J. Steroid Biochem. Molec. Biol. Vol. 38, No. 2, pp. 213–218, 1991
Printed in Great Britain

0960-0760/91 $3.00 + 0.00
Pergamon Press plc

EFFECT OF KETOCONAZOLE ON HUMAN OVARIAN $C_{17,20}$-DESMOLASE AND AROMATASE

M. M. WEBER, A. WILL, B. ADELMANN and D. ENGELHARDT*
Ludwig-Maximilians-Universität München, Medizinische Klinik II, Klinikum Großhadern, Marchioninistrasse 15, 8000 München 70, Germany

(Received 6 June 1990)

Summary—Ketoconazole, an imidazole antimycotic drug, inhibits steroid biosynthesis in adrenal and testicular tissue by blocking cytochrome *P*-450 dependent enzymes. To study the effect of ketoconazole on steroid biosynthesis in the human ovary we incubated human ovarian tissue (mainly theca cells) or granulosa cells with radiolabeled precursors and increasing concentrations of ketoconazole. After incubation, steroids were extracted and separated by thin layer chromatography (TLC). Activity of $C_{17,20}$-desmolase and aromatase was estimated by measuring the amount of their radioactive products with liquid scintillation counting. After incubation of ovarian tissue with [^{3}H]17-hydroxyprogesterone the production of [^{3}H]androstenedione was reduced by increasing concentrations of ketoconazole (0–200 μM) to a minimum of 31% of basal production. This indicates a strong inhibition of ovarian $C_{17,20}$-desmolase by ketoconazole with a 50% inhibiting concentration (IC_{50}) of 23 μM. After incubation of human granulosa cells with ketoconazole (0–2000 μM) and [^{3}H]androstenedione the production of [^{3}H]estrone and [^{3}H]estradiol was suppressed to minimally 37 and 35% of basal values, indicating a significant inhibition of ovarian aromatase. IC_{50}-values were 105 μM ketoconazole for estradiol and 130 μM for estrone. In conclusion, ketoconazole was shown to inhibit human ovarian $C_{17,20}$-desmolase and aromatase *in vitro*. As in human adrenals and testes ovarian $C_{17,20}$-desmolase seems to be most sensitive to the inhibitory effect of ketoconazole.

INTRODUCTION

The orally active, broad-spectrum antimycotic drug ketoconazole blocks ergosterol biosynthesis in fungi at picomolar concentrations by interaction with the cytochrome *P*-450 component of the fungal C_{14}-demethylase [1]. At higher, micromolar concentrations, ketoconazole also inhibits various mammalian cytochrome *P*-450 dependent enzyme systems. In the human, the most sensitive enzyme to the inhibitory effect of ketoconazole seems to be adrenal and testicular $C_{17,20}$-desmolase [2–5]. This cytochrome *P*-450 dependent microsomal enzyme converts 17OH-progesterone to androstenedione and is a key enzyme of androgen biosynthesis. Furthermore, ketoconazole blocks a number of human adrenal and testicular steroidogenic enzymes [2–8].

Due to its inhibitory effect on human androgen and corticosteroid biosynthesis, ketoconazole has been used as a therapeutic agent in the treatment of advanced prostatic cancer [9, 10], precocious puberty [11] and various forms of Cushing's syndrome [12–15]. While the size of action on steroid biosynthesis in human testis and adrenals has been extensively investigated *in vitro* and *in vivo*, the effect of ketoconazole on estrogen biosynthesis in the human ovary is unclear. In contrast to *in vitro* findings in rat ovarian and human placental microsomes that ketoconazole blocks the $C_{17,20}$-desmolase as well as the aromatase, an inhibitory effect of ketoconazole on these enzyme systems in the human ovary has not been shown. *In vivo* serum estrogen levels remain unchanged [16–18] or show only a mild decline after the administration of high doses of ketoconazole [19–23]. The aim of this study was to investigate the *in vitro* effects of ketoconazole on human ovarian $C_{17,20}$-desmolase and aromatase, both important cytochrome *P*-450 dependent microsomal enzyme systems.

