J. Enzyme Inhibition, 1989, Vol. 2, pp. 261–268
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COMPARATIVE EFFECTS OF KETOCONAZOLE ON RAT, DOG AND HUMAN TESTICULAR STEROIDOGENESIS

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(Received 6 May, 1988)

Ketoconazole is an antifungal azole derivative which also inhibits the cytochrome P-450$_{17\alpha}$, catalyzing the conversion of progestins into androgens. The effects of ketoconazole on human, dog and rat testosterone biosynthesis were compared using short term incubations of dispersed testicular cells. The results showed that ketoconazole inhibited androgen biosynthesis at lower concentrations in dispersed human testicular cells (IC_{50}: 0.08 μmol/l) than in canine (IC_{50}: 0.1 μmol/l) and rat cells ($IC_{50} \geqq 0.2$ μmol/l). Furthermore, they demonstrated that ketoconazole first inhibited the 17,20-lyase activity and then the 17-hydroxylation in rat and dog cells whereas only the 17-hydroxylation was affected in human cells.

KEY WORDS: Ketoconazole, human, dog and rat testicular steroidogenesis.

INTRODUCTION

Ketoconazole is an orally active broad-spectrum antifungal agent. At low (nanomolar) concentrations, it blocks the cytochrome P-450 dependent 14-demethylase, catalyzing the synthesis of ergosterol in yeast and fungi.[1] At higher concentrations (0.1 to 10 μmolar), this imidiazole derivative has been shown to inhibit several mammalian cytochrome P-450 dependent enzymes, mainly those involved in steroidogenesis.[2,3]

In man, the drug prevents the testicular and adrenal androgen production.[4–6] A partial blockade of the glucocorticoid biosynthesis has also been reported, but clinical adrenal dysfunction occurs rarely.[6–9] These results prompted studies that suggested ketoconazole high dose therapy (i.e. 400 mg t.i.d.) to be of value in the first or second line management of patients with prostatic cancer.[9–11]

In mammalian systems, the most sensitive enzyme to ketoconazole is the cytochrome P-450$_{17\alpha}$ which converts progestins to androgens.[3,12–14] This enzyme catalyses at least two successive reactions, i.e. the 17-hydroxylation and the 17,20-lyase reaction, and it is not clear which one is preferentially blocked by the drug.

The purpose of this paper was to compare the effects of ketoconazole on the androgen biosynthesis using short term incubations of rat, dog and human testicular cells.

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MATERIALS AND METHODS

Testis Tissue

Human testis tissue was obtained from 5 patients (median age 72, range 64–81) who underwent orchiectomy as primary therapy for prostatic carcinoma. None of the patients had been taking any specific medication. All underwent general anaesthesia with pentothal.

The canine testes were obtained from 6 adult beagle dogs (median age: 19 months, range 12–36), within 5 minutes after induction of anaesthesia by an etomidate analogue (R 8110, 4 mg/kg) and fentanyl (0.015 mg/kg). The former was used because it is less potent than etomidate in inhibiting steroid biosynthesis[15] and has no effect on androgen production in dogs.[16]

The rat testes were obtained from 25 adult Wistar rats weighing 250 g, immediately after sacrifice by decapitation.

Cell Suspensions

Short term primary cultures of dispersed testicular cells were performed by modification of the method described by Dufau *et al.*[17] The testes were decapsulated, sliced with scissors and placed in Tissue Culture Medium 199 (Gibco, NY, USA), containing 2 mg/ml of BSA (fraction V of Cohn, Sigma, St. Louis, USA) and 200 IU/ml of collagenase (type I, Worthington, Freehold, USA). Dispersion of the cells was obtained after 30 minutes of incubation at 37°C under constant agitation followed by mechanical dispersion by means of a Pasteur pipette. A second incubation with fresh collagenase was needed to achieve a complete disruption of the human tissue. The tubular mass was allowed to settle and the supernatant was passed through a nylon screen (pore size 0.08 mm). The cells were collected by centrifugation (100 g, 15 min), washed twice and counted in a haemocytometer. After determination of the viability by the Trypan blue method, the cell suspension, was diluted to 10^6 viable cells/ml and divided into aliquots of 1 ml in multidishes containing 8 wells.

