Inhibitory Effect and Interaction of Stanozolol with Pig Testicular Cytochrome P-450 (17α-Hydroxylase/$C_{17,20}$-Lyase)[1)]

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The inhibitory effect of an anabolic steroid, stanozolol, on testicular microsomal cytochrome P-450 (17α-hydroxylase/$C_{17,20}$-lyase) (P-450$_{17\alpha/lyase}$) and the nature of the interaction were compared with those of other anabolic steroids, furazabol and mestanolone. Stanozolol markedly inhibited Δ^{16}-C_{19}-steroid synthesizing activity, 17α-hydroxylase and $C_{17,20}$-lyase activities, which were mediated by oxygenase activities of testicular microsomal cytochrome P-450$_{17\alpha/lyase}$. In addition, stanozolol was a competitive inhibitor of 17α-hydroxylase (K_i = 6.31 μM) and $C_{17,20}$-lyase (K_i = 1.30 μM) activities in the reconstituted enzyme system.

The interaction of cytochrome P-450$_{17\alpha/lyase}$ with stanozolol induced a type I difference spectrum (peak at 387 nm and trough at 418 nm) with a dissociation constant (K_s) of 1.47 μM.

Keywords anabolic steroid; stanozolol; furazabol; mestanolone; pig testicular microsome; cytochrome P-450; P-450 (17α-hydroxylase/$C_{17,20}$-lyase); oxygenase activity; competitive inhibition; type I difference spectrum

It is well known that some steps in the synthesis of testicular androgens are catalyzed by cytochromes P-450. In the conversion of C_{21} steroids (pregnenolone or progesterone to C_{19} steroids (dehydroepiandrosterone or androstenedione), 17α-hydroxylase and $C_{17,20}$-lyase activities are catalyzed by a single cytochrome P-450 in testicular microsomes (cytochrome P-450$_{17\alpha/lyase}$).[2)] On the other hand, it has been reported that Δ^{16}-C_{19}-steroids, such as androstadienol or androstadienone, are synthesized by pig testicular tissue.[3)] We have reported that Δ^{16}-C_{19}-steroid synthesizing activity is catalyzed by cytochrome P-450$_{17\alpha/lyase}$, participating with cytochrome b_5 as an essential component of electron transport systems.[4)] We thus considered that the cytochrome P-450$_{17\alpha/lyase}$ is one of the most important enzymes in the synthesis of testicular androgens.

Stanozolol[5)] is an anabolic steroid. It is designated as a doping agent by the International Olympic Committee because of its misuse by sportsmen. Rendić and Ruf[6)] reported the interaction of stanozolol with liver microsomal cytochrome P-450 and the inhibition of *O*-dealkylation of 7-ethoxycoumarin in liver microsomes.

In the present study, we examined the inhibitory effects of stanozolol on testicular microsomal cytochrome P-450$_{17\alpha/lyase}$ and the nature of the interaction in comparison with those of other anabolic steroids, furazabol and mestanolone (Fig. 1).

Materials and Methods

Chemicals [4-^{14}C]Progesterone (57.2 mCi, 2.12 GBq/mmol) and 17α-hydroxy[4-^{14}C]progesterone (53.0 mCi, 1.96 GBq/mmol) were purchased from New England Nuclear Corp., (Boston, Mass., U.S.A.) and their purity was checked by thin layer chromatography (TLC), developed with ethyl acetate–hexane (3:7, v/v) before use. Progesterone, 17α-hydroxyprogesterone, androstenedione, stanozolol, mestanolone, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and nicotinamide adenine dinucleotide phosphate [reduced from (NADPH)] were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Furazabol was a generous gift from Daiichi Seiyaku Co. (Tokyo, Japan). Other reagents were of the best grade available from Iwai Chemicals (Tokyo, Japan). Androstadienone was synthesized by the method of Barton *et al.*[7)]

