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INHIBITORY EFFECT OF SOME IMIDAZOLE ANTIFUNGAL COMPOUNDS ON THE SYNTHESIS OF 16-ENE-C_{19}-STEROID CATALYZED BY PIG TESTICULAR MICROSOMES

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Summary—The activity of the enzyme (16-ene-C_{19}-steroid synthesizing enzyme) responsible for the conversion of C_{21}-steroids to 16-ene-C_{19}-steroids, which was localized on pig testicular microsomes, was inhibited by some typical imidazole antifungal compounds such as clotrimazole, econazole, miconazole and ketoconazole which are known to be universal inhibitors of cytochrome *P*-450-dependent enzymes. The 50% inhibitory concentrations of clotrimazole, econazole and miconazole were 0.29, 0.36 and 1.25 μM, respectively for 16-ene-C_{19}-steroid synthesizing enzyme activity. Clotrimazole was the most powerful inhibitor of all the compounds examined, which shows the competitive inhibition for 16-ene-C_{19}-steroid synthesizing enzyme activity. The K_i-value was 0.26 μM for its activity. The degree of the inhibition by these imidazole compounds was very similar to the inhibition of 17α-hydroxylase and $C_{17,20}$-lyase activities on pig testicular microsomes.

INTRODUCTION

The 16-ene-C_{19}-steroids are very hydrophobic steroids which are volatile in nature, have a musk-like odor, are now known to be sex attractants in the pig and are synthesized in the testis [1, 2]. 16-ene-C_{19}-steroids, such as androstadienone or androstadienol, which are synthesized by the side-chain cleavage of C_{21}-steroids (progesterone or pregnenolone) and the enzyme (16-ene-C_{19}-steroids synthesizing enzyme) responsible for conversion of C_{21}-steroids to 16-ene-C_{19}-steroids, are found in the microsomes of pig testis [2, 3]. On the other hand, the synthesis of androgens by the testis involves the side-chain cleavage of C_{21}-steroid (i.e. progesterone or pregnenolone) to give C_{19}-steroids (i.e. androstenedione or dehydroepiandrosterone, respectively) by a microsomal enzyme. The enzyme is the cytochrome *P*-450-linked oxygenase system and we have previously purified a microsomal *P*-450 from neonatal pig testis [4]. The purified protein is capable of catalyzing 17α-hydroxylation followed by $C_{17,20}$-lyase cleavage reactions [5].

We previously reported the first evidence that the 16-ene-C_{19}-steroids synthesizing enzyme catalyzed by pig testicular microsomes is also the cytochrome *P*-450-linked oxygenase system including cytochrome *P*-450, cytochrome *P*-450 reductase and cytochrome b_5 [6, 7].

In the present paper, we have further evidence that the 16-ene-C_{19}-steroids synthesizing enzyme is a cytochrome *P*-450-linked oxygenase system, as judged by inhibition by some imidazole antifungal agents, which are known to be universal inhibitors of cytochrome *P*-450-dependent enzymes.

EXPERIMENTAL

Materials

[4-^{14}C]Progesterone (2.12 GBq, 57.2 mCi/mmol) and 17α-hydroxy[4-^{14}C]progesterone (1.96 GBq, 53.0 mCi/mmol) were purchased from New England Nuclear Corp. (Boston, Mass, U.S.A.), and their purity was checked by TLC, developed with ethylacetate–hexane (3:7, v/v) before use. Progesterone, 17α-

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Abbreviations: androstadienol, androsta-5,16-dien-3β-ol; androstadienone, androsta-4,16-dien-3-one; clotrimazole, 1-[(2-chlorophenyl)diphenylmethyl]-1H-imidazole; econazole, 1-[2-[(4-chlorophenyl)methoxy]-2-(2,4-dichlorophenyl)ethyl]-1H-imidazole; miconazole, 1-[2-(2,4-dichlorophenyl)-2-[(2,4-dichlorophenyl)methoxy]ethyl-1H-imidazole; urocanic acid, 3-(1H-imidazol-4-yl)-2-propenoic acid, KPB, potassium phosphate buffer; TLC, thin-layer chromatography.

hydroxyprogesterone, clotrimazole (±)-miconazole nitrate, econazole, cimethidine and NADPH were purchased from Sigma Chemical Co. (St Louis, Mo, U.S.A.). Ketoconazole was a generous gift from Kyowa Hakko (Tokyo, Japan). Other reagents were of the best grade available and purchased from Iwai Chemicals (Tokyo, Japan).