Incubation of Cell Suspensions and Steroid Determination

In a first series of experiments, the effect of ketoconazole (10^{-7}–10^{-5} M) on the steroid biosynthesis from endogenous substrate was studied. The cell suspensions were preincubated with the drug (dissolved in dimethylsulfoxide, 0.1% v/v) for 45 min at 37°C in an atmosphere of 5% CO_2 in air. The enzymatic reactions were then enhanced by addition of human chorionic gonadotropin (0.5 IU/ml, UCB, Brussels, Belgium). The rat testicular cells were further incubated for 2 h, whereas for the dog and human testicular cells a 15 h incubation period was used. The production of steroids was quantified directly on incubation medium in which cells were included, using a battery of specific steroid antisera and radioimmunoassays, ^{3}H-labelled steroids (specific activity of 38 to 70 Ci/mmol, New England Nuclear, Dreieich, W.-Germany) and activated charcoal for the separation of bound and free ligand. Characterization and specificity of these antisera have been published elsewhere: testosterone,[18] androstenedione,[19] 17α-hydroxyprogesterone[20] and progesterone.[21] Pregnenolone was measured with an antiserum raised in rabbit to pregnenolone-3-monohemisuccinate-

TABLE 1
Concentrations of steroids measured by radioimmunoassay in the supernatant of rat, dog and human testicular cell suspensions, after hCG stimulation. The results are expressed as nmol/l or pmol/10^6 viable cells (mean ± SEM).

	Rat (n = 10)	Dog (n = 6)	Human (n = 5)
Testosterone	113 ± 13	47.8 ± 9.7	72.7 ± 20.4
Androstenedione	27.2 ± 4.1	8.5 ± 1.3	6.9 ± 1.5
DHEA	< 0.5	12.9 ± 4.0	16.5 ± 2.0
17α-hydroxyprogesterone	1.5 ± 0.3	5.2 ± 1.1	21.6 ± 3.7
Progesterone	3.4 ± 0.6	1.5 ± 0.2	8.4 ± 1.2
Pregnenolone	3.1 ± 0.2	3.0 ± 0.9	40.5 ± 5.8

human serum albumin (Radioassay Systems Laboratories, Carson, USA) which showed the following main cross-reactions: pregnenolone sulphate: 50%, 17α-hydroxypregnenolone: 2%, progesterone: 1.5% and an interassay coefficient of variation of 11.4%. Dehydroepiandrosterone (DHEA) was measured with an antiserum raised in rabbit to 15β-carboxyethylmercapto-DHEA (Radioassay System Laboratories, Carson, USA) which mainly cross-reacted with DHEA-sulphate (1.2%) and androstenedione (0.32%). The interassay coefficient of variation reached 12.4%.

In a second set of experiments, the cell suspensions were incubated in the presence of ketoconazole and 0.8 μmol/l of ^{14}C-pregnenolone (specific activity 55 mCi/mmol, New England Nuclear, Germany). After the incubation period, the cell suspensions were loaded on silicagel columns (Extrelut, Merck, Darmstadt, W.-Germany) and the steroids were extracted with 15 ml of ether. The ether extract was dried under nitrogen, the steroids were dissolved in 600 μl of n-hexane/isopropanol (9/1, v/v) and 150 μl were loaded on a Varian 5560 Liquid Chromatograph equipped with a Perkin-Elmer ISS-100 automatic injector, a Vista 402 data system, a Varian UV-200 detector at 242 nm and a Berthold LB 504 radioactivity monitor. A Lichrosorb Diol column (250 × 4.6 mm, 5 μm, Merck, Darmstadt, W.-Germany) was eluted with n-hexane-isopropanol in a stepwise linear gradient at a flow rate of 1.3 ml/min. The radioactivity present in the column effluent was measured directly with pico-fluor (Packard, Downers Grove, USA) as scintillant (2.3 ml/min).

The system was calibrated using standard mixture of UV-detectable or radioactive steroids. The final recovery was always found to be higher than 88%.