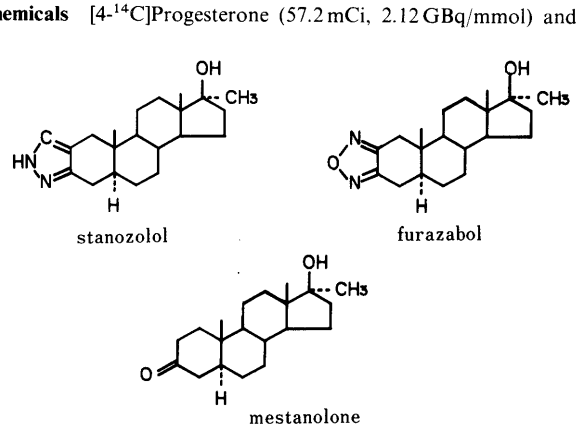

Fig. 1. Chemical Structures of Anabolic Steroids

Preparation of Testicular Microsomes Neonatal pig testes (10 d of age) were obtained at castration. The testes were decapsulated and homogenized in 0.15 M KCl–0.1 mM ethylenediaminetetraacetic acid (EDTA) with a Waring blender. The homogenate was centrifuged at 9000 × *g* for 60 min and the resulting supernatant was again centrifuged at 105000 × *g* for 60 min. After being washed with 100 mM potassium phosphate buffer (KPB)–1.0 mM EDTA, pH 7.4, the microsomal pellet was suspended 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.

Purification of Cytochrome P-450$_{17\alpha/lyase}$ Cytochrome P-450$_{17\alpha/lyase}$ was purified from pig testicular microsomes according to the method of Nakajin and Hall.[4a)] Cytochrome P-450-reductase was purified from pig liver microsomes according to the published method.[8)] These purified proteins were stored at −80 °C.

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

The substrate was incubated with microsomes (320 μg) or purified cytochrome P-450$_{17\alpha/lyase}$ with P-450-reductase (300 mU) in the presence of NADPH (240 nmol) in a total volume of 1 ml of 100 mM KPB (microsomes, pH 7.0 or purified cytochrome P-450, pH 7.25) at 37 °C. After the incubation (microsomes, 10 min or purified cytochrome P-450, 20 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 CH_2Cl_2 and subjected to TLC (plate: Kodak, 13181 silica gel), developed with ethyl acetate–hexane (3:7, v/v). After the observation of the TLC plate under ultraviolet (UV) light and/or radioautography (Fuji X-ray film, Rx), the relevant radioactive areas of chromatograms were cut off and ^{14}C was measured with a liquid scintillation counter (Packard Tri-Carb 460C).

Δ^{16}-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 pre-

vious report,[4a] was determined as the total radioactivity in 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 enzyme activities against the reciprocals of the concentration of substrate (2.0 to 10.0 μM).

Spectral Studies Purified cytochrome P-$450_{17\alpha/lyase}$ was dissolved in 100 mM KPB–20% glycerol–0.1 mM EDTA, pH 7.4. Optical difference spectra were recorded using a spectrophotometer (Hitachi 228) at 37 °C. The anabolic steroids were dissolved in ethanol (the concentration of solvent in the mixture never exceeded 2% (v/v)). We confirmed that a concentration of 2% (v/v) ethanol had no effect on the optical difference spectra. Upon spectral titration the dissociation constant, K_s (μM) and ΔA_{max} (absorbance/nmol P-450) were calculated from the double reciprocal plots [1/(concentration of anabolic steroid) *versus* $1/\Delta A$]. ΔA was equivalent to the absorbance peak minus absorbance through values.

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

Inhibitory Effect of Stanozolol on Testicular Microsomal Oxygenase Activities The inhibitory effects of anabolic steroids, stanozolol, furazabol and mestanolone, on testicular microsomal oxygenase activities were examined by incubation with microsomes. Figure 2 shows the inhibitory effects of each steroid on Δ^{16}-C_{19}-steroid synthesizing activity, 17α-hydroxylase and $C_{17,20}$-lyase activities. Stanozolol markedly inhibited Δ^{16}-C_{19}-steroid synthesizing activity, 17α-hydroxylase and $C_{17,20}$-lyase activities, the 50% inhibitory concentrations (IC_{50}) being 2.45, 2.90 and 0.74 μM, respectively. Furazabol and mestanolone also exhibited inhibitory effects on $C_{17,20}$-lyase activity (IC_{50} was 33.3 μM in both cases), but not on Δ^{16}-C_{19}-steroid synthesizing activity or 17α-hydroxylase activity. The inhibitory effects of furazabol were virtually the same as those of mestanolone. The K_m value of progesterone for Δ^{16}-C_{19}-steroid synthesizing activity of testicular microsomes was 0.51 μM and the V_{max} was 0.22 nmol/min/mg protein. The K_m value of progesterone for 17α-hydroxylase activity was 0.50 μM and the V_{max} was 0.40 nmol/min/mg protein. The K_m value of 17α-hydroxyprogesterone for $C_{17,20}$-lyase was 10.7 μM and the V_{max} was 2.16 nmol/min/mg protein. For each of the enzyme activities, the control values were 0.10 nmol/min/mg protein for Δ^{16}-C_{19}-steroid synthesizing activity, 0.29 nmol/min/mg protein for 17α-hydroxylase activity and 0.60 nmol/min/mg protein for $C_{17,20}$-lyase activity.

