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IN VITRO INHIBITION BY KETOCONAZOLE OF HUMAN TESTICULAR STEROID OXIDOREDUCTASES

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Summary—An oral antimycotic agent, ketoconazole has been demonstrated to be an inhibitor of cytochrome *P*-450-dependent monooxygenases. To investigate its effect on steroid oxidoreductases, *in vitro* studies were carried out using subcellular fractions of human testes. Ketoconazole competitively inhibited activities of 3β-hydroxy-5-ene-steroid oxidoreductase/isomerase and NADH-linked 20α-hydroxysteroid oxidoreductase for steroid substrate and the K_i values were 2.9 and 0.9 μM, respectively. In contrast, ketoconazole inhibited neither 17β-hydroxysteroid oxidoreductase nor NADPH-linked 20α-hydroxysteroid oxidoreductase, indicating that the two 20α-hydroxysteroid oxidoreductases are distinct. Further, ketoconazole inhibited non-competitively the above enzyme activities for the corresponding cofactors of NAD and NADH. From the binding mode of ketoconazole to cytochrome *P*-450 and the present findings, it appears likely that the agent binds to a site which is different from that of steroids or pyridine nucleotides.

INTRODUCTION

An antifungal imidazole derivative, ketoconazole (cis-1-acetyl-4-{4-[[2-(2,4-dichlorophenyl)-2-(1H-imidazole-1-ylmethyl)-1,3-dioxalan-4-yl]methoxy]phenyl}piperazine) inhibits the production of ergosterol in the yeast cell by blocking the 14α-demethylation of lanosterol [1]. It has been reported to inhibit various enzymes which belong to cytochrome *P*-450-dependent monooxygenases in rodents and humans such as side-chain cleavage of cholesterol and 11β-hydroxylase in the rat adrenal gland [2, 3] and also 17α-hydroxylase and C17–20 lyase in rat and human testes [4–7]. Recently, the authors suggested that the agent binds directly to cytochrome *P*-450 on the basis of steroid kinetic studies and spectrophotometric studies using human and rat testes, human and rat adrenal tissues and rat liver [7]. The current study describes the effect of ketoconazole on oxidoreductases which are also important enzymes in steroid biosynthesis in the human testis.

MATERIALS AND METHODS

Chemicals

[4-^{14}C]Pregnenolone (SA, 57 mCi/mmol), [4-^{14}C]-progesterone (SA, 57 mCi/mmol), [4-^{14}C]androstenedione (SA, 52 mCi/mmol) and Econofluor were purchased from New England Nuclear Corp. (Boston, Mass). Non-radioactive steroids, NAD, NADH and NADPH were products of Sigma Chemical Co. (St Louis, Mo.). Ketoconazole was supplied from Kyowa Hakko Co. (Tokyo, Japan). All other reagents were of analytical grade and all organic solvents were redistilled before use.

Tissue preparations

Human testicular tissue was obtained from patients at orchiectomy for treatment of prostatic carcinoma. None had received any previous hormonal treatment.

The removed tissue was immediately frozen in liquid nitrogen and stored at −70°C until use. The frozen tissue was allowed to thaw at 4°C, and then homogenized with a Ultra Turrax homogenizer (Ika-Werk Co., West Germany) in 5 volumes of Tris–sucrose buffer solution (pH 7.4) containing 0.25 M sucrose, 50 mM Tris, and 5 mM $MgCl_2$.

The microsomal and soluble fractions were prepared by conventional differential centrifugation at 10,000–105,000 *g* for 60 min. The centrifugation was repeated twice and thus obtained supernatant and precipitate resuspended in ice-cold Tris–sucrose buffer solution were used as enzyme preparations. All procedures were performed at 0–4°C. Glucose-6-phosphate phosphohydrolase [8] and glucose-6-phosphate dehydrogenase [9] were measured as respective marker enzymes of microsomal and soluble fractions to verify the purity of the tissue preparation.

Assay of enzymes related to steroid biosynthesis

Incubations. All incubations were carried out in duplicate in the presence or absence of ketoconazole.

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The incubation medium contained one of tissue preparations, a ^{14}C-labeled steroid substrate, 0.25 M sucrose, 50 mM Tris (pH 7.4), 5 mM $MgCl_2$ and corresponding pyridine nucleotide. Concentrations of each steroid substrate and ketoconazole were indicated in legends to figures. The final volume of incubation medium was adjusted to 3 ml. The specific radioactivity of each steroid substrate was adjusted with corresponding authentic non-radioactive steroids to obtain the desired substrate concentration. Incubations were carried out at 37°C for 20 min under room air. Enzyme reactions were started by adding the tissue preparation and terminated by vigorous shaking of the incubation flask after adding 10 ml methylene dichloride.