Androstadienone was synthesized by the method of Barton *et al.* [8].

Preparation of testicular microsomes

Immature pig testes (10 days of age) were obtained at castration. The testes were decapsulated and homogenized in 0.15 M KCl–0.1 mM EDTA using a Waring blender. The homogenate was centrifuged at 9000 *g* for 60 min and the resulting supernatant was again centrifuged at 10,500 *g* for 60 min. After being washed with 100 mM KPB–1.0 mM EDTA, pH 7.4, the microsomal pellet was resuspended in 20 mM KPB–20% (v/v) glycerol–0.1 mM EDTA, pH 7.4 (22 mg protein/ml). The microsomes were stored at −80°C. Specific contents of cytochromes or electron carriers were 0.43 nmol/mg protein for cytochrome *P*-450, 2.2 nmol/mg protein for cytochrome b_5, 0.06 U/mg protein for cytochrome *P*-450-reductase and 10.1 U/mg protein for cytochrome b_5-reductase

Enzyme assay

16-ene-C_{19}-Steroid synthesizing enzyme activity and 17α-hydroxylase activity were measured by determining the amount of radioactive material formed from [4-^{14}C]progesterone (5 nmol, 370 Bq/10 μl of ethanol solution). The measurement of $C_{17,20}$-lyase activity was carried out using 17α-hydroxy[4-^{14}C]progesterone (5 nmol, 370 Bq/10 μl of ethanol solution) as the substrate.

The substrate was incubated with microsomes (320 μg) in the presence of NADPH (240 nmol) in a total volume of 1 ml of 100 mM KPB, pH 7.0 at 37°C. After the incubation (10 min), the reaction was stopped by the addition of methylene dichloride (10 ml) and the steroids were extracted. Then, as carrier steroids, progesterone, 17α-hydroxyprogesterone, androstenedione and androstadienone (5 μg each, in ethanol) were added to the extract. The solvent was evaporated off and a portion of the residue was redissolved in a small amount of methylene dichloride and subjected to TLC (plate: Kodak, 13181 silica gel), being developed with ethylacetate–hexane (3:7, v/v). After the observation of the TLC plate under u.v. light and/or radioautography (Fuji X-ray film, Rx), the relevant radioactive areas of the chromatograms were cut off and ^{14}C was measured with a liquid scintillation counter (Packard Tri-Carb 460C).

16-ene-C_{19}-Steroid synthesizing activity was determined as ^{14}C present in the fractions corresponding to androstadienone as the product from the substrate, progesterone. 17α-Hydroxylase activity, according to the previous report [4], was determined as the total radioactivity in the 17α-hydroxyprogesterone and androstenedione fractions. $C_{17,20}$-Lyase activity was determined as the radioactivity in the fractions corresponding to androstenedione as the product from the substrate, 17α-hydroxyprogesterone. The enzyme activities were corrected for recovery based on the ratio of total ^{14}C counts of all areas of the TLC plate to the ^{14}C counts of radioactive steroid added to the incubation medium as the substrate.

The kinetic parameters were calculated from Lineweaver–Burk plots which were obtained by plotting the reciprocals of the apparent enzyme activities against the reciprocals of the concentration of substrate (2–10 μM).

Miscellaneous

Protein concentrations were estimated by the method of Lowry *et al.* [9] using crystalline bovine serum albumin (Armour Pharmaceutical Co., Fraction V) as a standard. Cytochrome *P*-450 and cytochrome b_5 were measured as described by Omura and Sato [10] and Strittmatter *et al.* [11], respectively. Cytochrome *P*-450 reductase was measured by the method of Omura and Takesue [12] and cytochrome b_5-reductase was measured by the method of Takesue and Omura [13].