Inhibitory Effect of Stanozolol on the Oxygenase Activities of Reconstituted Enzyme System Inhibitory effects of anabolic steroids were examined in a reconstituted system of testicular cytochrome P-$450_{17\alpha/lyase}$ with P-450-reductase. Figure 3 shows the Lineweaver–Burk plots for 17α-hydroxylase activity and $C_{17,20}$-lyase activity in the reconstituted system in the absence and presence of the anabolic steroid, stanozolol. It can be seen that stanozolol shows competitive inhibition of 17α-hydroxylase and $C_{17,20}$-lyase activities.

Although the data are not shown, inhibitions of 17α-hydroxylase and $C_{17,20}$-lyase activities by furazabol and mestanolone were also the competitive.

Table I shows the K_i values of the anabolic steroids for 17α-hydroxylase and $C_{17,20}$-lyase activities in the reconstituted enzyme system. The K_i values of stanozolol were

Table I. K_i Values of Anabolic Steroids on the Oxygenase Activities of Cytochrome P-$450_{17\alpha/lyase}$

Drug	K_i Value (μM)	
	17α-Hydroxylase	$C_{17,20}$-Lyase
Stanozolol	6.31	1.30
Furazabol	19.1	11.5
Mestanolone	14.9	7.89

Table II. Spectral Characteristics of Anabolic Steroids with Cytochrome P-$450_{17\alpha/lyase}$

Drug	Spectral type	Spectral parameters	
		K_s (μM)	ΔA_{max} (nmol P-450)$^{-1}$
Stanozolol	I	1.47	0.020
Furazabol	I	69.9	0.024
Mestanolone	I	74.0	0.034

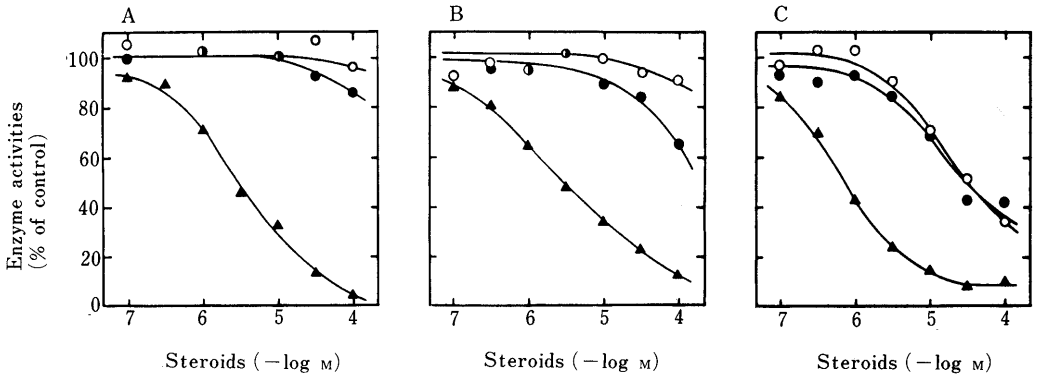

Fig. 2. Effect of Anabolic Steroids on Δ^{16}-C_{19}-Steroid Synthesizing (A), 17α-Hydroxylase (B) and $C_{17,20}$-Lyase (C) Activities of Pig Testicular Microsomes

[4-^{14}C]Progesterone (10 nCi, 370 Bq/5 nmol) of 17α-hydroxy[4-^{14}C]progesterone (10 nCi, 370 Bq/5 nmol) was incubated with pig testicular microsomes (320 μg) and NADPH (240 nmol) in the presence of various concentrations of the anabolic steroids. The assay mixture (1.0 ml) in 100 mM potassium phosphate buffer, pH 7.0, was incubated for 10 min at 37 °C. Further details are given in Materials and Methods. ▲, stanozolol; ○, furazabol; ●, mestanolone.

the lowest of all and the K_i value for 17α-hydroxylase activity was approximately a half of those of mestanolone and furazabol. The K_i value for $C_{17,20}$-lyase activity was approximately 5-fold lower than that of mestanolone, and 9-fold lower than that of furazabol. The K_m value of progesterone for 17α-hydroxylase activity was 2.50 μM and the V_{max} was 3.53 nmol/min/nmol P-450. The K_m value of 17α-hydroxyprogesterone for $C_{17,20}$-lyase activity was 2.31 μM and the V_{max} was 1.89 nmol/min/nmol P-450.