RESULTS

After collagenase treatment the viability of the cells was 85%, 82% and 72% for rat, dog and human preparations respectively.

In preliminary studies, the effect of increasing incubation times on the testosterone biosynthesis was studied. For the rat testicular cell suspension, a 2 h incubation period was sufficient to obtain maximal testosterone production, but 4 to 15 h were needed for dog and human testicular cell preparations. The concentrations of the androgens and their precursors under control conditions are shown in Table 1. Ketoconazole lowered testosterone production at a concentration of 0.2 μmol/l, 0.1 μmol/l and 0.08 μmol/l in rat, dog and human testicular cell suspensions respectively (Figure 1). Androstenedione and DHEA showed a similar inhibition. In rat testicular cell suspensions, 17α-hydroxyprogesterone and progesterone production rose to maximal values

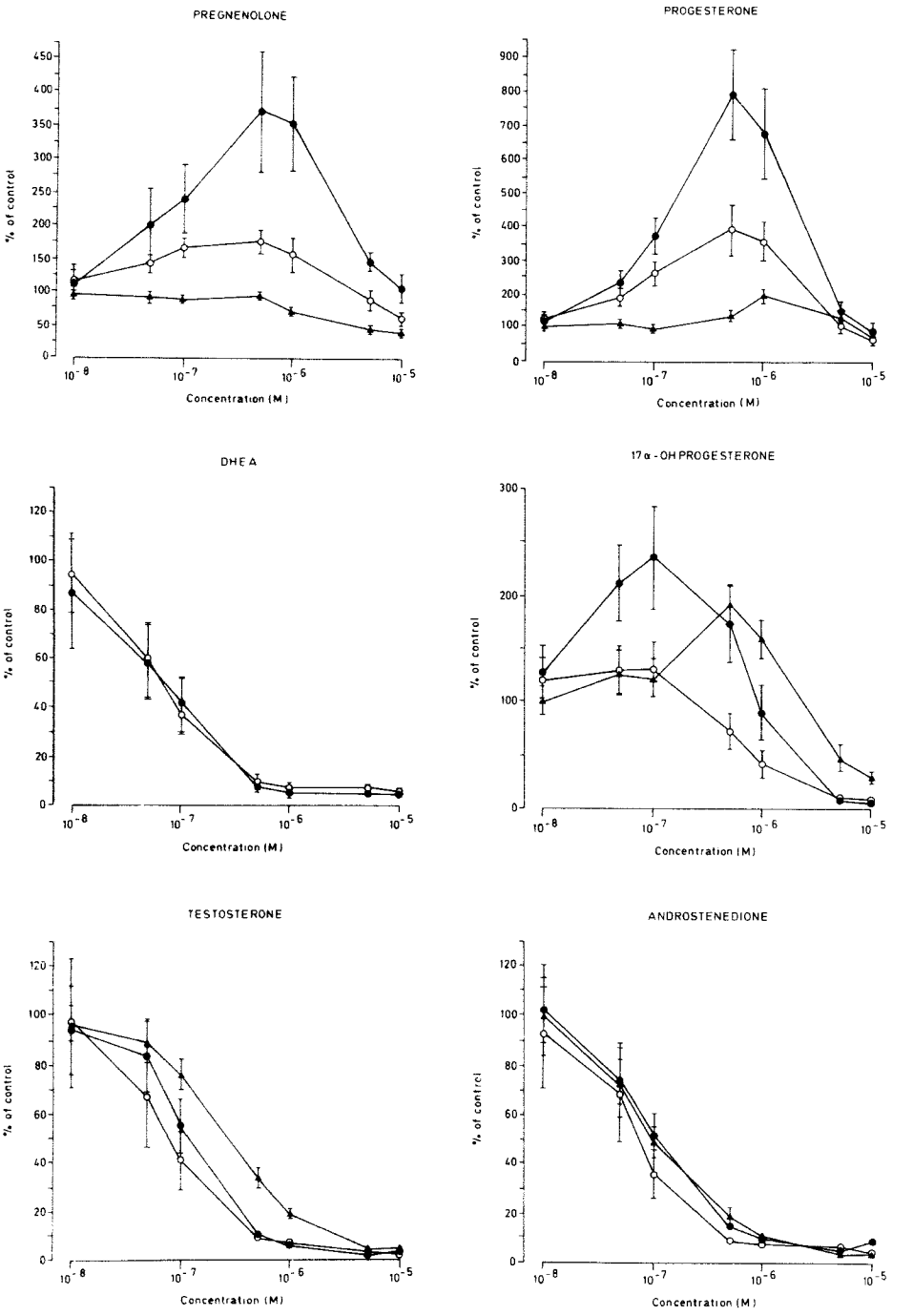

FIGURE 1 Effects of increasing ketoconazole concentrations on hCG-stimulated steroid production in short-term incubation of rat (▲), dog (●) and human (○) dispersed testicular cells. The results are expressed as mean ± SEM of 10 (rat), 6 (dog) and 5 (human) duplicate experiments.