Extraction, separation and identification of steroids. After adding non-radioactive carrier steroids (100 μg, each), extraction was repeated three times with 10 ml methylene dichloride. The combined extracts were dried over anhydrous sodium sulphate and concentrated at 40°C under reduced pressure. The residue was applied to an ascending thin-layer chromatography of silica gel and GF 254 (4:1, w/w) with the solvent system of benzene and acetone (4:1, v/v). Carrier and radioactive steroids on the thin-layer plate were detected under u.v. light (254 μm) and autoradiography, and exhaustively eluted from silica gel with a mixture of chloroform and ethanol (1:1, v/v).

Identification of metabolites was accomplished by demonstrating constant specific radioactivities through repeated recrystallization with corresponding authentic preparations. Once identification and purity of the products were established as above, the metabolites obtained in later experiments under similar conditions were identified using a combined method of chemical derivative formation and chromatographic mobilities [10].

Expression of enzyme activities. Enzyme activities were expressed as the amount of metabolite produced by its action on the substrate. When pregnenolone was incubated with microsomal fraction in the presence of NAD, the major metabolite detected on the autoradiogram was progesterone. Therefore, activities of NAD-linked 3β-hydroxy-5-ene-steroid oxidoreductase (3β-hydroxyteroid:NAD oxidoreductase) coupled with isomerase was expressed as the amount of progesterone produced from pregnenolone. Metabolites produced from progesterone in microsomal fraction was 20α-hydroxyprogesterone and 17α-hydroxyprogesterone in the presence of NADH. 20α-Hydroxyprogesterone was a sole detectable metabolite in soluble fraction in the presence of NADPH. The amount of each 20α-hydroxyprogesterone indicated the activities of NADH-linked 20α-hydroxysteroid oxidoreductase on microsome and NADPH-linked 20α-hydroxysteroid oxidoreductase in soluble fraction for progesterone. Since testosterone was a sole major metabolite from substrate androstenedione in human microsome fraction, its amount was employed as the activity of 17β-hydroxysteroid oxidoreductase (17β-hydroxysteroid:NADP 17β-oxidoreductase). Over 80% of substrate remained unconverted in each incubation flask, indicating saturation of enzymes by the substrate. Further, enzyme activities expressed as above showed linear relationships to either incubation time up to 30 min or added amount of tissue preparations under the conditions employed in the present study.

Quantitation of steroids and protein

Radioactivity of each steroid fraction was measured with a liquid scintillation spectrometer (Packard, TRI-CARB 460) in the scintillation cocktail Econofluor. The amount of each metabolite was calculated from its percent yield and the specific radioactivity of the substrate. Over 90% of the initial radioactivity was recovered.

The protein content in each tissue preparation was determined by the methods of Lowry *et al.* [11].

RESULTS

Effect of ketoconazole on activities of human testicular oxidoreductases

As shown in Fig. 1, ketoconazole markedly inhibited the activities of NAD-linked 3β-hydroxy-5-ene-steroid oxidoreductase for pregnenolone and NADH-linked 20α-hydroxysteroid oxidoreductase for progesterone. In contrast, no inhibition was observed for NADPH-linked 17β-hydroxysteroid

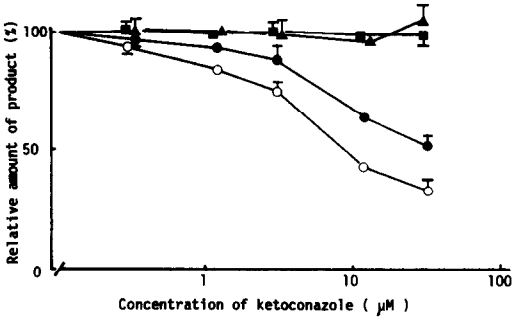

Fig. 1. Effect of ketoconazole on the human testicular steroid oxidoreductase activities. Microsome fractions and required pyridine nucleotides were incubated in duplicate with either 10.8 μM pregnenolone, 10.9 μM progesterone or 11.9 μM androstenedione in the presence of 0, 0.31, 1.3, 3.1. 12.5 or 31.3 μM ketoconazole. The soluble fraction and 10.9 μM substrate progesterone were incubated in the same manner. Experiments were repeated twice using enzyme preparations obtained from different patients. The protein concentration of microsome and soluble fractions for the first experiment were 6.0 and 7.3 mg/flask and for the second experiment were 5.8 and 7.0 mg/flask, respectively. Symbols show the mean value of duplicated incubations and bars represent standard errors obtained from two independent experiments. ○; progesterone (3β-hydroxy-5-ene-steroid oxidoreductase), ●; 20α-hydroxyprogesterone (NADH-linked 20α-hydroxysteroid oxidoreductase), ■; 20α-hydroxyprogesterone (NADPH-linked 20α-hydroxysteroid oxidoreductase) and ▲; testosterone (17β-hydroxysteroid oxidoreductase).