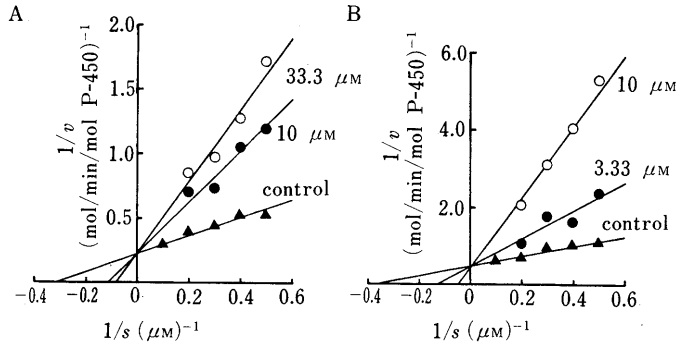

Fig. 3. Lineweaver–Burk Plots of Inhibition of 17α-Hydroxylase Activity (A) and $C_{17,20}$-Lyase Activity (B) by Stanozolol

The substrate, [4-^{14}C]progesterone or 17α-hydroxy[4-^{14}C]progesterone (10 nCi, 370 Bq/5 nmol), was incubated with a cytochrome P-450 enzyme system, which was reconstituted with purified pig testicular cytochrome P-$450_{17\alpha/lyase}$ (44 pmol), liver P-450-reductase (300 mU), and NADPH (240 nmol) in 1 ml of 100 mM potassium phosphate buffer, pH 7.25, without or with various concentrations of stanozolol. The mixture for enzyme assay was incubated for 20 min at 37 °C. The concentrations of stanozolol were as follows. In panel A: ○, 33.3 μM; ●, 10.0 μM; ▲, not added. In panel B: ○, 10 μM; ●, 3.33 μM; ▲, not added.

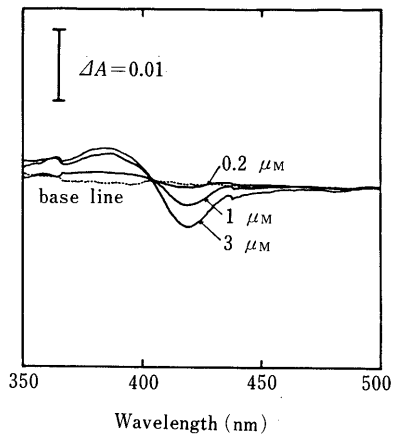

Fig. 4. Optical Difference Spectra of Purified Pig Testicular Cytochrome P-$450_{17\alpha/lyase}$ with Stanozolol

Difference spectra before (base line) and after the addition of stanozolol (0.2, 1.0 and 3.0 μM) were recorded at 37 °C. Each cuvette contained purified cytochrome P-$450_{17\alpha/lyase}$ (0.80 μM of P-450) in 100 mM potassium phosphate buffer–20% glycerol–0.1 mM EDTA, pH 7.4. The sample cuvette also contained an ethanol solution of stanozolol, but the reference cuvette received only ethanol without stanozolol. Further details are given in Materials and Methods.

Optical Difference Spectra of Cytochrome P-$450_{17\alpha/lyase}$ with Stanozolol To examine the interactions of anabolic steroids with cytochrome P-$450_{17\alpha/lyase}$, optical difference spectra of the purified form were recorded. Figure 4 shows optical difference spectra with stanozolol (data not shown in the case of furazabol and mestanolone) and purified cytochrome P-$450_{17\alpha/lyase}$. All of these anabolic steroids showed typical type I difference spectra (stanozolol and mestanolone, peak at 387 nm and trough at 418 nm; furazabol, peak at 390 nm and trough at 422 nm). Figure 5 shows double reciprocal plots of these data with the K_s values and the ΔA_{max}. Table II shows the K_s values and the ΔA_{max} of each anabolic steroid with purified cytochrome P-$450_{17\alpha/lyase}$. The K_s value of stanozolol was the lowest, being approximately 50-fold lower than that of furazabol or mestanolone.

Discussion

In the present study, we examined the inhibitory effect of anabolic steroids on purified pig testicular cytochrome P-$450_{17\alpha/lyase}$, a single kind of cytochrome P-450. We compared the effect of stanozolol with those of furazabol and mestanolone. All three steroids have the basic 17β-hydroxy-17-methyl-5α-androstane structure.

