생명과학회지
Korean J. Life Science, 8(6), 695-701, 1998

Comparison of Characteristics of Hepatic Microsomal Cytochrome P450-dependent Monooxygenases from Snake and Rat

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Abstract

This study was carried out to investigate levels of the components of microsomal mixed function oxidase (MFO) system and activities of the hepatic microsomal cytochrome P450 (P450)-dependent monooxygenases of grass snake (*Natrix tigrina Lateralis*) and to compare with those of rat. The levels of P450 and cytochrome b_5 (b_5) of snake were much lower than those in rat. NADPH-cytochrome *c* reductase activity in the snake was also only 40% of that in the rat. Activities of 7-ethoxycoumarin *O*-deethylase (ECOD) and benzphetamine *N*-demethylase (BPDM) of snake hepatic microsomes, when compared with those of rat, were markedly low. But, aryl hydrocarbon hydroxylase (AHH) and testosterone hydroxylase (TSH) activities were nearly the same or higher than those of the rat. Of the P450-dependent TSHs measured, 7α-hydroxylase activity was the highest in snake, whereas, 6β-hydroxylase activity was the highest in rat. However, stereoselectivity of the enzyme from the snake to C2 and C6 positions of testosterone was the same as rat. The result of radioimmunoassay (RIA) for the identification of five P450 isozymes with MAbs shows that relatively high content of ethanol-inducible P450 isozyme, CYP2E1, exists in the rat, whereas MC-inducible P450 isozyme, CYP1A1/1A2, does in the snake. From the analyses of SDS-PAGE and RIA of partially purified P450, we suggest the possibility of the presence of a certain P450 isozyme(s) in hepatic microsomes of snake different from those of rat.

Key words : *Cytochrome P450-dependent Monooxygenase, Snake, Rat*

Introduction

Cytochromes P450 (P450s) compose a superfamily of hemoproteins which function as terminal oxidases of an enzymatic system catalyzing the metabolism of xenobiotic as well as endogenous compounds.[1,2] Wide species differences in the metabolism of xenobiotic compounds including drugs have been reported and are attributed, among other factors, to different levels and forms of the liver microsomal enzymes.[3,4] Considerable progress of biochemical study on the characteristics of the P450 enzyme systems has been made for the last few decades in laboratory mammalian species, but very little study has undertaken in the enzyme systems of reptile species.[5,6] P450-dependent hepatic monooxygenase systems of reptiles undoubtedly play a similar role in detoxification or activation of the numerous chemicals that contaminate the environment. However, the ability of reptile species to metabolize drugs and xenobiotic compounds differs from that observed in mammals.[5,6]

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In the present study, we characterized the levels of the components of hepatic microsomal mixed function oxidase (MFO) system and activities of several P450-dependent monooxygenases of grass snake (*Natrix tigrina Lateralis*) and compared them with those of the rat, the laboratory animal species most commonly used for xenobiotic metabolism studies.

Materials and Methods

Chemicals

Benzo(a)pyrene, benzphetamine, sodium dithionite, cytochrome *c*, testosterone, 16α-hydroxytestosterone, and NADPH were purchased from Sigma Chemicals Co. (St. Louis, USA). Silica gel plate (F254), 7-ethoxycoumarin, and ^{14}C-testosterone (52 mCi/mmol) were purchased from Merck Co. (Darmstadt, Germany), Aldrich Chemical Co. (Milwaukee, USA) and New England Nuclear, respectively ; 2α-, 2β-, 6α-, 6β- and 7α-Hydroxytestosterone were gifts of professor D.N. Kirk (Queen Mary College, University of London). Monoclonal antibodies, NBS1-48-5, PB2-66-3, MC1-7-1, MC1-36-1, PCN2-13-1, and EtOH1-98-1-1-2, were kind gifts of Dr. Sang-Shin Park (National Cancer Institute, Bethesda, Maryland, USA). All other chemicals used were of the highest grade quality commercially available.

Animals

Male grass snakes (*Natrix tigrina Lateralis*, 4−6 years old) were collected from Taejon region area in August 1997 for this study. Sprague Dawley rats (male, 180-200 g) were provided from the animal breeding center at Korea Ginseng & Tobacco Research Institute.