RESULTS

The effect of imidazole ring-containing compounds on the 16-ene-C_{19}-steroid synthesizing enzyme, 17α-hydroxylase and $C_{17,20}$-lyase activities were examined by incubation with the microsomes. Table 1 shows the effect of these compounds on the enzyme activities at the same 10 μM concentration. Imidazole antifungal compounds, such as clotrimazole, econazole, miconazole and ketoconazole markedly inhibited the 16-ene-C_{19}-steroid synthesizing enzyme, 17α-hydroxylase and $C_{17,20}$-lyase activities, but

Table 1. Effect of imidazole ring-containing compounds on 16-ene-C_{19}-steroid synthesizing enzyme, 17α-hydroxylase and $C_{17,20}$-lyase activities of pig testicular microsomes

Compound (100 μM)	Enzyme activities (% of control)		
	16-ene-[a]	OHase[b]	Lyase[c]
Clotrimazole	<1.0	1.9	7.0
Econazole	<1.0	3.4	4.8
Miconazole	<1.0	4.0	6.7
Ketoconazole	2.6	7.7	8.2
Imidazole	96.0	98.0	102.2
Urocanic acid	100.7	95.5	88.0
Benzimidazole	109.2	99.5	93.8
Cimethidine	113.6	94.0	97.6

[a]16-ene-C_{19}-steroid synthesizing enzyme activity.
[b]17α-hydroxylase activity.
[c]$C_{17,20}$-lyase activity.
The details of the enzyme assay are given in the text.

other compounds did not. The inhibitory effect of some imidazole antifungal compounds were further examined at various concentrations, as shown in Fig. 1. The degree of inhibition of 16-ene-C_{19}-steroid synthesizing enzyme activity by clotrimazole, econazole or miconazole was similar to that of 17α-hydroxylase and $C_{17,20}$-lyase activities.

The 50% inhibitory concentrations (IC_{50}) of clotrimazole were 0.29 μM for the 16-ene-C_{19}-steroid synthesizing enzyme, 0.37 μM for 17α-hydroxylase, and 0.20 μM for $C_{17,20}$-lyase. Furthermore, the IC_{50} of econazole and miconazole were 0.36 and 1.25 μM, respectively for the 16-ene-C_{19}-steroid synthesizing enzyme, 0.59 and 1.48 μM for 17α-hydroxylase, and 0.18 and 0.54 μM for $C_{17,20}$-lyase. For each of the enzyme activities, the control values were 0.1 nmol/min/mg protein for 16-ene-C_{19}-steroid synthesizing enzyme activity, 0.29 nmol/min/mg protein for 17α-hydroxylase activity and 0.60 nmol/min/mg protein for $C_{17,20}$-lyase activity.

Figure 2 shows the Lineweaver–Burk plots for the 16-ene-C_{19}-steroid synthesizing enzyme, 17α-hydroxylase and $C_{17,20}$-lyase activities of pig testicular microsomes in the absence or presence of clotrimazole. It can be seen that clotrimazole shows competitive inhibition of these enzyme activities.

The K_i-values of the clotrimazole were 0.26 μM for the 16-ene-C_{19}-steroid synthesizing enzyme. 0.31 μM for 17α-hydroxylase and 0.18 μM for $C_{17,20}$-lyase. Although the data are not shown, the inhibition of econazole and miconazole were also competitive. The km value of the 16-ene-C_{19}-steroid synthesizing enzyme of pig testicular microsomes was 0.51 μM, V_{max} was 0.22 nmol/min/mg protein, and 0.50 μM and 0.4 nmol/min/mg protein for 17α-hydroxylase. The K_m-value of 17α-hydroxyprogesterone for $C_{17,20}$-lyase were 10.7 μM and the V_{max} was 2.16 nmol/min/mg protein.

DISCUSSION

Gower and coworkers have investigated the formation of the 16-ene-C_{19}-steroids *in vitro* using mature pig testicular tissue and proposed the pathway such that pregnenolone and progesterone were precursors of the 16-ene-C_{19}-steroids, such as androstadienol and androstadienone [2, 3]. This pathway is the first step in forming the various 16-ene-C_{19}-steroids, and the enzyme catalyzing this pathway is considered to be one of the lyases responsible for the side-chain cleavage of the C_{17}—C_{20} bond of the C_{21}-steroid. However, the properties of this enzyme had been not clarified until our experimental evidence was reported. We previously found that the microsomes prepared from immature pig testis also catalyzed the formation of androstadienol or androsta-