EXPERIMENTAL

Materials

[1,2-^{3}H]17α-hydroxyprogesterone (41.5 Ci/mmol), [6,7-^{3}H]androstenedione (45 Ci/mmol),

*To whom correspondence should be addressed.

[4-^{14}C]17α-hydroxyprogesterone (50 mCi/mmol), [4-^{14}C]androstenedione (52 mCi/mmol), [4-^{14}C]-estradiol (56 mCi/mmol) and [4-^{14}C]estrone (50 mCi/mmol) were purchased from New England Nuclear, Boston, MA, U.S.A. Unlabeled steroids and calf thymus DNA were obtained from Sigma, D-8028 Taufkirchen, F.R.G., and thin layer chromatography (TLC) plates (Kieselgel 60, F_{254}), from Merck, D-6100 Darmstadt, F.R.G. Follicle stimulating gonadotropin (FSH, Fertinorm®) and human menopausal gonadotropin (HMG, Pergonal®) were purchased from Serono, D-7800 Freiburg, F.R.G., human chorionic gonadotropin (HCG, Predalon®) from Organon, D-8042 Oberschleißheim, F.R.G., ketoconazole (Nizoral®) from Janssen, D-4040 Neuss, F.R.G., Dulbeco's Modified Eagle's Medium (DMEM) as well as magnesium- and calcium-free Earle's Balanced Salt Solution (EBSS) from Gibco, Grand Island, NY, U.S.A.

Preparation and incubation of human ovarian stroma

Normal ovarian tissue was obtained from five female patients who underwent hysterectomy due to carcinoma or myoma of the uterus. Mean age of patients was 41 yr (range 37–48 yr), all women were menstruating regularly and except one in the first-half of their cycle (patient No. 1–4 on day 9–12 p.m., patient No. 5 on day 21 p.m.). All ovaries were macroscopically and histologically normal. After removal of larger visible follicles, slices of 50 mg wet wt from ovarian stroma (containing mainly theca cells) were prepared at 4°C and transferred to incubation vessels containing 2 ml of DMEM. Steroid biosynthesis was stimulated by adding FSH and hCG in a concentration of 200 ng/ml each. In preceding studies we found a two- to five-fold stimulation of steroid biosynthesis by addition of gonadotropins as compared to the basal steroid production. For metabolism studies [^{3}H]17-hydroxyprogesterone (135 pmol) was added and incubated with ketoconazole in various concentrations (0–200 μM) for 3 h at 37°C in a shaking waterbath. All incubation studies were done in triplicate.

Preparation and incubation of human granulosa cell suspension

Granulosa cells were obtained from 10 regularly menstruating women (mean age 27, range 24–32 yr) in whom an *in vitro* fertilization (IVF) or an gamete intrafallopian transfer (GIFT) was performed due to sterility. All women were given a standardized therapy with hMG (1000 units i.m./day) to stimulate multiple follicular development. As soon as estrogen plasma levels reached 400 pg/ml and follicle size was greater than 15 mm, hCG (10,000 units i.m.) was administered and follicle aspiration was performed laparoscopically 36 h later (day 11–13 of the menstrual cycle). From the aspirated ovulatory follicles oocyte–cumulus complexes were separated for IVF or GIFT treatment. Granulosa-lutein cells were separated from the aspirates by centrifugation for 10 min at 1000 *g* and washing the sediment with 20 ml of EBSS. The granulosa cells were purified from cellular debris and red blood cells on a continuous Percoll density gradient as described previously [24]. The cell DNA content was measured by the method of Labarca and Paigen [25] which is based on the enhancement of fluorescence after binding of bisbenzimidazole to DNA. The cell pellet then was suspended in DMEM incubation medium and aliquots of 2 ml containing 8 μg cell DNA (1 μg = 1.67×10^5 cells) were put into incubation vessels. Viability of cells was found to be $70 \pm 4\%$ ($n = 8$) as determined by the trypan blue exclusion test. Steroid biosynthesis was stimulated by FSH in a concentration of 200 ng/ml. After addition of radiolabeled precursor [^{3}H]androstenedione (80 pmol) and ketoconazole (0–2000 μM), the cell suspension was incubated for 3 h at 37°C in a shaking waterbath. Preliminary experiments showed that the amount of estrogens formed was related linearly to the amount of granulosa cell DNA and time. All incubation studies were done in triplicate and reaction was stopped by freezing at −40°C.