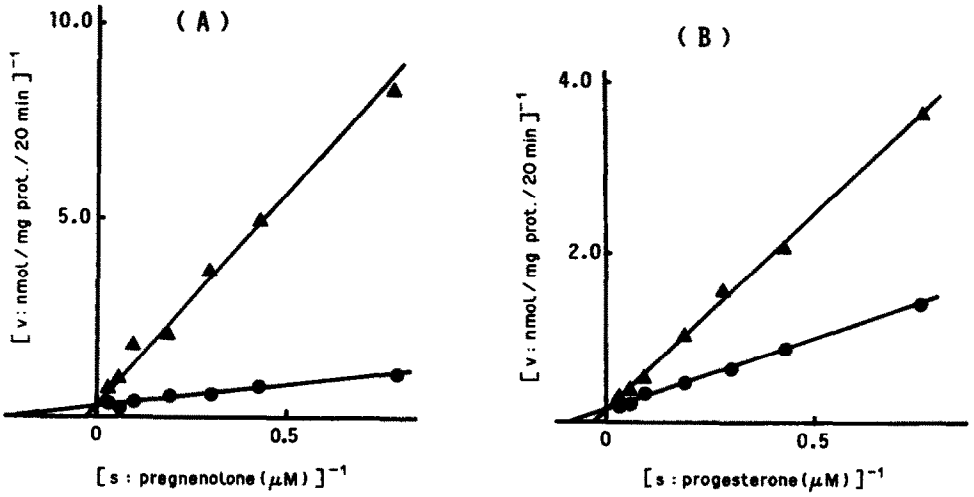

Fig. 2. Competitive inhibition by ketoconazole of NAD-linked 3β-hydroxy-5-ene-steroid oxidoreductase activity for pregnenolone (A) and NADH-linked 20α-hydroxysteroid oxidoreductase for progesterone (B). Microsome fractions containing 5.6 mg protein/flask were incubated with each substrate in the absence (●) or presence (▲) of 31.3 μM ketoconazole. The concentrations of NAD (A) and NADH (B) were 0.50 and 0.47 mM, respectively.

oxidoreductase for androstenedione and 20α-hydroxysteroid oxidoreductase for pregesterone by ketoconazole at concentrations examined.

Mode of inhibition by ketoconazole of oxidoreductase activities for steroid substrates and for pyridine nucleotides

Figure 2 shows competitive inhibition by ketoconazole of NAD-linked 3β-hydroxy-5-ene steroid oxidoreductase activity for pregnenolone and NADH-linked 20α-hydroxysteroid oxidoreductase activity for progesterone by the Lineweaver–Burk plot. The Michaelis constant (K_m) and the inhibition constant (K_i) calculated from Fig. 2 were tabulated in Table 1. ketoconazole non-competitively inhibited the above two oxidoreductase activities for pyridine-nucleotides (Fig. 3). The difference in V_{max} between Figs 2 and 3 might be ascribed to different storage period of tissue preparations. K_i of ketoconazole against 3β-hydroxy-5-ene-steroid oxidoreductase for NAD and 20α-hydroxysteroid oxidoreductase for NADH were 23 and 730 μM, respectively (Table 2).

DISCUSSION

Present study demonstrates that ketoconazole inhibits NAD-linked 3β-hydroxy-5-ene-steroid oxidoreductase for pregnenolone and NADH-linked 20α-hydroxysteroid oxidoreductase for progesterone in the human testis. The inhibition of 3β-hydroxy-5-ene-steroid oxidoreductase by ketoconazole is consistent with the suppression of rat testicular 3β-hydroxy-5-ene-steroid oxidoreductase *in vivo* [12] and of human ovarian and adrenal 3β-hydroxy-5-ene-steroid oxidoreductase *in vitro* [13, 14]. Some authors, however, report that ketoconazole did not inhibit human placental or rat testicular 3β-hydroxy-5-ene-steroid oxidoreductase activity [6, 15]. The controversy appears to be caused by organ specificity of the enzyme or conditions to measure enzyme activities.

Human testicular monooxygenase activities are reduced to less than 50% by 3.1 μM ketoconazole and K_i values of ketoconazole to the monooxygenases for steroid substrates are in the order of 10^{-8} M [7]. Since K_i values of ketoconazole to oxidoreductase are in the order of 10^{-7}–10^{-6} M, ketoconazole appears to have less affinity to oxidoreductases than to monooxygenases. The difference in K_i values between the two enzyme groups with similar K_ms for steroid substrate further suggests different binding sites for ketoconazole and steroid substrate on oxidoreductase as observed in monooxygenases.

