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Species Difference among Experimental Rodents in Induction of P450IA Family Enzymes by 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine

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Rats, mice, hamsters and guinea pigs were given an i.p. injection of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), a protein-derived pyrolysate component present in cooked foods, and inductions of cytochrome P450 (P450) in the liver and kidney of these animals were examined. The activity and amount of P450s corresponding to the rat P450IA1 and P450IA2 were assessed by means of a bacterial mutation test using 3 carcinogenic heterocyclic aromatic amines including PhIP as substrates and by Western blotting with a monoclonal antibody reactive with both P450IA1 and P450IA2. In rats, PhIP induced P450IA1, P450IA2 and a new but unspecified P450 isozyme in the liver, and induced P450IA1 in the kidney. However, PhIP induced none of these P450 isozymes in mice, hamsters and guinea pigs.

Key words: 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine, PhIP — P450-induction — Species difference — Rodent

Most environmental carcinogens display carcinogenic and mutagenic activities after undergoing metabolic activation catalyzed by cytochrome P450 (P450). P450 consists of multiple isozymes and the proportion and/or induction pattern of each isozyme are different in different organs, and in animals of different species or sex.[1–8] The activity and/or induction rate of P450 enzyme(s) responsible for the bioactivation of a carcinogen are believed to be related to the susceptibility of animals to that carcinogen.[1,9,10] As we have reported previously, all carcinogenic aromatic amines examined possess activity for induction of P450IA family isozymes, especially P450IA2, responsible for bioactivation of the amines in the rat liver.[11,12] The induction rate of each P450 isozyme by the amines is, however, different in different organs, and in animals of different species or sex.[1–3,9,10]

2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), a pyrolysis product of protein, is considered to be an important environmental carcinogen, because it is present in cooked foods[13–15] as well as in cigarette smoke[16] and because it induces lymphoma in mice[17] and colon and mammary tumors in rats.[18] Like other carcinogenic aromatic amines, PhIP is activated by P450IA family isozymes[19] and induces P450 isozymes, including an unspecified P450 isozyme, in the rat liver.[20] It has also been shown that PhIP given to rats is distributed selectively in the liver and kidney as a cell-bound form.[21]

To further examine the carcinogenic risk of PhIP, we examined the species difference of experimental rodents in the induction of P450IA family isozymes in the liver and kidney by a PhIP treatment. We show that PhIP allowed the selective induction of P450IA family isozymes in rats but not in mice, hamsters and guinea pigs, and that the induction patterns in the rat liver and kidney are different.

MATERIALS AND METHODS

Chemicals PhIP and 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp P-2) were purchased from Nard Institute, Osaka, and 2-amino-6-methyldipyrido[1,2-*a*;3′,2′-*d*]-imidazole (Glu P-1) from Katsura Kagaku, Tokyo. 3-Methylcholanthrene (MC) was obtained from Tokyo Kasei Kogyo, Co., Osaka.

Treatment of animals with PhIP or MC and preparation of hepatic microsomes F344 rats, BALB/c mice, golden hamsters and Hartley guinea pigs were obtained from Japan SLC, Inc., Shizuoka. All animals were male and used at 7–9 weeks of age. They were kept in an air-conditioned room with free access to the basal diet, CE-2 (CLEA Japan) for rats, mice, and hamsters or RC4 (Oriental Yeast Co.) for guinea pigs.

Animals were given an i.p. injection of aqueous solution of PhIP and were killed 6, 10, 24 or 48 h after the treatment. Because we found in a preliminary experiment the PhIP could induce a significant amount of P450 isozymes in rats at a dose of 100 mg (0.38 mmol)/kg but not at lower doses and because another aromatic amine present in protein pyrolysis products, 2-amino-3-methyl-

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9*H*-pyrido[2,3-*b*]indole acetate (MeAαC), induced P450 in the hamster at a dose of 50 mg/kg,[1] we adopted this dose (100 mg/kg) of PhIP throughout the present experiments.

Liver or kidney of animals was homogenized in 1.15% KCl solution to obtain 25% (w/v) homogenate. The supernatant fraction (S-9) was obtained from the homogenates by centrifugation at 9,000*g* for 20 min and then the S-9 was recentrifuged at 105,000*g* for 60 min. The resultant precipitates were resuspended in 1.15% KCl solution and used as a microsome preparation. Amounts of protein and P450 in subcellular fractions were measured by the methods of Lowry *et al.*[22] and Omura and Sato,[23] respectively.