Analysis of steroid metabolism

After incubation, ovarian tissue was homogenized mechanically in the medium. Traces of ^{14}C-steroids (10^5 cpm) were added to the incubation mixture for recovery calculations and extracted together with the tritium-labeled steroids by chloroform. After distribution between methanol and *n*-heptane (2:1, v/v), the steroid extracts were evaporated under nitrogen, dissolved in dichlormethane–methanol (1:1, v/v) and applied to TLC plates with the following systems: chloroform–ethylacetate (8:2, v/v) and butyl-acetate–ethylacetate (1:1, v/v). The R_f-values of the separated metabolites were: 0.1 (19-hydroxyandrostenedione), 0.3 (17-hydroxyprogesterone), 0.32 (estradiol), 0.41 (androstenedione), 0.48 (estrone) in the first

phase and 0.2 (19-hydroxyandrostenedione), 0.49 (androstenedione), 0.51 (17-hydroxyprogesterone), 0.57 (estradiol), 0.67 (estrone) in the second phase. Location of labeled steroids on TLC plates was assessed with short-wave u.v. light by adding unlabeled steroids chromatographed on the same plate. After scraping the areas of silical gel containing labeled steroids separately into counting vials, quantitative analysis was made by liquid scintillation counting. The yields of ^3H products in the absence or presence of ketoconazole were corrected for manipulative loss using the recovery of ^{14}C-labeled steroids. The recoveries were found to be equal for all steroids measured ($72 \pm 2\%$, $n = 15$), the crossover of ^{14}C counts into the ^3H channel was $9 \pm 1\%$ ($n = 15$). Activity of $C_{17,20}$-desmolase was estimated by measuring the amount of [^{3}H]androstenedione formed from [^{3}H]17-hydroxyprogesterone by ovarian stroma, containing mainly theca cells. Activity of aromatase was estimated by measuring the amount of [^{3}H]estrone and [^{3}H]estradiol formed from [^{3}H]androstenedione by purified human granulosa cells. Similarly, the activity of the first hydroxylation step of the aromatase, the 19-hydroxylase was assessed by the amount of [^{3}H]19-hydroxyandrostenedione formed from [^{3}H]androstenedione.

Statistical analysis

Statistical analysis was performed using the Student's t-test. Comparisons were made between the activity of [^{3}H]product at different concentrations of ketoconazole vs control activity in the basal incubation without ketoconazole.

RESULTS

Metabolism of [^{3}H]17-hydroxyprogesterone

In the absence of ketoconazole the mean production rate of [^{3}H]androstenedione from the added precursor [^{3}H]17-hydroxyprogesterone was 17 ± 4.5 fmol/mg tissue/min of incubation. When rising concentrations of 2, 20 and 200 μM ketoconazole were added, a significant decline in [^{3}H]androstenedione formation to 67, 51 and 32% of the basal production was observed (Fig. 1). The concentration of unmetabolized precursor [^{3}H]17-hydroxyprogesterone rose maximally 1.5 fold in a dose dependent way. This shows a significant inhibitory effect of ketoconazole on human ovarian $C_{17,20}$-desmolase, with a 50% inhibiting concentration of 23 μM ketoconazole.

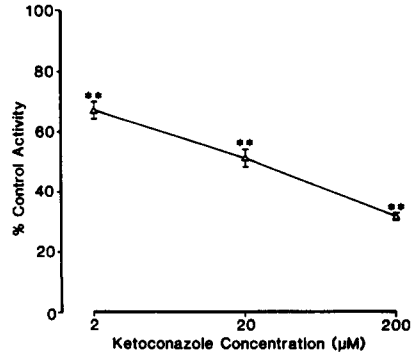

Fig. 1. Effect of increasing concentrations of ketoconazole on the formation of [^{3}H]androstenedione by human ovarian tissue incubated with [^{3}H]17-hydroxyprogesterone. 100% control activity represents a production of 17 ± 4.5 fmol [^{3}H]androstenedione/mg tissue/min of incubation without ketoconazole. Values are means $\pm$ SEM of five independent incubation experiments (** = $P < 0.01$).