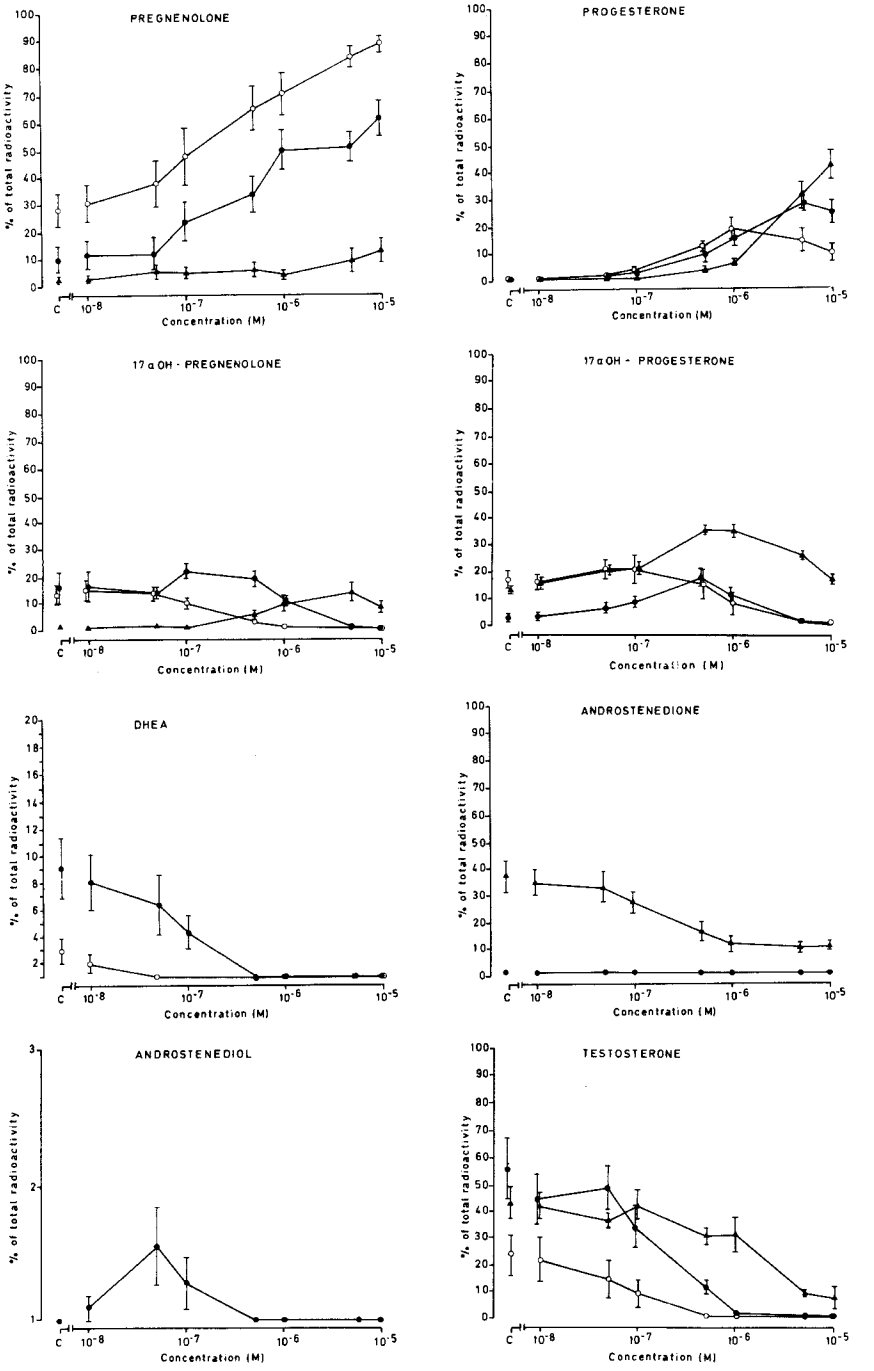

FIGURE 2 Effects of increasing ketoconazole concentrations on hCG-stimulated ^{14}C-pregnenolone metabolism by short-term incubation of rat (▲), dog (●) and human (○) dispersed testicular cells. The results are expressed as mean ± SEM of 8 (rat), 6 (dog) and 5 (human) duplicate experiments.

of 192% and 197% of their basal levels at ketoconazole concentrations of 0.5 μmol/l and 1 μmol/l, respectively, whereas pregnenolone production remained almost unchanged (Figure 1).

In canine testicular cell suspensions, 17α-hydroxyprogesterone, progesterone and pregnenolone production increased to 242%, 786% and 350% of their initial value at ketoconazole concentrations of 0.1 μmol/l, 0.5 μmol/l and 0.5 μmol/l, respectively (Figure 1). In human cell suspensions, progesterone and pregnenolone production only accumulated to 168% and 388% at 0.5 μmol/l of ketoconazole.

At concentrations higher than 1 μmol/l, all the steroids measured in the culture medium of rat, dog and human cells fell below their control levels.

Figure 2 shows the effects of rising ketoconazole concentrations on the metabolism of ^{14}C-pregnenolone in the cell suspensions of the three different species. In control conditions 98%, 87% and 72% of the labeled pregnenolone was metabolized by rat, dog and human cells respectively. In the rat testicular cell incubations, only steroids of the Δ^4 pathway could be detected with the exception of 17α-hydroxypregnenolone. In contrast, pregnenolone metabolism occurred via both Δ^5 and Δ^4 pathways in dog and human testicular cells with some prevalence of the Δ^5 steroids, especially DHEA.

