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INHIBITION MECHANISM OF RECONSTITUTED CYTOCHROME *P*-450scc-LINKED MONOOXYGENASE SYSTEM BY ANTIMYCOTIC REAGENTS AND OTHER INHIBITORS

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Summary—The effects of various antimycotic reagents and some other reagents on a cytochrome *P*-450-linked monooxygenase system were investigated with respect to the activities of NADPH–ferricyanide reductase. NADPH–cytochrome *c* reductase of NADPH–adreno-ferredoxin reductase from NADPH to cytochrome *c* via adreno-ferredoxin, NADPH–cytochrome *P*-450-phenylisocyanide complex reductase, and the cholesterol side chain cleavage of the cytochrome *P*-450scc-linked monooxygenase system. No reagents inhibited the NADPH–ferricyanide reductase activity. Only cloconazole inhibited about 50% of NADPH–cytochrome *c* reductase activity. Cloconazole, econazole, clotrimazole, etomidate and ketoconazole inhibited both NADPH–cytochrome *P*-450-phenylisocyanide complex reductase and the side chain cleavage activity of cholesterol of the cytochrome *P*-450scc-linked monooxygenase system. Cloconazole, econazole, etomidate and ketoconazole behaved like non-competitive inhibitors for NADPH–cytochrome *P*-450-phenylisocyanide reductase activities and their K_i values were 10^{-4}–10^{-6} M. Cloconazole was a non-competitive inhibitor of NADPH–cytochrome *c* reductase and its K_i value was 8.3×10^{-4} M. Cloconazole, clotrimazole, econazole, etomidate, ketoconazole and mitotane completely inhibited the side chain cleavage activity of cholesterol.

INTRODUCTION

The cytochrome *P*-450scc-linked monooxygenase system consists of three components, NADPH–adreno-ferredoxin reductase, adreno-ferredoxin and cytochrome *P*-450scc (*P*-450XIA1). It acts in the first step of the biosynthesis of steroid hormones [2]. It is known that antimycotic reagents, such as econazole, clotrimazole and ketoconazole block microsomal steroidogenesis [3–5]. These reagents also block mitochondrial steroidogenesis [6–8], and activation of vitamin D_3 [9]. Etomidate, used for induction of anesthesia or long-term sedation, suppressed adrenorcortical function [10, 11]. The enzymatic reactions of the reconstituted cytochrome *P*-450scc-linked monooxygenase system of adrenocortical steroidogenesis were studied. Particularly, the inhibitory effects of various reagents, on the activities of NADPH–cytochrome *c* reductase, NADPH–cytochrome *P*-450scc-phenylisocyanide reductase, and the cholesterol side chain cleavage enzyme (cytochrome *P*-450scc).

MATERIALS AND METHODS

Chemicals

Etomidate and mitotane were purchased from Janseen Pharmaceutica N.N. and Nippon Co. Ltd., respectively. Econazole (Ohtsuka Chemical Co.), clotrimazole (Bayer Pharmaceutica Ltd.), cloconazole (Shionogi Chemical Co.), ketoconazole (Kyowa Hakko Ind. Co.) were generous gifts from those companies.

Each reagent was dissolved in ethanol at a concentration of 20 mM. Horse heart cytochrome *c* (type III) and NADPH were purchased from the Sigma Co. Phenylisocyanide was synthesized by the method of Schmidt and Stern [12]. Other reagents were purchased from Wako Pure Chemicals Industries Ltd, and were

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Abbreviations: SDS, sodium dodecyl sulfate: HPLC, high pressure liquid chromatography. The term adreno-ferredoxin (adrenal ferredoxin) will be used synonymously with the popular term adrenodoxin in accordance with the recommendations of the IUPAC IUB Communication on Biochemical Nomenclature [1]

of the highest commercially available grade. Potassium phosphate buffer was 10 mM and pH 7.4.

Materials

Bovine adrenal glands were obtained from a local slaughterhouse. They were frozen at −20°C and transported to our laboratory. Cytochrome *P*-450scc was purified from bovine adrenocortical mitochondria by the method of Tsubaki *et al.* [13]. NADPH–adreno-ferredoxin reductase and adreno-ferredoxin were purified from adrenocortical mitochondria by the method of Hiwatashi *et al.* [14, 16] and Ichikawa [15], respectively. These purified proteins gave single protein bands by SDS–polyacrylamide gel electrophoresis.