Metabolism of [^{3}H]androstenedione

In the absence of ketoconazole the mean production rate of radiolabeled estrogens from [^{3}H]androstenedione was 4909 ± 838 fmol per 10^5 cells and minute of incubation for [^{3}H]estradiol, and 2674 ± 402 fmol per 10^5 cells and minute for [^{3}H]estrone. The mean formation rate of [^{3}H]19-hydroxyandrostenedione was 1874 ± 311 fmol per 10^5 cells and minute of incubation. Under the influence of ketoconazole a dose dependent and significant decrease of estrogen formation was observed. In the presence of 2, 20 and 200 μM ketoconazole formation of [^{3}H]estrone was suppressed to 90, 62 and 46% of the basal production rate, formation of [^{3}H]estradiol was suppressed to 94, 65 and 35% (Fig. 2). Inversely, the activity of the

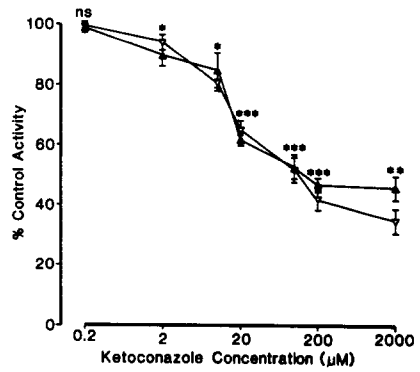

Fig. 2. Effect of increasing concentrations of ketoconazole on the formation of [^{3}H]estradiol (▽) and [^{3}H]estrone (▲) by human granulosa cells incubated with [^{3}H]androstenedione. 100% control activity represents a production of 4909 ± 838 fmol [^{3}H]estradiol 2674 ± 402 fmol [^{3}H]estrone per 10^5 cells and minute of incubation without ketoconazole. Values are means $\pm$ SEM of 10 independent incubation experiments (NS = not significant, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$).

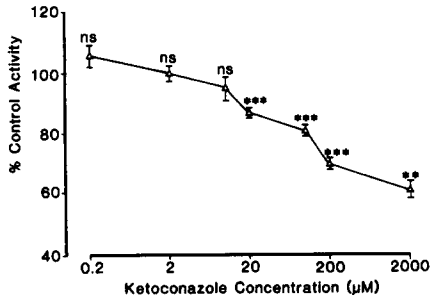

Fig. 3. Effect of increasing concentrations of ketoconazole on the formation of [^{3}H]19-hydroxyandrostenedione by human granulosa cells incubated with [^{3}H]androstenedione. 100% control activity represents a production of 1874 ± 311 fmol [^{3}H]19-hydroxyandrostenedione per 10^5 cells and minute of incubation without ketoconazole. Values are means $\pm$ SEM of 10 independent incubation experiments (NS = not significant, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$).

unmetabolized precursor [^{3}H]androstenedione rose in a dose-dependent way to a maximum of 232% of basal activity. A 50% inhibition of estrogen biosynthesis was observed at 105 μM ketoconazole for [^{3}H]estradiol and at 130 μM for [^{3}H]estrone. In the same experiments the formation of [^{3}H]19-hydroxyandrostenedione was suppressed to 87, 70 and 61% of the basal formation rate by 20, 200 and 2000 μM ketoconazole, respectively (Fig. 3). This indicates a significant inhibitory effect of ketoconazole on the first step of the aromatase, the 19-hydroxylase.