Enzyme activity for mutagenic activation of aromatic amines Trp P-2, Glu P-1 and PhIP were selected as substrates for assessment of the catalytic activity of P450 isozymes, because mutagenic activations of Glu P-1 and PhIP are catalyzed efficiently by P450IA2 but marginally by P450IA1, whereas that of Trp P-2 is mediated efficiently by either P450IA1 or P450IA2.[17, 24, 25] The activations of these chemicals were found to be hardly mediated by phenobarbital-inducible P450 isozymes.[24]

Trp P-2 (2 nmol/plate), Glu P-1 (2 nmol/plate) and PhIP (100 nmol/plate) were dissolved in 50 μl of dimethylsulfoxide (DMSO) and used as substrates. Activities of hepatic microsomes and renal S-9 for the mutagenic activation of Trp P-2 and Glu P-1 were assessed by means of the Ames test using *Salmonella typhimurium* TA 98 as described previously.[12] Throughout the present experiments, the number of spontaneous revertant colonies was in the range of 10 to 40. Data shown are the values obtained by subtracting the spontaneous revertant colonies from the total number of colonies developed. The number of revertants given by each substrate increased linearly with increase of the amount of subcellular fraction (50–200 μg protein/plate of hepatic microsomes and 0.5–4 mg protein/plate of renal S-9).

Western blot analysis of P450 isozymes APL-2 monoclonal antibody (MoAb) raised against rat P450IA1[26] was used for Western blot analysis. APL-2 MoAb is reactive with both P450IA1 and P450IA2 but much more so with the former. The MoAb is also cross-reactive with MC-inducible P450 isozyme(s) in mice, hamsters and guinea pigs but is hardly reactive with phenobarbital-inducible P450 isozymes of these animals and rats.[1, 24]

Western blotting of a microsome preparation was carried out according to the method described in our previous paper.[12] Briefly, microsomes dissolved in sodium dodecyl sulfate (SDS) were subjected to SDS-polyacryl amide gel electrophoresis (SDS-PAGE). The separated components on a gel sheet were transferred to a nitrocellulose sheet and immunostained using a protein A-mediated enzyme-linked immunosorbent assay (protein A-ELISA) kit and 0.05% 3,3′-diaminobenzidine tetrachloride (Sigma).

RESULTS

Induction of enzymes for mutagenic activation of aromatic amines by PhIP treatment Rats, mice, hamsters and guinea pigs were given an i.p. injection of PhIP, and changes in the activities of the hepatic and renal enzymes responsible for bioactivation of Trp P-2, Glu P-1 and PhIP were determined by means of a bacterial mutation test. The results are summarized in Tables I and II. In rats, the activity of hepatic microsomes for the mutageneses of Glu P-1, Trp P-2 and PhIP increased as early

Table I. Effects of PhIP on the Amount of P450 and the Activity for Mutagenic Activation of Carcinogenic Aromatic Amines in Hepatic Microsomes of Experimental Rodents

Animal	Time (h) after PhIP treatment[a]	P-450 content	Microsomal activities: ratio to the corresponding controls Substrates		
			PhIP	Trp P-2	Glu P-1
Rat	0	0.50	1.0	1.0	1.0
	6	0.44	1.9[b]	2.8[b]	2.7[b]
	10	0.43	6.8[b]	11.5[b]	7.5[b]
	24	0.45	3.7[b]	3.8[b]	3.4[b]
	48	0.43	1.2	1.9[c]	1.3
Mouse	0	0.60	1.0	1.0	1.0
	10	0.39[b]	0.7[d]	0.5[b]	0.4[b]
	24	0.49[d]	0.8	0.6[b]	0.5[b]
	48	0.55	1.1	0.7	0.7
Hamster	0	0.71	1.0	1.0	1.0
	10	0.44[b]	0.8	0.9	1.0
	24	0.40[b]	0.8	0.8	0.8
	48	0.57	0.7	0.8	0.9
Guinea pig	0	0.51	1.0	1.0	1.0
	10	0.26[b]	0.8	0.9	0.7
	24	0.49	1.2	1.2	1.3
	48	0.44	0.9	1.0	1.2

All assays were performed using hepatic microsomes (each 200 μg protein/plate) from the pooled livers of 3 animals at each time. The data shown represent the means of triplicate samples. In all experiments, the standard error of the mean was less than 15% of the mean.