NADPH–ferricyanide reductase activity

NADPH–ferricyanide reductase activity was measured at 25°C in 1.1 ml phosphate buffer containing 0.2 μM NADPH–adreno-ferredoxin reductase and 0.6 mM ferricyanide in the absence and presence of inhibitors. The reaction was started by the addition of 10 μl 20 mM NADPH dissolved in potassium phosphate buffer to the 3 ml reaction mixture which was incubated for 5 min at 25°C, and its NADPH–ferricyanide reductase activity was measured by the decrease in absorbance at 420 nm with a Shimadzu spectrophotometer, Model UV-240. All assays were performed independently in triplicate.

Analytical procedure

The concentrations of NADPH–adreno-ferredoxin reductase, adreno-ferredoxin and NADPH were determined spectrophotometrically with the molar extinction coefficients 11,300 $M^{-1}cm^{-1}$ at 450 nm [16], 9800 $M^{-1}cm^{-1}$ at 414 nm [15] and 6300 $M^{-1}cm^{-1}$ at 340 nm [17], respectively. Cytochrome *P*-450scc was measured from the CO-difference spectrum of the reduced cytochrome *P*-450scc with the molar extinction coefficient 97,800 $M^{-1}cm^{-1}$ at 448 – 490 nm [13].

NADPH–cytochrome c reductase activity

NADPH–cytochrome *c* reductase activity was measured at 25°C in 1.1 ml phosphate buffer containing 0.2 mM ferri-cytochrome *c*, 0.02 μM NADPH–adreno-ferredoxin reductase, 1 μM adreno-ferredoxin. NADPH–cytochrome *c* reductase activity was measured spectrophotometrically by the increase of the absorbance at 550 nm at the α-band of reduced cytochrome *c*.

NADPH–cytochrome P-450scc-phenylisocyanide reductase

NADPH–cytochrome *P*-450scc-phenylisocyanide complex reductase activity was measured at 25°C in 1.1 ml phosphate buffer containing 0.1 mM phenylisocyanide, 0.02 μM NADPH–adreno-ferredoxin reductase, 1 μM–adreno-ferredoxin, and 0.84 μM cytochrome *P*-450scc. The NADPH–cytochrome *P*-450scc-phenylisocyanide complex reductase reaction was started by the addition of 20 μl 10 mM NADPH in the absence and presence of inhibitors and the activity was measured by the increase of absorbance at 455 nm in reduced form of cytochrome *P*-450scc-phenylisocyanide complex [18].

Calculation of K_i values

The components of the enzyme solution and the method of measurement of NADPH–cytochrome *P*-450scc-phenylisocyanide complex reductase activity were as described above and the assays were performed with four different concentrations of NADPH (0.5–5.0 μM) in the presence of inhibitors.

From these assays, the reciprocal of the increase of absorbance per min and the concentration of NADPH were calculated. These reciprocals were plotted, and the K_i values were calculated.

Cholesterol side chain cleavage activity of cytochrome P-450scc

A cytochrome *P*-450scc-linked monooxygenase system was prepared with each inhibitor at various concentrations (50, 5 and 0.5 μM) in a solution containing 0.3 μM NADPH–adreno-ferredoxin reductase, 5 μM adreno-ferredoxin, 0.84 μM cytochrome *P*-450scc and a NADPH-generating system (5 mM glucose-6-phosphate, 4 mM $MgCl_2$ and glucose-6-phosphate dehydrogenase; 0.5 IU) in the absence and presence of inhibitors in 1.1 ml phosphate buffer. The sample solution was incubated for 5 min at 37°C and the reaction was started by addition of 10 μl 20 mM NADPH and incubation was continued with shaking for 10 min. The reaction was terminated by adding 1 ml iced methanol. After adding 20 nmol deoxycorticosterone acetate as internal standard, pregnenolone (the enzymatic product) was extracted twice with 4 ml isopropyl alcohol. The tubes were vigorously

shaken for 1 min and centrifuged at 3500 *g* for 5 min. The isopropyl alcohol layers were collected and dried under nitrogen. The residues were dissolved in 20 μl 60% methanol and analyzed by HPLC (GILSON, Model 302, GILSON 115 UV detector, and on a Wakopak C^{18} column (4.6 mm × 250 mm, flow rate 1 ml/min) and the elutions were monitored by absorbance at 205 nm. The enzymatic activities were confirmed to be proportional to the incubation time and the concentration of cytochrome *P*-450scc under the experimental conditions.