DISCUSSION

Ovarian stroma, namely theca cells, are the major site of androgen biosynthesis in the human premenopausal ovary and therefore have a high activity of $C_{17,20}$-desmolase. This cytochrome *P*-450 dependent microsomal enzyme converts 17-hydroxyprogesterone to androstenedione and thus supplies granulosa cells with androgens for aromatization to estrogens. After incubation of ovarian stroma with ketoconazole we found a strong and dose-dependent reduction in the formation of androstenedione as well as a corresponding increase in unmetabolized 17-hydroxyprogesterone. This suggests a strong inhibitory effect of the imidazole drug on the activity of human ovarian $C_{17,20}$-desmolase. The 50% inhibiting concentration (IC_{50}) of 23 μM found for human ovarian $C_{17,20}$-desmolase is only slightly higher than the IC_{50} of 0.5–5 μM reported for human testicular and adrenal $C_{17,20}$-desmolase [2, 3, 5, 6]. Up to now an inhibition of ovarian $C_{17,20}$-desmolase by ketoconazole has been reported only in rat ovaries [26]. Our results are in accordance to *in vivo* findings in female patients with ovarian androgen excess who showed significant reduction in serum androgen levels after treatment with 600–1000 mg of ketoconazole per day [17, 18, 27].

The principal site of ovarian aromatase activity is the granulosa cell of the preovulatory follicle [28, 29, 30]. This unique, cytochrome *P*-450 and NADPH dependent enzyme system converts androgens to estrogens by a three step hydroxylation beginning at the C_{19} position [31, 32, 33]. In our incubation experiments with human granulosa cells we found a dose-dependent decrease in the formation of estrogens as well as a concomitant increase in unmetabolized androstenedione under the influence of ketoconazole. This significant inhibition of human ovarian aromatase is in accordance to findings in human placental microsomes as well as in rat ovarian and testicular tissue [2, 23, 26, 31, 33–35]. For aromatase in human placental microsomes Mason *et al.* and France *et al.* found an IC_{50} of 60–65 μM ketoconazole. This is only slightly higher than the IC_{50} values of 105–130 μM found for human ovarian aromatase in our studies [33, 36]. In contrast Ayub *et al.* observed a much stronger inhibition of human placental aromatase by ketoconazole with an IC_{50} of 2 μM [31].

These findings are discordant to the results of DiMattina *et al.* who in 1988 performed the only other study on the effect of ketoconazole on human ovarian steroid biosynthesis [37]. Besides a strong inhibition of the 17α-hydroxylase and, surprisingly of the cytochrome *P*-450 independent 3β-hydroxysteroid dehydrogenase, a significant inhibitory effect of ketoconazole on the microsomal aromatase could not be found, except at the highest concentration of 5000 μM ketoconazole. Ovarian 17,20-desmolase activity was not tested in this study [37]. The reason for this discrepancy between the results of DiMattina's study and ours as well as the findings in animal ovarian and human placental aromatase are not entirely clear. One possible explanation might be the low number of experiments with ovarian microsomes of only two patients in DiMattina's study. Furthermore, the time course of ketoconazole-induced inhibition of 17α-hydroxylation and aromatization may be different so that the short incubation time of 15–30 min in DiMattina's experiments may not have been long enough to sufficiently block aromatase activity. In accordance with Khalil

et al. who found a significant inhibition of 19-hydroxylase in porcine granulosa cells our incubation experiments with human granulosa cells showed a minor but significant and dose dependent decrease in formation of 19-hydroxyandrostenedione under the influence of ketoconazole [38]. The fact that the inhibition of the first hydroxylation of androstenedione at the C_{19} position was not as strong as the overall inhibition of estrogen biosynthesis points to a further inhibitory effect of ketoconazole on the following steps of the aromatase enzyme system. This is in accordance to the results of Watanabe *et al.* who found an inhibition of all three sequential hydroxylation steps in rat ovarian tissue aromatase [35].