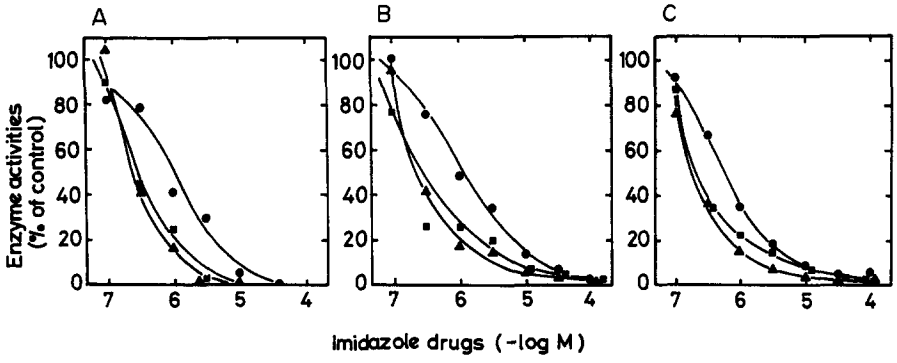

Fig. 1. Inhibitory effect of imidazole antifungal compound on 16-ene-C_{19}-steroid synthesizing enzyme (A), 17α-hydroxylase (B) and $C_{17,20}$-lyase (C) activities of pig testicular microsomes. [4-^{14}C]progesterone (5 μM) or 17α-hydroxy[4-^{14}C]progesterone (5 μM) was incubated with immature pig testicular microsomes (320 μg/ml) and NADPH (240 μM) in the presence of various concentration of clotrimazole (▲), econazole (■) or miconazole (●). Further details are given in the text.

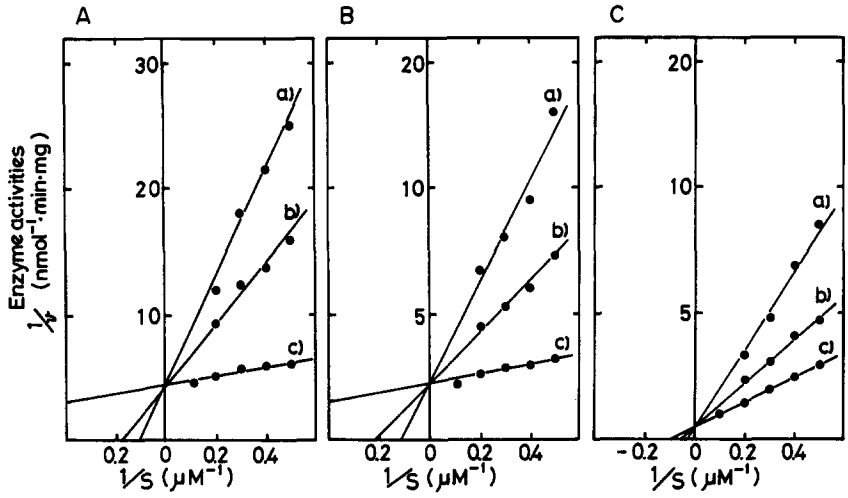

Fig. 2. Lineweaver–Burk plots of inhibition of 16-ene-C_{19}-steroid synthesizing enzyme (A), 17α-hydroxylase (B) and $C_{17,20}$-lyase (C) activities of pig testicular microsomes by clotrimazole. The concentration of clotrimazole was as follows; in panels A, B and C: (a) 5 μM; (b) 2.5 μM; and (C) not added.

dienone from pregnenolone or progesterone, respectively, and we reported the first evidence that the enzyme responsible for the formation of the 16-ene-C_{19}-steroid is the cytochrome *P*-450-linked oxygenase system [6, 7]. Recently, further evidence for involvement of cytochrome *P*-450 in the synthesis of androstadienol from pregnenolone was reported by another worker [14].

Many studies have been carried on the effect of various imidazole antifungal compounds on testicular *P*-450 (17α-hydroxylase/$C_{17,20}$-lyase) [15–18]. In this paper, we are the first to report that the 16-ene-C_{19}-steroid synthesizing enzyme activity was inhibited by some typical imidazole antifungal compounds and the degree of inhibition can be compared with those of 17α-hydroxylase and $C_{17,20}$-lyase activities of pig testicular microsomes. As a result, some imidazole antifungal compounds, clotrimazole, econazole, miconazole and ketoconazole markedly inhibited 16-ene-C_{19}-steroid synthesizing enzyme activity with clotrimazole the most powerful inhibitor of all the compounds examined. Furthermore, the inhibition of the 16-ene-C_{19}-steroid synthesizing enzyme activity by clotrimazole was competitive. The degree of inhibition by them was similar to that of 17α-hydroxylase and $C_{17,20}$-lyase activities.

In conclusion, the results presented here strongly suggest that the 16-ene-C_{19}-steroid synthesis is catalyzed by a cytochrome *P*-450-linked oxygenase which is the analog of 17α-hydroxylase/$C_{17,20}$-lyase.

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