Preparation of liver microsomes

Microsomes were prepared from the livers with 30 mM Hepes buffer, pH 7.4, containing 150 mM KCl by differential centrifugation after removing blots and other subcellular fractions as described previously.[7] The microsomes were frozen immediately in liquid nitrogen and stored in −70℃ until use.

Biochemical assays

The activity of NADPH cytochrome *c* reductase was determined by measuring the reduction rate of exogenous cytochrome c at 550 nm.[8] Aryl hydrocarbon hydroxylase (AHH) activity was determined by a modified method of Nebert and Gelboin[9] as described previously.[10] 7-Ethoxycoumarin *O*-deethylase (ECOD) activity was measured by the method of Greenlee and Poland.[11] Benzphetamine N-demethylase (BPDM) activity was measured according to the method described elsewhere.[10] Testosterone hydroxylase (TSH) activity was measured by the method of Lee and Park.[12]

SDS-PAGE

The hepatic microsomal protein was subjected to polyacrylamide gel electrophoresis by conventional procedures[13] as follows : Hepatic microsomes were boiled for 4 min with gel loading buffer (50 mM Tris/10% SDS/10% glycerol/10% 2-mercaptoethanol/2 mg of bromophenol blue per ml) in a ratio of 1 : 1 and centrifuged at 10,000×g for 10 min. Total protein equivalents for each sample were separated on SDS/10% polyacrylamide minigels (Bio-Rad) using the Laemmli buffer system. After electrophoresis, the gels were stained with Coomassie Brilliant Blue G-250 by the method of Fairbanks *et al.*[14]

Other assays

Microsomal protein concentrations were determined by the method of Lowry *et al.*[15] Bovine serum albumin was used as the standard. The contents of microsomal P450 and b_5 were measured as described by Omura and Sato.[16] Radioimmunoassay (RIA) for the identification of P450 isozymes was performed according to the method of Park *et al.*[17] using Mabs (NBS 1-48-5p 24, PB 2-66-3, MC 1-7-1 and MC 1-36-1) raised agai-

nst the P450 isozymes purified from rat liver, and [^{35}S] labeled anti-mouse IgG as a secondary antibody.

Results

Components of MFO systems

We compared the components of hepatic MFO systems from rat and snake (Table 1). The level of P450 and b5 in microsomes from snake was 0.41 and 0.14 nmol/mg protein, which corresponds to 51 and 39% of that of the rat, respectively. The difference of carbon monoxide-reduced spectra of the P450 from two species were qualitatively similar and had a maximum absorption at 450 nm. The b5 spectrum was also similar in two species with a maximum absorption at 426 nm. NADPH-cytochrome *c* reductase activity in the snake was 40% of the activity in rat. The P450 content of hepatic microsomes from the garter snake, *Thamnophis*, observed by Schwen and Mannering[5] was nearly equal that found in the species of snake used in our studies (*Natrix tigrina Lateralis*). However, the b5 content in grass snake was 1.7-fold higher than in garter snake. The difference in the content may be due to species difference of snakes (*Natrix tigrina Lateralis and Thamnophis*).

Activities of P450-dependent monooxygenases

The reaction rates for the metabolism of B(a)P, 7-ethoxycoumarin, and benzphetamine were also determined in snake and rat (Table 1). The reaction rate for each substrate was lower in the snake microsomes than those for the rat. Especially, BPDM activity in snake was only 48% of the activity in the rat. AHH and ECOD activities were 89 and 74% of those of the rat, respectively. To compare the metabolic activity of the snake liver microsomes for testosterone, the amounts of 6 metabolic products, 2α-, 2β-, 6α-, 6β-, 7α-, and 16α-monohydroxytestosterone, were determined (Table 2). Total activity of TSH was higher in snake than in rat, which was contrast with the results of ECOD and BPDM. 6β-Hydroxylation rate of testosterone showed the highest in both snake and rat. The hydroxylation rates of testosterone at all 5 positions, except 6β-position, were all similar in the snake, but were much variable in the rat. These activities were higher in the snake than in the rat. Especially, the 6α-hydroxylation rate of the substrate in the rat was the lowest among the 6 positions and were also lower than that in the snake. There were relatively marked differences in the 6α- and 6β-hydroxylation rates between snake and rat. The stereoselectivity in testosterone metabolism was also observed to compare the specificity of P450 isozymes between snake and rat (Table 3). The stereoselectivity was evaluated by calculating the ratios of two-epimers of monohydroxytestosterone (C2 and C6) from the data presented in Table 2. The stereoselectivity on C2 position of testosterone of snake was similar that of rat as 1 : 1 ratio. However, higher stereoselectivity on C6 position of testosterone was observed in rat than in snake ; the ratio of 6α- to 6β-hydroxy forms was 1 : 2 and 1 : 21, respectively.