In analogy to rat and ovine testicular aromatase, human ovarian aromatase seems to be less sensitive to an inhibition by ketoconazole than the enzymes of androgen biosynthesis, namely the $C_{17,20}$-desmolase [1, 2]. While the human ovarian $C_{17,20}$-desmolase activity in our *in vitro* experiments is blocked significantly at concentrations that are well in the range of plasma levels during high dose treatment with ketoconazole [39], the human ovarian aromatase is blocked 50% only at nearly millimolar concentrations of ketoconazole. Therefore it remains open whether the slight decrease in serum estrogen levels observed in humans treated with ketoconazole is due to a direct inhibitory effect of ketoconazole on the aromatase or only secondary to the decrease in their androgen precursors [19–23]. Other authors found no significant decrease or even a slight increase in serum estrogen levels after the administration of ketoconazole [16–18]. This different sensitivity of androgen and estrogen biosynthesis to the blocking effect of ketoconazole is the most likely reason for an elevated estrogen/androgen ratio, observed in some patients who developed gynecomasty during ketoconazole treatment [16].

In comparison to 4-hydroxyandrostenedione, a well-known aromatase inhibitor, ketoconazole is much less effective *in vitro* as well as *in vivo* [36, 38, 40]. Thus a therapeutical use of the antimycotic drug as an aromatase inhibitor does not seem to be very promising. This is in accordance to the findings of Harris *et al.* who treated 14 postmenopausal women with advanced breast cancer with ketoconazole 1200 mg/day [20]. While serum testosterone levels were suppressed significantly, there was only a small decrease in serum estradiol levels and estrone concentration remained unchanged. A partial clinical response was found only in one patient treated in combination with aminoglutethimide.

In contrast to the weak inhibitory effect of ketoconazole on estrogen biosynthesis, the strong suppressing effect of ketoconazole on androgen biosynthesis in human adrenals and testes has been used successfully in the treatment of advanced prostatic cancer [9, 10], androgen producing adrenal tumors [27, 41] and precocious puberty [11]. With regard to our results of a strong inhibition of androgen biosynthesis also in the human ovary, further studies on the treatment of hyperandrogenic states of ovarian origin with ketoconazole are encouraged.

In conclusion, our findings have shown for the first time that ketoconazole blocks human ovarian $C_{17,20}$-desmolase and aromatase [42]. As in human adrenals and testis, ovarian $C_{17,20}$-desmolase seems to be most sensitive to an inhibitory effect of ketoconazole.

REFERENCES

1. Vanden Bossche H., Willemsens G., Bellens D., Roels I. and Janssen P. A.: From 14α-demethylase inhibitors in fungal cells to androgen and estrogen biosynthesis inhibitors in mammalian cells. *Biochem. Soc. Trans.* **18** (1990) 10–13.
2. Albertson B. D., Frederick K. L., Maronian N. C., Feuillan P., Schorer S., Dunn J. F. and Loriaux D. L.: The effect of ketoconazole on steroidogenesis: I. Leydig cell enzyme activity *in vitro*. *Res. Commun. Chem. Path. Pharmac.* **61** (1988) 17–26.
3. Ayub M. and Levell M. J.: Inhibition of human adrenal steroidogenic enzymes *in vitro* by imidazole drugs including ketoconazole. *J. Steroid Biochem.* **32** (1989) 515–524.
4. Rajfer J., Sikka S. C., Rivera F. and Handelsmann D. J.: Mechanism of inhibition of human testicular steroidogenesis by oral ketoconazole. *J. Clin. Endocr. Metab.* **63** (1986) 1193–1198.
5. Weber M. M., Abedinpour F., Luppa P. and Engelhardt D.: Inhibition of adrenal androgen biosynthesis by ketoconazole *in vivo* and *in vitro*. *Acta Endocr. Copenh.* **120** (Suppl. 1) (1989) 114.
6. Albertson B. D., Maronian N. C., Frederick K. L., DiMattina M., Feuillan P., Schorer S., Dunn J. F. and Loriaux D. L.: The effect of ketoconazole on steroidogenesis: II. Adrenocortical enzyme activity *in vitro*. *Res. Commun. Chem. Path. Pharmac.* **61** (1988) 27–34.
7. Miksch T. and Engelhardt D.: Inhibition of human adrenal 17α-hydroxylase and 16α-hydroxylase by ketoconazole. *Acta Endocr. Copenh.* **109** (Suppl. 270) (1985) 39.
8. Zeller M. and Engelhardt D.: Inhibition of $C_{17,20}$desmolase-, 17α-hydroxylase- and 16α-hydroxylase activity in human testis by ketoconazole. *Acta Endocr. Copenh.* **108** (Suppl. 267) (1985) 99–100.
9. Trachtenberg J. and Pont A.: Ketoconazole therapy for advanced prostatic cancer. *Lancet* **II** (1984) 433–435.
10. Williams G., Kerle D. J., Ware H., Doble A., Dunlop H., Smith C., Allen J., Yeo T. and Bloom S. R.: Objective responses to ketoconazole therapy in patients with relapsed progressive prostatic cancer. *Br. J. Urol.* **58** (1986) 45–51.