RESULTS

Effects of various steroidgenic inhibitors on activities of NADPH–ferricyanide reductase, NADPH–cytochrome c reductase and NADPH–cytochrome P-450scc-phenylisocyanide complex reductase

The activities of the cytochrome *P*-450scc-linked monooxygenase system reconstituted with NADPH–adreno-ferredoxin reductase, adreno-ferredoxin, and cytochrome *P*-450scc, were examined with various inhibitors such as ketoconazole, cloconazole and econazole (Figs 1 and 2). The activity of the NADPH–cytochrome *P*-450scc-phenylisocyanide reductase was inhibited non-competitively by these inhibitors. However, the activity of

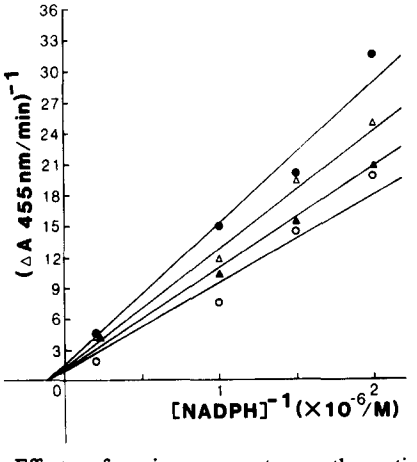

Fig. 1. Effects of various reagents on the activity of NADPH–cytochrome *P*-450scc-phenylisocyanide complex reductase. In the double-reciprocal plot of kinetic data, the vertical axis shows the reciprocal of the increase of absorbance at 455 nm/min of the reduced cytochrome *P*-450scc-phenylisocyanide complex. The lateral axis shows the reverse of the concentration of NADPH. Each point is the mean of triplicate assays, with the concentration of the reagent at 0.05 mM. ●, Ketoconazole; △, cloconazole; ▲, econazole; and ○, control.

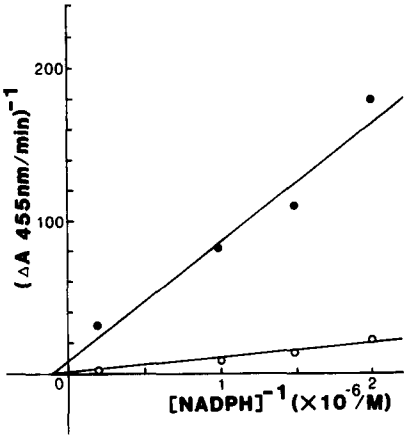

Fig. 2. Effects of etomidate on the activity of NADPH–cytochrome *P*-450scc-phenylisocyanide complex reductase. The vertical axis shows the reciprocal of the increase in absorbance at 455 nm/min of the reduced cytochrome *P*-450scc-phenylisocyanide complex, and the lateral axis shows the reciprocal of the concentration of NADPH with the concentration of the reagents at 0.05 mM. ●, Etomidate; and ○, control without reagent.

NADPH–ferricyanide reductase of NADPH–adreno-ferredoxin reductase was only slightly inhibited. Cloconazole and clotrimazole apparently inhibited 30% of the activity of NADPH–ferricyanide reductase. The activity of NADPH–cytochrome *c* reductase of the complex NADPH–adreno-ferredoxin reductase with adreno-ferredoxin was inhibited 50% by cloconazole, in contrast, other reagents did not inhibit the activity. However, in Fig. 1, the activity of NADPH–cytochrome *P*-450scc-phenylisocyanide complex reductase was inhibited by the reagents: clotrimazole, cloconazole, econazole, etomidate and ketoconazole. The findings indicated that the reduction from adreno-ferredoxin to cytochrome *P*-450scc is non-competitively inhibited 10–40% by these reagents. The order of inhibition by various inhibitors of the activities of NADPH–ferricyanide reductase of NADPH–adreno-ferredoxin reductase and NADPH–cytochrome *c* reductase of the complex NADPH–adreno-ferredoxin reductase with adreno-ferredoxin and NADPH–cytochrome *P*-450scc-phenylisocyanide reductase are summarized in Table 1. The results suggest that clotrimazole inhibits the electron transfer from adreno-ferredoxin to cytochrome *P*-450scc in particular. The mitotane did not inhibit the reducing activity of the cytochrome *P*-450scc-linked monooxygenase system. The inhibitions of these reagents on the activities of NADPH–cytochrome *P*-450scc-phenylisocyanide reductase and NADPH–

Table 1. Inhibition of the activities of NADPH–ferricyanide reductase, NADPH–cytochrome *c* reductase and NADPH–cytochrome *P*-450 phenylisocyanide reductase by various reagents