In this experiment, testicular microsomes were used for measuring the inhibitory effect on testicular cytochrome P-450 oxygenase activities, because the cytochrome P-$450_{17\alpha/lyase}$ activity is more stable and the Δ^{16}-C_{19}-steroid synthesizing activity is higher in the native microsomes than in the reconstituted system.[4b] But we considered that the reconstituted system should be used to clarify the direct effect on cytochrome P-450. Figure 3 shows that all of these anabolic steroids exhibited competitive inhibition of 17α-hydroxylase and $C_{17,20}$-lyase activities. Figure 4 shows that stanozolol induced typical type I difference spectra. It is considered that the binding of these anabolic steroids is very similar to substrate binding (*i.e.*, pregnenolone, progesterone, 17α-hydroxypregnenolone and 17α-hydroxyprogesterone) to testicular cytochrome P-$450_{17\alpha/lyase}$.[14] However, Redić and Ruf reported that stanozolol induced type II difference spectra by coordination of the nitrogen atom of the pyrazole ring of stanozolol as the ligand to

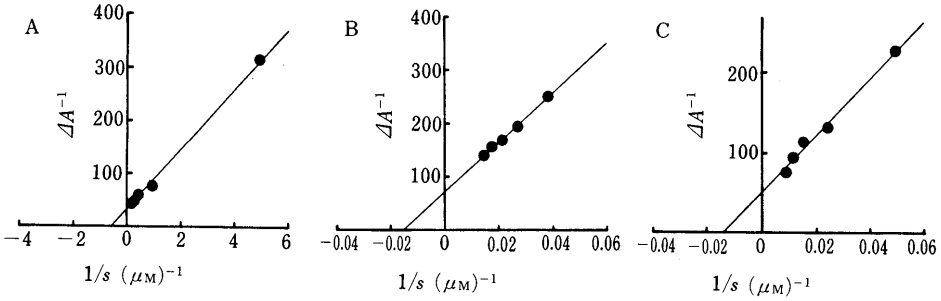

Fig. 5. Double Reciprocal Plots of Difference Spectra of Cytochrome P-$450_{17\alpha/lyase}$ with Stanozolol (A), Furazabol (B) and Mestanolone (C)

Purified cytochrome P-$450_{17\alpha/lyase}$ (A, 0.80 μM; B, 0.61 μM; C, 0.63 μM of P-450) dissolved in 100 mM potassium phosphate buffer–20% glycerol–0.1 mM EDTA, pH 7.4, was titrated with anabolic steroid; 0.2, 1.0, 2.0, 3.0 and 4.0 μM (in panel A), 26, 36, 46, 56 and 66 μM (in panel B) and 10, 20, 30, 40 and 50 μM (in panel C). The conditions used were as described in the legend to Fig. 4.

cytochrome P-450.[6] Our results suggest that the high affinity of stanozolol to pig testicular cytochrome P-$450_{17\alpha/lyase}$ originates more from the steroid structure than from the pyrazole ring.

The inhibitory effects of stanozolol on testicular microsomal oxygenase activities were remarkably strong, compared to those of furazabol and mestanolone, and the K_i values for oxygenase activities in the reconstituted enzyme system and the K_s value on purified cytochrome P-$450_{17\alpha/lyase}$ were markedly lower than those of the other steroids. The inhibitory effects and kinetic and spectral parameters of furazabol were virtually the same as those of mestanolone (Fig. 1, Tables I and II). It is considered that the existence of the pyrazole ring of stanozolol increase the affinity for cytochrome P-450. However, the furazan ring of furazabol seems to have little influence in view of the similarity of properties to those of mestanolone, which has no corresponding ring structure.

It seems likely that the anabolic steroid stanozolol will interact with other kinds of cytochrome P-450 in other organs, and affect cytochrome P-450 mediated oxgenase reactions involved in steroidogenesis.

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References and Notes

1) Abbreviations: dehydroepiandrosterone, 3β-hydroxyandrost-5-en-17-one; androstenedione, androst-4-ene-3,17-dione; androstadienol, androst-5,16-dien-3β-ol; androstadienone, androst-4,16-dien-3-one; stanozolol, 17β-hydroxy-17α-methyl-5α-androstano[3,2-*c*]pyrazole; furazabol, 17β-hydroxy-17α-methyl-5α-androstano[2,3-*c*]furazan; mestanolone, 17β-hydroxy-17α-methyl-5α-androstan-3-one; P-450 (17α-hydroxylase/$C_{17,20}$-lyase), P-$450_{17\alpha/lyase}$.
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