this case, cyproterone). Similar results are found using intact immature female rats.

Evaluation

Dose–response curves were established for increasing doses of the antiandrogen at a given dose of testosterone propionate or for increasing doses of testosterone propionate at a given dose of the antiandrogen, cyproterone.

Critical Assessment of the Method

The method is time consuming and was directed to the specific investigation of cyproterone and cyproterone acetate in precocious puberty and androgen-dependent disorders.

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Intrauterine Feminizing/Virilizing Effect

Purpose and Rationale

From clinical observations as well as from experimental data (Neumann and Junkmann 1963; Neumann and Kramer 1964), it is well known that the external genitalia of female fetuses can be masculinized by tumors secreting endogenous androgens or by steroids with androgenic activity. This effect can be antagonized by an antiandrogen.

The method is of interest for assessment of reproductive toxicology.

Procedure

Adult female Sprague–Dawley rats are mated, and the beginning of pregnancy is determined by vaginal smears. From day 16 to day 19 of pregnancy, the antiandrogens are administered in various doses subcutaneously in sesame oil. Testosterone propionate is used in doses between 1.0 and 10.0 mg as the androgenic stimulus. The dams are sacrificed on the 20th day of pregnancy and the external genitalia of the female embryos examined. The sex of the embryos is recognized by the presence of ovaries and uterus. A dose of 10 mg testosterone propionate leads to total masculinization of female embryos with the loss of

female and the appearance of male sex characteristics. The anogenital distance in female rat fetuses measured macroscopically and microscopically is dose-dependently increased by testosterone propionate. This characteristic androgen effect is diminished by an antiandrogen.

Evaluation

The androgen-dependent decrease of the anogenital distance in female fetuses by various doses of the antiandrogen is expressed as percentage inhibition of the testosterone-induced virilization.

Modifications of the Method

Feminization of male rats was induced by treatment of pregnant rats during the second half of gestation and of the newborn fetuses during weeks 1–3 postpartum with an antiandrogen, e.g., cyproterone acetate (Neumann and Elger 1966; Nishino et al. 1988; review by Neumann 1994). A decrease of the anogenital distance in the male fetuses of antiandrogen-treated rats is expressed as percentage inhibition relative to fetuses from untreated mothers.

In feminized male rats, nipples and associated glandular tissues develop after birth as in normal female rats (Neumann and Elger 1967).

Feminization of male rats treated in utero was also observed with nonsteroidal antiandrogens and a 5α-reductase inhibitor (Imperato-McGinley et al. 1992).

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Antiandrogenic Activity on Sebaceous Glands

Purpose and Rationale

Bioassays for topical antiandrogens are based on inhibition of sebum secretion. Sebum production is increased by endogenous or exogenous androgens in many species including humans. In the mouse (Lapière and Chèvremont 1953; Neumann and Elger 1966), the Mongolian gerbil (Mitchell 1965), and the golden hamster (Hamilton and Montagna 1950), the male sex hormone stimulates sebum production and sebaceous gland growth. Morphometric evaluation by light microscopy in the rat has shown that castration causes a large reduction in the volume of the glands (Sauter and Loud 1975). The administration of testosterone over several days produces an enlargement of the sebaceous glands. This effect is used for the morphometric evaluation of topical antiandrogens.

The method is described in detail in chapter "▶ Vitiligo Models".

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Antiandrogenic Activity in the Hamster Flank Organ

Purpose and Rationale

This is another bioassay preferably for topical antiandrogens. The flank organs of Syrian golden hamsters are located on each flank of the animal consisting mainly of sebaceous tissue. Like sebaceous glands in other species, these pigmented spots respond to androgens by an increase in size. This proliferation is inhibited by systemic or topical antiandrogens.

The method is described in detail in chapter "▶ Vitiligo Models".

Effect of 5α-Reductase Inhibitors on Plasma and Tissue Steroid Levels

Purpose and Rationale

5α-Reductase inhibitors change the ratio of plasma testosterone to dihydrotestosterone (DHT) as well as the tissue concentrations particularly in the prostate tissue.

Procedure

Treatment of Animals

Male Sprague–Dawley rats are treated subcutaneously with the 5α-reductase inhibitor or vehicle beginning on postnatal day 3 until the age of 4 or 7 weeks. After sacrifice, blood is withdrawn for testosterone and DHT determinations (George et al. 1989). Moreover, intraprostatic concentrations of testosterone and DHT are determined as an index of antiproliferative activity (di Salle et al. 1993).

Radioimmunoassay for Testosterone and Dihydrotestosterone

Serum testosterone and DHT are measured by radioimmunoassay (RIA) in serum or serum extracts using specific antisera without prior chromatography (Falvo and Nalbandov 1974). Serum samples of 0.5 ml may be extracted with 2 ml of freshly purified, peroxide-free diethyl ether by shaking for 60 s on a vortex mixer. The aqueous phase is frozen at −70 °C, then the ether phase containing steroids is transferred to conical test tubes, and evaporated under a stream of dry nitrogen. The dry residue is redissolved in BSA/phosphate buffer (1 % BSA = bovine serum albumin) for RIA. [1,2,6,7-^{3}H]-Testosterone or [1,2,6,7-^{3}H]-dihydrotestosterone and specific antisera are added and tubes incubated over a period of 24 h at +4 °C under nonequilibrium conditions. Bound hormone and free hormone are separated by adsorption on dextran-coated charcoal. The activity of each sample is determined by beta-spectrometry.

Commercially available RIA kits can be used with suitable validation.

Evaluation

The hormone concentrations in the sample are calculated from a standard curve by a computer program (e.g., RIA-Calc, LKB), using appropriate control sera. The ratios of testosterone to DHT in rats treated with different doses of 5α-reductase inhibitors are compared with those of vehicle-treated intact control rats.

Modifications of the Method

di Salle et al. (1998) measured prostatic concentrations of testosterone and 5α-dihydrotestosterone in rats by specific RIAs after treatment with a dual type I and II 5α-reductase inhibitor. Similar measurements of tissue testosterone to DHT ratios have been performed in dogs, in the

context of pituitary downregulation of androgen secretion by luteinizing-hormone-releasing hormone (LHRH) agonists.

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Castration of Male Rats (Orchiectomy)

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