In the 3 systems, ketoconazole prevented the androgen synthesis, producing 50% inhibition of testosterone formation at 2, 0.2 and 0.07 μmol/l in rat, dog and human cells, respectively. Furthermore, the variations of the progestins confirmed the species differences observed in the first set of experiments. In rat and dog, low concentrations of ketoconazole induced a rise of 17-hydroxylated steroids. At concentrations higher than 5 μmol/l, these 17-hydroxylated steroids fell, whereas progesterone and unmetabolized pregnenolone accumulated. In human cell suspensions, the 17-hydroxylated steroids almost followed the profile of the androgens. Only a slight increase of 17α-hydroxyprogesterone was detected, changing from 18 to 21% of total radioactivity in the presence of 0.1 μmol/l of ketoconazole. At 10 μmol/l pregnenolone accumulated more than in rat and dog systems, reaching 88% of non-metabolized product. At the latter concentration, a decrease in progesterone was also demonstrated.

In 1 out of 6 incubations of rat testicular cells, about 1.5% of total radioactivity was eluted with a retention time similar to that of the 4-pregnene-17α,20α-diol-3-one. In dog and human testicular cell incubations, 4 unidentified metabolites (i.e. metabolites not corresponding to ^{3}H-markers) were observed. In dog testicular cell suspensions, one of these metabolites was detected at 0.1 μmol/l of ketoconazole (1% of total radioactivity). This metabolite, less polar than progesterone, accumulated to 16.2 ± 2.7% of total radioactivity at 10 μmol/l. Its elution time was similar to that of 4,16-androstadien-3-one. Futhermore, another very polar metabolite, following the pattern of testosterone was found in 5 out of 6 incubations (5% ± 3.2% of total radioactivity in control incubations). In human testicular cell suspensions, 2 unidentified metabolites were detected in control incubations (13.6% ± 3% and 1.8 ± 0.3% of total radioactivity). In the presence of ketoconazole, they fell similarly to testosterone and their elution time corresponded to those of 4,16-androstadien-3-one and 5,16-androstadien-3β-ol, respectively.

