Inhibitory Effect and Interaction of Stanozolol with Pig Testicular Cytochrome P-450 (17α-Hydroxylase/C17,20-Lyase)\textsuperscript{11}

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The inhibitory effect of an anabolic steroid, stanozolol, on testicular microsomal cytochrome P-450 (17α-hydroxylase/C17,20-lyase) (P-450\textsubscript{17α/lyase}) and the nature of the interaction were compared with those of other anabolic steroids, furazabol and mestanolone. Stanozolol markedly inhibited \(17α\)-CYP-stereoid synthesizing activity, 17α-hydroxylase and C17,20-lyase activities, which were mediated by oxygenase activities of testicular microsomal cytochrome P-450\textsubscript{17α/lyase}. In addition, stanozolol was a competitive inhibitor of 17α-hydroxylase (\(K_i=6.31\) \(\mu\)M) and C17,20-lyase (\(K_i=1.30\) \(\mu\)M) activities in the reconstituted enzyme system.

The interaction of cytochrome P-450\textsubscript{17α/lyase} with stanozolol induced a type I difference spectrum (peak at 387 nm and trough at 418 nm) with a dissociation constant (\(K_i\)) of 1.47 \(\mu\)M.

Keywords: anabolic steroid; stanozolol; furazabol; mestanolone; pig testicular microsome; cytochrome P-450; P-450 (17α-hydroxylase/C17,20-lyase); oxygenase activity; competitive interaction; 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 C19 steroids (pregnenolone or progesterone to C18 steroids (dehydroepiandrosterone or androstenedione), 17α-hydroxylase and C17,20-lyase activities are catalyzed by a single cytochrome P-450 in testicular microsomes (cytochrome P-450\textsubscript{17α/lyase}).\textsuperscript{2,3} On the other hand, it has been reported that \(17α\)-CYP-steroids, such as androstadienol or androstenadienone, are synthesized by pig testicular tissue.\textsuperscript{3} We have reported that \(17α\)-CYP-steroid synthesizing activity is catalyzed by cytochrome P-450\textsubscript{17α/lyase}, participating with cytochrome \(b_2\) as an essential component of electron transport systems.\textsuperscript{4} We thus considered that the cytochrome P-450\textsubscript{17α/lyase} is one of the most important enzymes in the synthesis of testicular androgens.

Stanozolol\textsuperscript{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 Rulf\textsuperscript{6} reported the interaction of stanozolol with liver microsomal cytochrome P-450 and the inhibition of \(\delta\)-dealkylation of 7-ethoxycoumarin in liver microsomes.

In the present study, we examined the inhibitory effects of stanozolol on testicular microsomal cytochrome P-450\textsubscript{17α/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-\textsuperscript{14}C]Progestosterone (57.2 mCi, 2.12 GBq/mmol) and 17α-hydroxy\textsuperscript{14}C]progestosterone (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-hydroxyprogestosterone, 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 Daichi Sanyaku 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.\textsuperscript{7}

Preparation of Testicular Microsomes: Neonatal pig testes (10-12 days 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 \(\times g\) for 60 min and the remaining supernatant was again centrifuged at 105,000 \(\times g\) for 60 min. After being washed with 100 mm potassium phosphate buffer (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 \(-80^\circ\)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 \(b_2\), 0.06 \(\mu\)g U/mg protein for cytochrome P-450-reductase and 10.1 \(\mu\)g protein for cytochrome \(b_2\)-reductase.

Purification of Cytochrome P-450 \textsubscript{17α/lyase}: Cytochrome P-450\textsubscript{17α/lyase} was purified from pig testicular microsomes according to the method of Nakajin and Hall.\textsuperscript{8} Cytochrome P-450-reductase was purified from pig liver microsomes according to the published method.\textsuperscript{9} These purified proteins were stored at \(-80^\circ\)C.

Enzyme Assays: \(17α\)-CYP-stereoid synthesizing activity and 17α-hydroxylase activity were measured by determining the amount of radioactive material formed from [4-\textsuperscript{14}C]progestosterone (5 mmol, 105 Ci, 370 Bq/10 \(\mu\)l of ethanol solution) and the measurement of C17,20-lyase activity was carried out by using 17α-hydroxy\textsuperscript{14}C]progestosterone (5 mmol, 105 Ci, 370 Bq/10 \(\mu\)l of ethanol solution) as the substrate.

The substrate was incubated with microsomes (320 \(\mu\)g) or purified cytochrome P-450\textsubscript{17α/lyase}, with P-450-reductase (300 \(\mu\)U) in the presence of NADPH (240 \(\mu\)mol) 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 androstandienone (5 \(\mu\)g each, in ethanol) were added to the extract. The solvent was evaporated off and a portion of the residue was dissolved in a small amount of CH2Cl2 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 \(\textsuperscript{14}C\) was measured with a liquid scintillation counter (Packard Tri-Carb 460C).