	Enzymatic activity (mmol/min/nmol adrenodoxin reductase)		
	Electron acceptor		
Inhibitor	Ferricyanide	Cytochrome *c*	*P*-450scc
None	6.14 (100.0)	0.605 (100.0)	0.409 (100.0)
Mitotane	4.58 (74.6)	0.57 (94.3)	0.361 (88.4)
Clotrimazole	4.18 (68.1)	0.479 (79.3)	0.0 (0.0)
Cloconazole	3.83 (62.3)	0.254 (42.0)	0.145 (35.4)
Econazole	4.41 (71.7)	0.482 (79.8)	0.145 (35.4)
Etomidate	5.39 (87.7)	0.514 (85.0)	0.012 (3.0)
Ketoconazole	5.12 (83.8)	0.459 (76.0)	0.176 (43.1)

Ferricyanide, NADPH–ferricyanide reductase activity; cytochrome *c*, NADPH–cytochrome *c* reductase activity; *P*-450scc, NADPH–cytochrome *P*-450scc-phenylisocyanide complex reductase activity. The concentration of inhibitors was 0.5 mM. These activities of NADPH–ferricyanide reductase, NADPH–cytochrome *c* reductase, and NADPH–cytochrome *P*-450scc-phenylisocyanide complex reductase were expressed as percentage of the activities in the absence of the inhibitors. Parentheses indicate the percentage of the enzymatic activities in the absence of the inhibitor.

Table 2. Enzymatic activity of NADPH–cytochrome *P*-450-phenylisocyanide reductase and NADPH–cytochrome *c* reductase

	K_i values	
Inhibitor	NADPH–cytochrome *P*-450-phenylisocyanide reductase	NADPH–cytochrome *c* reductase
Cloconazole	1.7×10^{-4} M	8.3×10^{-4}
Econazole	5.0×10^{-4} M	No inhibition
Etomidate	7.0×10^{-6} M	No inhibition
Ketoconazole	8.3×10^{-5} M	No inhibition

cytochrome *c* reductase were non-competitive and the K_i values are summarized in Table 2. The effect of cloconazole on the activity of the NADPH–cytochrome *c* reductase of the complex of NADPH–adreno-ferredoxin reductase with adreno-ferredoxin was examined (Fig. 3). The K_i values of cloconazole was 0.83 mM at 25°C.

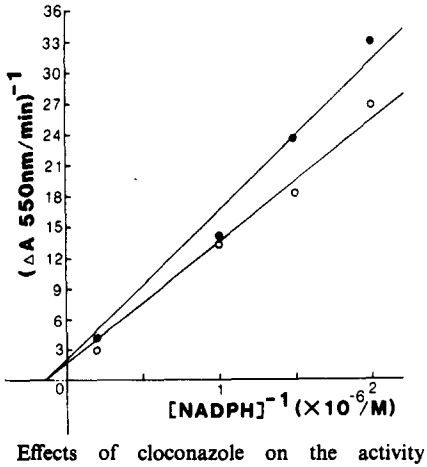

Fig. 3. Effects of cloconazole on the activity of NADPH–cytochrome *c* reductase on NADPH–adreno-ferredoxin reductase complex with adreno-ferredoxin. The vertical axis shows the reciprocal of the increase in absorbance at 550 nm/min of the ferri-cytochrome *c*, and the lateral axis shows the reciprocal of the concentration of NADPH. The concentration of the reagents was 0.5 mM. ●, Cloconazole; and ○, control without reagent.

Effects of various reagents on the activity of side chain cleavage of cholesterol of the reconstituted cytochrome P-450scc-linked monooxygenase system

The activity of side chain cleavage of cholesterol in the reconstituted cytochrome *P*-450scc-linked monooxygenase system was inhibited by every reagent (Table 3). The concentration of

Table 3. Inhibition of cholesterol side chain cleavage activities of the reconstituted cytochrome *P*-450scc-linked monooxygenase system by various reagents

Reagent concentrations (μM)	Amount of pregnenolone (nmol)	Specific activity (nmol/min/nmol *P*-450)
Control		
(0)	27.4	5.54 (100)
Mitotane		
(50)	9.79	1.98 (35.7)
(5)	17.4	3.51 (63.4)
Econazole		
(50)	13.6	2.74 (49.5)
(5)	22.5	4.54 (81.9)
Clotrimazole		
(5)	7.12	1.44 (26.0)
(0.5)	15.4	3.11 (56.1)
Clonconazole		
(5)	9.23	1.87 (33.8)
(0.5)	23.2	4.69 (84.7)
Etomidate		
(5)	3.45	0.70 (12.6)
(0.5)	10.8	2.18 (39.4)
Ketoconazole		
(5)	16.4	3.31 (59.7)
(0.5)	16.8	3.40 (61.4)

Parentheses indicate the percentage of the enzymatic activity.