DISCUSSION

The metabolism of labeled pregnenolone in testicular cell suspensions confirms

previous studies showing that in the rat testosterone is formed via the Δ^4 pathway, whereas both Δ^4 and Δ^5 pathways, with some prevalence of the Δ^5 one are used in dog and man.[22–24] These results also confirm that ketoconazole blocks testicular androgen biosynthesis, mainly by inhibiting the cytochrome P-$450_{17\alpha}$.[3,12–14,25–28] This single enzyme catalyses 2 successive reactions: the 17-hydroxylation and the 17,20-lyase activity, at least in cow, guinea pig and pig, and probably also in man.[26]

However, conflicting results have been reported concerning the potency of ketoconazole (IC_{50} varying from 0.3 to 12 μmol/l) and the site of its inhibition (17-hydroxylation or 17,20-lyase activity). The preferential inhibition by ketoconazole of the 17,20-lyase activity has been shown by several groups in rat[3,12,28,29] and human[30] tissue. Other investigators found similar effects of ketoconazole on both 17-hydroxylation and 17,20-lyase activity in rat[13,14] and human testis,[27] or even an inhibition of the 17-hydroxylation alone after oral administration of ketoconazole in rats.[25] These discrepancies might result from differences in methodologies. The use of subcellular or cellular fractions of different species, incubated in the presence of various concentrations of labeled precursors (0.3 to 60 μmol/l) and cofactors, the measurement of testicular enzymatic activities or steroid production in the presence of ketoconazole *in vitro*, *ex vivo* and after oral administration of the drug, may explain some of these differences.

Our results, obtained for similar cell suspensions of rat, dog and human testicular cells, in the presence of the same concentration of ^{14}C-pregnenolone (0.8 μmol/l) or without any added steroid stress some important species differences. Testosterone biosynthesis in human cells is more sensitive to ketoconazole than in dog and rat cells, especially in incubations with ^{14}C-pregnenolone. Furthermore, the 17,20-lyase activity, catalyzing the conversion of 17α-hydroxyprogesterone and pregnenolone into androstenedione and DHEA, is most affected in rat and canine systems, whereas only the 17α-hydroxylation, catalyzing the conversion of progesterone and pregnenolone to 17α-hydroxylated steroids, is inhibited in human cell suspensions.

These results are in keeping with the data reported in animal and human *in vivo* studies. Oral doses of 24 mg/kg are needed to suppress plasma toestosterone concentrations in rats,[31] whereas 10–15 mg/kg and 5 mg/kg are needed in dogs[20,32] and humans,[4] respectively. Furthermore, a rise of plasma 17α-hydroxyprogesterone and of progesterone levels has been described in dogs after ketoconazole treatment[20,32] whereas in man, only plasma progesterone concentrations are increased.[5,10]

The fall of all the steroids measured by radioimmunoassay in the supernatant of the cell suspensions, including pregnenolone, is in keeping with the inhibition of cytochrome P-450_{scc} as described in mitochondrial fractions of rat[12] and pig[33] testis. It might also be due to inference of high concentration of ketoconazole with the cholesterol biosynthesis.[34]

Finally, three out of 4 unidentified metabolites isolated from the cell suspension showed elution times which corresponded to 16-androstenes. These latter steroids are synthetized from androgens of progesterone. These have been particularly studied in pigs,[23] but have also been identified in rat[35] and human[24] testis.

The results of the present study stress some important species specific differences in the effect of ketoconazole on testicular steroidogenesis and point out that homologous *in vitro* systems might be of great value to evaluate inhibitors of androgen biosynthesis.

Acknowledgments

The authors are grateful to the whole staff of the Department of Urology (University Hospital of Antwerp). They also wish to thank L. Leijssen and colleagues and L. Geentjens who prepared the manuscript.

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