11. Holland F. J., Fishman L., Bailey J. D. and Fazekas A. T. A.: Ketoconazole in the management of precocious puberty not responsive to LHRH-analogue therapy. *N. Engl. J. Med.* **312** (1985) 1023–1028.
12. Engelhardt D., Jacob K. and Doerr H. G.: Different therapeutic efficacy of ketoconazole in patients with Cushing's syndrome. *Klin. Wochenschr.* **67** (1989) 241–247.
13. Loli P., Berselli M. A. and Tagliaferri M.: Use of ketoconazole in the treatment of Cushing's syndrome. *J. Clin. Endocr. Metab.* **63** (1986) 1365–1371.
14. McCance D. R., Hadden D. R., Kennedy L., Sheridan B. and Atkinson A. B.: Clinical experience with ketoconazole as a therapy for patients with Cushing's syndrome. *Clin. Endocr.* **27** (1987) 593–599.
15. Sonino N., Boscaro M., Merola G. and Mantero F.: Prolonged treatment of Cushing's disease by ketoconazole. *J. Clin. Endocr. Metab.* **61** (1985) 718–722.
16. Heyns W., Drochmans A., vander Schueren E. and Verhoeven G.: Endocrine effects of high-dose ketoconazole therapy in advanced prostatic cancer. *Acta Endocr. Copenh.* **110** (1985) 276–283.
17. Martikainen H., Heikkinen J., Ruokonen A. and Kauppila A.: Hormonal and clinical effects of ketoconazole in hirsute women. *J. Clin. Endocr. Metab.* **66** (1988) 987–991.
18. Pepper G. M., Poretsky L., Gabrilove J. L. and Ariton M. M.: Ketoconazole reverses hyperandrogenism in a patient with insulin resistance and acanthosis nigricans. *J. Clin. Endocr. Metab.* **65** (1987) 1047–1052.
19. De Coster R., Caers I., Haeltermann C. and Debroye M.: Effect of a single administration of ketoconazole on total and physiologically free plasma testosterone and 17β-oestradiol levels in healthy male volunteers. *Eur. J. Clin. Pharmac.* **29** (1985) 489–493.
20. Harris A. L., Cantwell B. M. J. and Dowsett M.: High dose ketoconazole: endocrine and therapeutic effects in postmenopausal breast cancer. *Br. J. Cancer* **58** (1988) 493–496.
21. Medda F., Puligheddu P., Parodo G., Marcello C., Aste N., Pascalis P., Pia G. and Lecca U.: Short term treatment with ketoconazole: effects on gonadal and adrenal steroidogenesis in women. *Clin. Exp. Obstet. Gynec.* **14** (1987) 161–166.
22. Pont A., Goldmann E. S., Sugar A. M., Siiteri P. K. and Stevens D. A.: Ketoconazole-induced increase in estradiol–testosterone ratio. *Arch. Int. Med.* **145** (1985) 1429–1431.
23. Wouters W., de Coster R., Goeminne N., Beerens D. and van Dun J.: Aromatase inhibition by the antifungal ketoconazole. *J. Steroid Biochem.* **30** (1988) 387–389.
24. Kolena J., Kiss A. and Channing C. P.: Purification of porcine granulosa cells by continuous Percoll gradient. *Experientia* **39** (1983) 908–909.
25. Labarca C. and Paigen K.: A simple, rapid and sensitive DNA assay procedure. *Anal. Biochem.* **102** (1980) 344–352.
26. Malozowski S., Mericq V. and Cassorla F.: Effects of ketoconazole on rat ovarian steroidogenic enzymatic activities. *Res. Commun. Chem. Path. Pharmac.* **61** (1988) 27–34.
27. Verhelst J. A., Druwé P., van Erps P., Denis L. and Mahler C.: Use of ketoconazole in the treatment of a virilizing adrenocortical carcinoma. *Acta Endocr. Copenh.* **121** (1989) 229–234.