Patterns of P450 isozymes

Hepatic microsomal P450 consists of multiple forms

Table 1. Hepatic microsomal MFO systems and P450-dependent monooxygenase activities of snake and rat

	P450[1]	b_5[1]	NADPH-cyto. *c* reductase[2]	AHH[3]	ECOD[3]	BPDM[3]
Snake	0.41	0.17	0.06	0.08	7.91	3.94
Rat	0.81	0.43	0.15	0.09	10.63	8.24

Unit : [1]nmol/mg protein ; [2]μmol/min/mg protein ; [3]nmol/min/mg protein

Table 2. Activities of cytochrome P450-dependent testosterone hydroxylase in hepatic microsomes from snake and rat

	Testosterone hydroxylase						
	2α-	2β-	6α-	6β-	7α-	16α-	Total
Snake	0.97	0.85	0.93	1.98	0.93	0.95	6.61
Rat	0.43	0.42	0.14	2.90	0.32	0.54	4.75

Unit : nmol/min/mg protein

Table 3. Comparison of stereoselectivity in testosterone hydroxylation catalyzed by P450 in hepatic microsomes from snake and rat

Species	Ratio of monohydroxytestosterone epimers	
	2α/2β	6α/6β
Snake	1 : 1	1 : 2
Rat	1 : 1	1 : 21

of isozyme.[18] The P450 isozyme patterns beween the two species were also compared by RIA and SDS-PAGE analyses. As shown in Fig. 1, the specific binding of ethanol-inducible P450 isozyme to MAb EtOH1-98-1-1-2 was higher in rat than in snake, whereas that of the P450 isozyme specific to MAb MC1-7-1 was in the snake. SDS-PAGE analysis shows that, within the molecular weight range of P450 isozymes, approximately 43,000-56,000 daltons, snake had greatly different protein patterns from those of rat (Fig. 2). These results suggest that a certain P450 isozyme(s) different from those of rat may exist in the liver of snake.

Discussion

Although the majority of the species have the capacity to metabolize xenobiotic compounds, there are known interspecies differences in the metabolism of xenobiotics by P450-dependent monooxygenase systems. The present study provides a profile of the characteristics of some hepatic microsomal P450-dependent monooxygenase systems in snake and rat. Our data show that the snake possesses microsomal MFO system, but that the

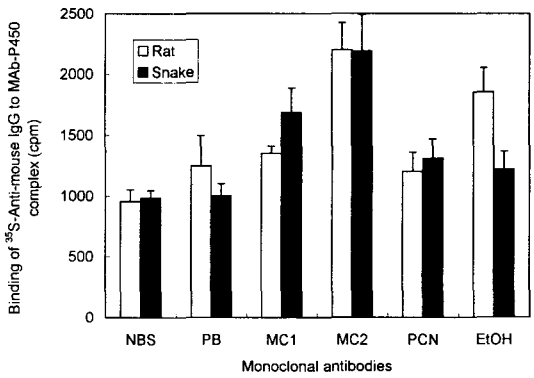

Fig. 1. Specific binding of MAbs at 5μg to hepatic microsomal cytochrome P450 isozymes of snake and rat. Abbreviations : NBS (NBS1-48-5P-24) ; Non-specific hybrids, PB (PB 2-66-3) ; MAb specific to CYP2B1 and 2B2 isozymes, MC 1 and MC2 (MC1-7-1 and MC1-36-1, respectively) ; MAbs specific to CYP1A1 and 1A2, PCN (PCN2-13-1) ; MAb specific to CYP3A1 and 3A2, EtOH (EtOH1-98-1-1-2) ; MAb specific to CYP2E1.