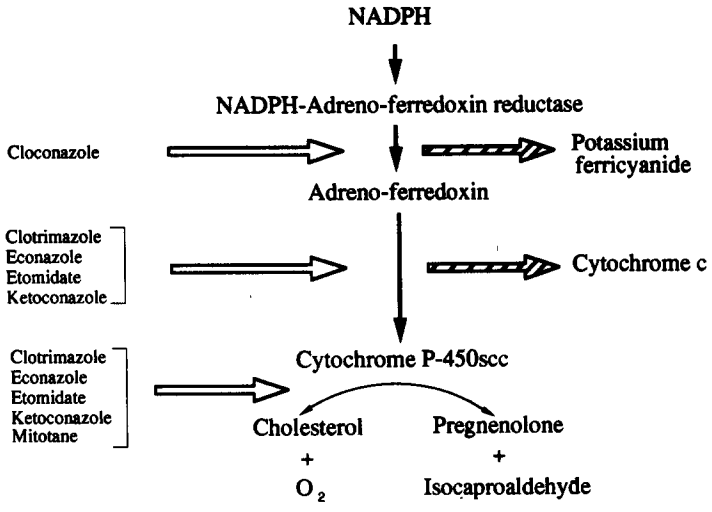

Fig. 4. Effects of various reagents on the cytochrome *P*-450scc-linked monooxygenase system. Solid arrows indicate the physiological electron flow and enzymatic lyase reaction. Open arrows indicate the sites of inhibition of the reagents. Hatched arrows indicate non-physiological electron flow.

the reagents which was effective was only 50 μM. Among the reagents, etomidate was the strongest inhibitor. These antimycotic reagents inhibited the active site of cytochrome *P*-450scc rather than the electron transport from adreno-ferredoxin to cytochrome *P*-450scc.

DISCUSSION

The effects of various imidazole antimycotic reagents such as ketoconazole and clotrimazole on the activity of individual components of the reconstituted cytochrome *P*-450scc-linked monooxygenase system were investigated with respect to the inhibition mechanism in each step of the electron transport system. The activity of NADPH–ferricyanide reductase on NADPH–adreno-ferredoxin reductase was scarcely inhibited by those reagents (i.e. mitotane, clotrimazole, econazole, etomidate and ketoconazole) used. A similar result was obtained on NADPH–cytochrome *c* reductase of the complex of NADPH–adreno-ferredoxin reductase with adreno-ferredoxin. These results suggest that the electron transport from NADPH to adreno-ferredoxin via NADPH–adreno-ferredoxin reductase is probably not affected by those reagents. Similar results were also obtained using whole microsome [19, 20].

Furthermore, the electron transport from adreno-ferredoxin to cytochrome *P*-450scc was also examined with respect to the activity of NADPH–cytochrome *P*-450scc-phenylisocyanide reductase. Among the reagents used in this experiment, all reagents except mitotane, strongly inhibited the reducing activity of NADPH–cytochrome *P*-450scc-phenylisocyanide reductase. On the other hand, the side chain cleavage activity of cholesterol on the reconstituted cytochrome *P*-450scc-linked monooxygenase system was also strongly inhibited by the reagents used in this study. These findings suggest that blocking the electron transport from adreno-ferredoxin to cytochrome *P*-450scc may cause these inhibitory effects. In this context, this block of electron transport may be caused by the adrenodoxin binding site of cytochrome *P*-450scc that may be influenced conformationally by antimycotic reagents. Because, the activity of NADPH–cytochrome *P*-450scc-phenylisocyanide reductase, like microsomal cytochrome *P*-450, occurred aerobically in the absence of those inhibitors [18]. It is now well known that antimycotic reagents containing the imidazole group can be linked to the sixth coordination of cytochrome *P*-450 heme [21]. From this and our results, the inhibition of activity of cholesterol side chain cleavage was probably due not only to the adrenodoxin binding site but also the cholesterol binding site on cytochrome *P*-450scc influenced by antimycotic reagents. These mechanisms of inhibition are illustrated in Fig. 4.

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