a) Time 0 represents the values in untreated control animals, which are shown as 1.0. The net numbers of revertant colonies (per mg microsomal protein) for PhIP, Trp P-2 and Glu P-1 were as follows: rat; 1,100, 1,500 and 1,100: mouse; 2,000, 18,000 and 4,000: hamster; 1,600, 15,000 and 2,900: guinea pig; 4,500, 3,800 and 1,300, respectively.

b–d) Statistically significant differences from the corresponding controls (Student's *t* test): *b*, $P<0.001$; *c*, $P<0.01$; *d*, $P<0.05$.

as 6 h after the PhIP treatment and after 10 h, it reached the maximum and then decreased gradually, while the total amount of the microsomal P450 was not significantly altered by the PhIP treatment up to 48 h. In mice, hamsters and guinea pigs, the activity of the microsomes and total amount of P450 were both decreased 10 h after the PhIP treatment and then gradually returned to the normal levels.

Table II. Effect of PhIP on Renal S-9 Activities in Experimental Rodents for Mutagenic Activations of Trp P-2 and PhIP

Animal	Time (h) after PhIP treatment[a]	Renal S-9 activity: No. revertants/mg protein Substrates	
		Trp P-2	PhIP
Rat	0	<50	<50
	6	<50	<50
	10	550[b]	80[b]
	24	<50	<50
	48	<50	<50
Mouse	0	90	<50
	10	80	<50
	24	70	<50
	48	<50	<50
Hamster	0	<50	<50
	10	<50	<50
	24	<50	<50
	48	<50	<50
Guinea pig	0	<50	<50
	10	<50	<50
	24	<50	<50
	48	<50	<50

All assays were performed using S-9 preparations (each 2 mg protein/plate) from the pooled kidneys of three animals at each time. The data shown represent the means of triplicate samples. In all experiments, the standard error of the mean was less than 15% of the corresponding mean.

a) Lines of time 0 represent the values in untreated control animals.

b) Statistically significant difference from the corresponding controls (Student's *t* test): $P<0.001$.

As for kidney (see Table II), the S-9 preparations from all species of untreated animals except mice were inactive for the mutagenic conversion of Trp P-2 and PhIP. The PhIP treatment, however, resulted in the induction of renal enzyme(s) responsible for the activations of Trp P-2 and PhIP but only in rats, where the enzyme induction was slower (manifested 10 h after PhIP treatment) than that observed with the rat liver. PhIP did not induce the enzyme activity in the lung and small intestine of all species of animals used (data not shown).

Western blot analysis of PhIP-induced P450 Changes in the amount of P450IA family isozymes in rats, mice, hamsters and guinea pigs induced by PhIP treatment were examined by means of Western blot using APL-2 MoAb reactive with both P450IA1 and P450IA2. The blot profile is presented in Fig. 1. Hepatic microsomes from untreated rats (control) gave only one APL-2-reactive component at a position corresponding to a molecular weight (MW) of 54,000 (corresponds to

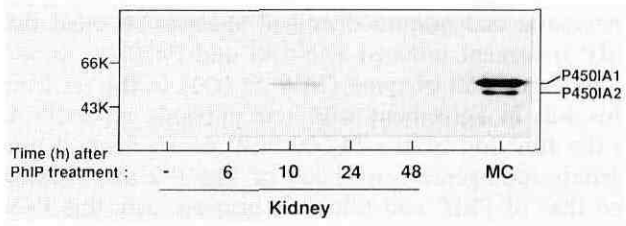

Fig. 2. Western blot profile of renal microsomes from a PhIP-treated rat. Renal microsome preparations (each 40 mg protein/lane) were subjected to Western blot analysis using APL-2 MoAb, as described in "Materials and Methods." MC, see the legend to Fig. 1.