\(17α\)-CYP-stereoid synthesizing activity was determined as \(\textsuperscript{14}C\)-label present in the fractions corresponding to androstandienone as the product from the substrate, progesterone. 17α-Hydroxylase activity, according to the pre-

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Results

Inhibitory Effect of Stanozolol on Testicular Microsomal Oxygenase Activities

The inhibitory effects of anabolic steroids, stanozolol, furazabol and mesterolone, on testicular microsomal oxygenase activities were examined by incubation with microsomes. Figure 2 shows the inhibitory effects of each steroid on $\Delta^{10}$-C$_{19}$-steroid synthesizing activity, 17$\alpha$-hydroxylase and C$_{17,20}$-lyase activities. Stanozolol markedly inhibited $\Delta^{10}$-C$_{19}$-steroid synthesizing activity, 17$\alpha$-hydroxylase and C$_{17,20}$-lyase activities, the 50% inhibitory concentrations (IC$_{50}$) being 2.45, 2.90 and 0.74 $\mu$m, respectively. Furazabol and mesterolone also exhibited inhibitory effects on C$_{17,20}$-lyase activity (IC$_{50}$ was 33.3 $\mu$m in both cases), but not on $\Delta^{10}$-C$_{19}$-steroid synthesizing activity or 17$\alpha$-hydroxylase activity. The inhibitory effects of furazabol were virtually the same as those of mesterolone. The $K_m$ value of progesterone for $\Delta^{10}$-C$_{19}$-steroid synthesizing activity of testicular microsomes was 0.51 $\mu$m and the $V_{\text{max}}$ was 0.22 nmol/min/mg protein. The $K_m$ value of progesterone for 17$\alpha$-hydroxylase activity was 0.50 $\mu$m and the $V_{\text{max}}$ was 0.40 nmol/min/mg protein. The $K_m$ value of 17$\alpha$-hydroxyprogesterone for C$_{17,20}$-lyase was 10.7 $\mu$m and the $V_{\text{max}}$ was 2.16 nmol/min/mg protein. For each of the enzyme activities, the control values were 0.10 nmol/min/mg protein for $\Delta^{10}$-C$_{19}$-steroid synthesizing activity, 0.29 nmol/min/mg protein for 17$\alpha$-hydroxylase activity and 0.60 nmol/min/mg protein for C$_{17,20}$-lyase activity.

Inhibitory Effect on 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 17$\alpha$-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 17$\alpha$-hydroxylase and C$_{17,20}$-lyase activities.

Although the data are not shown, inhibitions of 17$\alpha$-hydroxylase and C$_{17,20}$-lyase activities by furazabol and mesterolone were also the competitive.

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

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Table II. Spectral Characteristics of Anabolic Steroids with Cytochrome P-450, 17$\alpha$-Lyase

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Fig. 2. Effect of Anabolic Steroids on $\Delta^{10}$-C$_{19}$-Steroid Synthesizing (A), 17$\alpha$-Hydroxylase (B) and C$_{17,20}$-Lyase (C) Activities of Pig Testicular Microsomes

[1$\alpha$-C]Progesterone (10 nCi, 370 Bq/5 nmol) of 17$\alpha$-hydroxy- [4$\alpha$-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; ●, mesterolone.
the lowest of all and the \( K_i \) value for 17\( \alpha \)-hydroxylase activity was approximately a half of those of mestanolone and furazabol. The \( K_i \) value for C17,20\( \alpha \)-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 17\( \alpha \)-hydroxylase activity was 2.50 \( \mu \)M and the \( V_{\text{max}} \) was 3.53 nmol/min/\( \mu \)mol P-450. The \( K_m \) value of 17\( \alpha \)-hydroxyprogesterone for C17,20\( \alpha \)-lyase activity was 2.31 \( \mu \)M and the \( V_{\text{max}} \) was 1.89 nmol/min/\( \mu \)mol P-450.

**Optical Difference Spectra of Cytochrome P-450\( _{17\alpha\text{-lyase}} \) with Stanozolol**

To examine the interactions of anabolic steroids with cytochrome P-450\( _{17\alpha\text{-lyase}} \), 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\( _{17\alpha\text{-lyase}} \). 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 \( K_i \) values and the \( \Delta A_{\text{max}} \). Table II shows the \( K_i \) values and the \( \Delta A_{\text{max}} \) of each anabolic steroid with purified cytochrome P-450\( _{17\alpha\text{-lyase}} \). The \( K_i \) 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\( _{17\alpha\text{-lyase}} \), 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 17\( \beta \)-hydroxy-17-methyl-5a-androstene 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\( _{17\alpha\text{-lyase}} \) activity is more stable and the \( \Delta^{16}\)-C20-steroid synthesizing activity is higher in the native microsomes than in the reconstituted system.\(^{48}\) 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 17\( \alpha \)-hydroxylase and C17,20\( \alpha \)-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, 17\( \alpha \)-hydroxyprogrenenolone and 17\( \alpha \)-hydroxyprogesterone) to testicular cytochrome P-450\( _{17\alpha\text{-lyase}} \). However, Redic 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
cytochrome P-450.\(^6\) Our results suggest that the high affinity of stanozolol to pig testicular cytochrome P-450\(^{17a,17b}\) 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 \(K_i\) value on purified cytochrome P-450\(^{17a,17b}\) 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 increases 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 oxygenase reactions involved in steroidogenesis.

Acknowledgement We are grateful to Daiichi Seiyaku Co., Ltd. (Tokyo, Japan) for the generous supply of furazabol. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

References and Notes

1) Abbreviations: 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-androstane-3-one; P-450 (17α-hydroxylase/C17,20-lyase), P-450\(^{17a,17b}\).