Table 1. Mode of inhibition by ketoconazole of testicular steroid oxidoreductases for steroids

Oxidoreductases (tissue prep.)	Substrates	Cofactors	K_m (μM)	Inhibition by ketoconazole	
				K_i (μM)	Type
3β-Hydroxy-5-ene-steroid (microsome)	Pregnenolone	NAD	4.6	2.9	Competitive
20α-Hydroxysteroid (microsome)	Progesterone	NADH	11.8	0.9	Competitive
20α-Hydroxysteroid (soluble)	Progesterone	NADPH	n.d.		Not inhibited
17β-Hydroxysteroid (microsome)	Androstenedione	NADPH	n.d.		Not inhibited

n.d.: not determined.

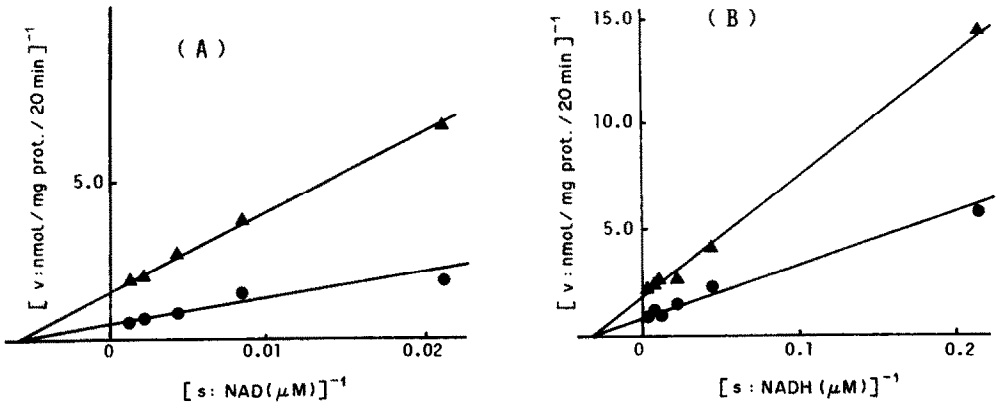

Fig. 3. Non-competitive inhibition by ketoconazole of 3β-hydroxy-5-ene-steroid oxidoreductase activity for NAD (A) and 20α-hydroxysteroid oxidoreductase activity for NADH (B). Microsome fraction containing 2.9 mg protein/flask were incubated with each substrate in the absence (●) or presence (▲) of 31.3 μM ketoconazole. The concentrations of pregnenolone (A) and progesterone (B) were 10.8 and 10.9 μM, respectively.

Table 2. Mode of inhibition by ketoconazole of testicular steroid oxidoreductases for pyridine nucleotides

Oxidoreductases (tissue prep.)	Substrates	K_m (μM)	Inhibition by ketoconazole	
			K_i (μM)	Type
3β-Hydroxy-5-ene-steroid (microsome)	NAD	170	23	Non-competitive
20α-Hydroxysteroid (microsome)	NADH	31	730	Non-competitive

In the present study, ketoconazole does not inhibit 17β-hydroxysteroid oxidoreductase activity for androstenedione. The result is consistent with a previous finding that ketoconazole has revealed no significant inhibition *in vitro* on 17β-hydrosteroid oxidoreductase activity in the minced rat testicular tissue [4] and in the human placental microsome [15]. However, oral [12] or intramuscular [16] administration of high-dose ketoconazole has resulted in a decrease of rat testicular 17β-hydroxysteroid oxidoreductase activity. The discrepancy appears to be attributable to the reduced amount of the enzyme after long exposure to ketoconazole.

It is of interest the ketoconazole has an inhibitory effect on human testicular microsomal 20α-hydroxysteroid oxidoreductase activity but not on the soluble one. The microsomal 20α-hydroxsteroid oxidoreductase has substrate affinity to pregnenolone and pregesterone and prefers NADH as the hydrogen donor [17], while 20α-hydroxysteroid oxidoreductase in the soluble fraction has substrate affinity to 17α-hydroxypregnen compounds in addition to the above two steroids and is NADPH-dependent [18]. The present result offers further evidence that two 20α-hydroxysteroid oxidoreductases are distinct.

The current study demonstrates competitive inhibition by ketoconazole of NAD-linked 3β-hydroxy-5-ene-steroid oxidoreductase and NADH-linked 20α-hydroxysteroid oxidoreductase activities for steroid substrates and non-competitive inhibition of these enzyme activities for pyridine nucleotides. Ketoconazole binds to cytochrome *P*-450 inducing type II difference spectra [7, 19] and inhibits cytochrome *P*-450-dependent monooxygenase activities in the same mode [7] as observed above. Since induction of type II difference spectra indicates tight binding of ketoconazole to cytochrome *P*-450 [7, 19], it appears likely that ketoconazole binds to a site different to those of both steroids and pyridine nucleotides on monooxygenases and some steroid oxidoreductases.

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