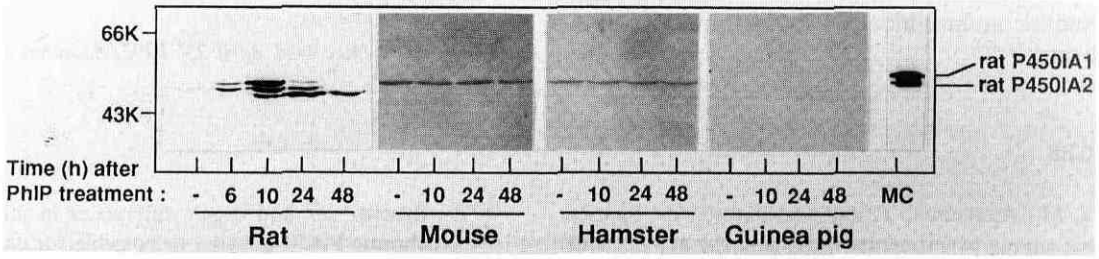

Fig. 1. Western blot profile of hepatic microsomes from PhIP-treated rodents. Rat, mouse, hamster and guinea pig were treated with PhIP and their hepatic microsomes (40 μg protein/lane) were subjected to Western blot analysis using APL-2 MoAb, as described in "Materials and Methods." MC, hepatic microsomes (20 μg protein/lane) from rats 24 h after treatment with 3-methylcholanthrene (30 mg/kg body weight).

P450IA2). When rats were treated with PhIP, 3 components showed time-dependent quantitative changes; the amount of the P450IA2 component reached maximum by 24 h, a component with MW 56,000 (corresponds to P450IA1) by 10 h, and a component with MW 51,000[20] by 24 h. The P450IA2 and P450IA1 components were undetectable 48 h after the PhIP treatment but the induced 51,000 MW component remained even at this time. Hepatic microsomes from mice and hamsters gave an APL-2-reactive component of MW 55,000 in the uninduced state and its amount was not significantly changed by the PhIP treatment of the animals. In guinea pigs, APL-2-reactive components were not detected from either uninduced or PhIP-induced hepatic microsomes.

As for kidney, microsome preparations from all species of untreated animals showed no APL-2-reactive components in the blot (Fig. 2). However, when animals were treated with PhIP, rats (see Fig. 2), but not other species of animals (data not shown), responded with induction of a component corresponding to P450IA1 within 10 h after the treatment.

DISCUSSION

This work demonstrated that there is species difference among experimental rodents in the induction by PhIP of P450IA family enzymes in hepatic and renal microsomes. Enzymatic and immunochemical analyses revealed that PhIP treatment induced P450IA1 and P450IA2 as well as another P450 isozyme (MW 51,000) in the rat liver. This was in agreement with our previous report.[20] As to the function of the 51,000 MW component, it may mediate mutagenic conversion of Trp P-2 and possibly also that of PhIP and Glu P-1, because only this P450 isozyme remained in the PhIP-induced microsomes 48 h after the PhIP treatment of rats (Fig. 1) and the microsomes mediated the mutagenic conversion of aromatic amines (Table I). It is noteworthy that the 51,000 MW component is inducible in the rat liver by PhIP treatment but not by other carcinogenic aromatic amines such as aminoazobenzene derivatives and several amino acid or protein pyrolysate components other than PhIP, though all these aromatic amines induce P450IA1 and P450IA2 in the rat liver.[1, 11, 12]

As demonstrated in the present work, PhIP showed clear species difference in terms of its activity for the induction of P450IA family isozymes. Although only one dose of PhIP (100 mg/kg) was used for the induction, this dose appeared to be relevant to investigate the effect of PhIP on the rodents used, because PhIP at this dose showed physiological effects on the total contents and activities of P450 of rats as well as all other species of animals tested, such as mice, hamster and guinea pigs (Table I), and because MeAαC, a protein pyrolysis product having a heterocyclic amine structure like PhIP, was found to induce P450 in hamsters at a lower dose (50 mg/kg).[1]

With regard to organ difference in the P450 induction, only liver and kidney of rats responded to PhIP. This is probably due to the different distribution or retention of PhIP given to rats, since PhIP is found to remain mainly in live and kidney as a cell-bound form.[21] The induction of P450IA1 and P450IA2 in the rat liver reached maximum 10 to 24 h after PhIP treatment and that of P450IA1 in the kidney was manifested only at 10 h of PhIP treatment and not later than 24 h. Such time-controlled induction of P450 may be due to rapid clearance of PhIP from, and stability of the induced P450 in, these organs, since PhIP given to rats by i.p. injection is cleared from rats within 48 h.[21]

In this work, we demonstrated species and organ differences in the induction by PhIP of P450 responsible for mutagenic or carcinogenic activation of PhIP in rodent animals. To establish the carcinogenic risk of PhIP, it is essential to investigate in further detail the metabolic fate of PhIP and its relation to the dynamic changes of P450 induced by PhIP in colon and mammary tissue, since these tissues have been demonstrated to be major target organs of PhIP carcinogenesis.[18]

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