28. Hillier S. G., van den Bogaard A. M. J., Reichert L. E. and van Hall E. V.: Intraovarian sex steroid hormone interactions and the regulation of follicular maturation: aromatization of androgens by human granulosa cells *in vitro*. *J. Clin. Endocr. Metab.* **50** (1980) 640–647.
29. McNatty K. P.: Hormonal correlates of follicular development in the human ovary. *Aust. J. Biol. Sci.* **34** (1981) 249–268.
30. Moon Y. S., Tsang B. K., Simpson C. and Armstrong D. T.: 17β-estradiol biosynthesis in cultured granulosa and theca cells of human ovarian follicles: stimulation by follicle-stimulating hormone. *J. Clin. Endocr. Metab.* **47** (1978) 263–267.
31. Ayub M. and Stitch S. R.: Effect of ketoconazole on placental aromatase, 3β-hydroxysteroid dehydrogenase-isomerase and 17β-hydroxysteroid dehydrogenase. *J. Steroid Biochem.* **25** (1986) 981–984.
32. Brodie A. M. H.: Recent advances in studies on estrogen biosynthesis. *J. Endocr. Invest.* **2** (1979) 445–460.
33. France J. T., Mason J. I., Magness R. R., Murry B. A. and Rosenfeld C. R.: Ovine placental aromatase: studies of activity levels, kinetic characteristics and effects of aromatase inhibitors. *J. Steroid Biochem.* **28** (1987) 155–160.
34. Mason J. I., Murry B. A., Olcott M. and Sheets J. J.: Imidazole antimycotics: inhibitors of steroid aromatase. *Biochem. Pharmac.* **34** (1985) 1087–1092.
35. Watanabe H. and Menzies J. A.: Inhibition of ovarian aromatase by ketoconazole. *Res. Commun. Chem. Pathol. Pharmac.* **54** (1986) 181–189.
36. Mason J. I., Carr B. R. and Murry B. A.: Imidazole antimycotics: selective inhibitors of steroid aromatization and progesterone hydroxylation. *Steroids* **50** (1987) 179–189.
37. DiMattina M., Maronian N., Ashby H., Loriaux D. L. and Albertson B. D.: Ketoconazole inhibits multiple steroidogenic enzymes involved in androgen biosynthesis in the human ovary. *Fert. Ster.* **49** (1988) 62–65.
38. Khalil M. W., Morley P., Glasier M. A., Armstrong D. T. and Lang T.: Formation of 4-oestrene-3,17dione (19-norandrostenedione) by porcine granulosa cells *in vitro* is inhibited by the aromatase inhibitor 4-hydroxyandrostenedione and the cytochrome *P*-450 inhibitors aminoglutethimide phosphate and ketoconazole. *J. Endocr.* **120** (1989) 251–260.
39. Van Tyle J. H.: Ketoconazole: mechanism of action, spectrum of activity, pharmacokinetics, drug interaction, adverse reactions and therapeutic use. *Pharmacotherapy* **4** (1984) 343–373.
40. Koos R. D., LeMaire W. J., Hung T. T. and Brodie A. M. H.: Comparison of the effect of 4-hydroxy-4-androstene-3,17-dione on aromatase activity in granulosa cells from preovulatory follicles of rats, rabbits and humans. *Steroids* **45** (1985) 143–150.
41. Weber M. M., Luppa P. and Engelhardt D.: Inhibition of human adrenal androgen secretion by ketoconazole. *Klin. Wochenschr.* **67** (1989) 707–712.
42. Weber M. M., Will A. and Engelhardt D.: Ketoconazole blocks estrogen biosynthesis in the human ovary. *J. Steroid Biochem.* **33** (Suppl.) (1989) 12S.