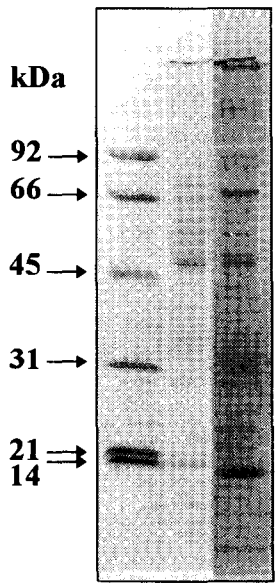

Fig. 2. SDS-polyacrylamide gel electrophoresis of cytochrome P450 purified partially from the hepatic microsomes of rat and snake. Lane 1, Molecular mass standards ; Lane 2, rat ; Lane 3, snake.

capacity of the system to metabolize xenobiotics is relatively lower than those of the rat, a laboratory animal species which has been used most commonly and extensively for the study on xenobiotic metabolism. In other words, rat had significantly higher levels of P450, b5 and NADPH-cytochrome *c* reductase than those of the snake.

Further characterization and comparison of xenobiotic-metabolizing capacity of the snake was made by determining the activities of P450-dependent monooxygenases such as AHH, ECOD, BPDM and TSH. ECOD and BPDM activities in the snake were relatively low as compared to those in the rat, which are consistent with the low levels of P450 and other components of the MFO system in the species.[5,6] However, AHH and TSH activities in the snake were similar or higher than in the rat. Particularly, different stereoselectivity in the hydroxylation rate of testosterone in liver microsomes of the snake and the rat was observed. Testosterone is the major male sex hormone and is oxidized to various hydroxytestosterones by P450-dependent monooxygenases in liver microsomes. Investigators[19-24] had reported that the profiles of testosterone metabolites are markedly altered by age, sex, species of animal, inducers, diet, and environmental factors. These results demonstrate that although it had relatively lower amount of P450 as well as b5 than that of the rat, the snake had a great AHH and TSH activities in the hepatic microsomes.

In conclusion, it is obvious from our data that rodents and reptiles have distinctly different potential capacities of metabolizing xenobiotics. Because poikilothermal animals such as a snake encounter a wide range of ambient temperatures throughout the course of year, they would be expected to possess enzyme systems that function at both relatively high and low temperatures. Busbee *et al.* [25] have reported studies which suggest that the optimal incubation temperatures may vary considerably among different species of amphibia. Therefore, our further studies are now in progress to investigate differences in the optimum conditions for P450-dependent monooxygenase activities between summer- and winter-snakes.

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초록 : 꽃뱀과 흰쥐의 간 마이크로좀에 존재하는 Cytochrome P450 의존성 Monooxygenases의 특성 비교

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한국산 꽃뱀(*Natrix tigrina Lateralis*)의 간 마이크로좀에 존재하는 mixed function oxidase system 구성성분들의 함량과 P450 의존성 monooxygenase의 활성도를 조사하고 이들을 흰쥐(Sp. D)의 것과 상호 비교하였다. 꽃뱀에서의 P450, b5 함량 및 NADPH-cytochrome *c* reductase 활성도는 흰쥐에서 보다 낮았으며, 7-ethoxycoumarin *O*-deethylase와 benzphetamine *N*-demethylase 활성도 역시 흰쥐에서 보다 상당히 낮았다. 그러나 aryl hydrocarbon hydroxylase와 testosterone hydroxylase 활성도는 흰쥐와 비교할 때 거의 비슷하거나 오히려 높았다. Testosterone의 수산화 반응에 대한 선택특이성을 조사한 결과, 꽃뱀은 7α 위치에서, 흰쥐는 6β 위치에서 가장 높은 수산화 반응물을 생성했다. 그러나 testosterone의 C2와 C3 위치에서의 수산화 반응에 대한 선택특이성은 꽃뱀과 흰쥐에서 비슷하였다. Radioimmunoassay (RIA)를 실시하여 5종 (CYP2B, CYP1A1, CYP1A2, CYP3A 및 CYP2E1)의 P450 동위효소의 구성비를 비교한 결과, 꽃뱀에서는 CYP1A1/1A2가, 흰쥐에서는 CYP2E1이 각각 비교적 많이 존재하였다. 부분정제한 P450을 SDS-PAGE와 RIA로 분석한 결과, 꽃뱀의 간 마이크로좀에 존재하는 P450중에는 흰쥐와는 다른 종류의 P450 동위효소가 존재함을 시사하였다.