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RENAL AND HEPATIC MICROSOMAL ENZYMES RESPONSIBLE FOR BIOACTIVATION OF 3-METHOXY-4-AMINOAZOBENZENE IN THE RODENT

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Abstract—Activities of the renal and hepatic microsomal enzymes responsible for the N-hydroxylation and mutagenic activation of 3-methoxy-4-aminoazobenzene (3-MeO-AAB) were examined in male mice, rats, hamsters and guinea pigs. In all these rodent species, hepatic microsomes showed definite N-hydroxylation of 3-MeO-AAB, whereas the renal activity was detected only in mice. The hepatic enzyme responsible for N-hydroxylation of 3-MeO-AAB (3-MeO-AAB *N*-hydroxylase) was induced in all species except mice by phenobarbital and selectively in mice and hamsters by 3-methylcholanthrene, whereas these cytochrome P450 inducers did not affect the renal enzyme in mice, rats or hamsters. In individual microsome samples, activities for N-hydroxylation and mutagenic activation of 3-MeO-AAB correlated well. These results indicate that the renal and hepatic enzymes responsible for the metabolic activation of 3-MeO-AAB differed among different species of rodent animals in terms of their activity and inducibility with cytochrome P450 inducers.

Cytochrome P450 (P450†) isozymes, an important family of genetically controlled isozymes responsible for the metabolism of endogenous and exogenous chemicals, are present in various organs, especially the liver, and show different substrate specificities. The P450 isozymes responsible for the metabolism of xenobiotics such as drugs and carcinogens have been known to be generally induced with the xenobiotics themselves [1]. Moreover, the induction patterns of P450 isozymes by xenobiotics are different in different animals and organs [1–3] and multiplicity of P450 isozymes is suggested as being a factor determining the pharmacological susceptibility of animals and their organs to drugs and carcinogenic chemicals [5–6].

Recently, we have found that mouse kidney contains an androgen-dependent microsomal P450 isozyme responsible for the N-hydroxylation of 3-methoxy-4-amino-azobenzene (3-MeO-AAB) which was named 3-MeO-AAB *N*-hydroxylase [7, 8]. This renal enzyme was different, in molecular property, to either a hepatic P450 isozyme showing 3-MeO-AAB *N*-hydroxylase activity or other androgen-dependent renal enzymes such as testosterone 15α-hydroxylase (cytochrome P450$_{15\alpha}$) and dimethylnitrosamine *N*-demethylase (P450IIE1). Moreover, mouse renal 3-MeO-AAB *N*-hydroxylase has been demonstrated to show little mutagenic activation of other carcinogenic aromatic amines such as amino acid-pyrolysate components [7]. Although carcinogenicity of 3-MeO-AAB has not been investigated in animals except rats [9] and mice [10], this unique enzyme may contribute as a marker enzyme to studies on the species, sex and organ differences in the metabolism of chemicals. In this context, we examined species and organ differences in the activity and induction of 3-MeO-AAB *N*-hydroxylase in male mice, rats, hamsters and guinea pigs.

We report herein that hepatic 3-MeO-AAB *N*-hydroxylase is present in all species of animals tested but its activity and inducibility with P450 inducers differ between species, and the renal 3-MeO-AAB *N*-hydroxylase is present only in mice.

MATERIALS AND METHODS

Chemicals. Phenobarbital sodium salt (PB) was obtained from the Tokyo kasei Kogyo Co., Tokyo, Japan and 3-methylcholanthrene (MC) from Wako Pure Chemical Industries, Osaka, Japan. 3-methoxy-4-aminoazobenzene (3-MeO-AAB), *N*-hydroxy-3-methoxy-4-aminoazobenzene (*N*-OH-3-MeO-AAB) and 4′-hydroxy-3-methoxy-4-aminoazobenzene (4′-OH-3-MeO-AAB) were synthesized in our laboratory [11, 12].

Animals. Male BALB/c mice, F344 rats and golden hamsters were purchased from the Shizuoka Agricultural Corporation for Laboratory Animals, Hamamatsu, Japan and male Hartley guinea pigs from Funabashi Farm, Funabashi, Japan. All animals were 7–10 weeks old. They were kept in an air-conditioned room and had free access to CE-2 diet (CLEA Japan, Tokyo) for mice, rats and hamsters and to GM-1 diet (Funabashi Farm) for guinea pigs. Some animals were treated with a single i.p. injection of either MC (0.11 mmol/kg) in corn oil or PB (0.22 mmol/kg) in physiological saline 24 hr before killing.

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† Abbreviations: P450, cytochrome P450; 3-MeO-AAB, 3-methoxy-4-aminoazobenzene; 3-MeO-AAB *N*-hydroxylase, microsomal enzyme responsible for N-hydroxylation of 3-MeO-AAB; *N*-OH-3-MeO-AAB, *N*-hydroxy-3-methoxy-4-aminoazobenzene; 4′-OH-3-MeO-AAB, 4′-hydroxy-3-methoxy-4-aminoazobenzene; PB, phenobarbital sodium salt; MC, 3-methylcholanthrene.

Table 1. Species difference in renal microsomal enzymes responsible for bioactivation of 3-MeO-AAB in the rodent

Species	Chemical treatment	P450 content (nmol/mg protein)	Mutagenic activity (No. of revertants/ nmol cytochrome P450)	Metabolites of 3-MeO-AAB (nmol/nmol cytochrome P450/15 min)		Metabolic pattern (*N*-OH/4′-OH)
				N-OH	4′-OH	
Mouse	None	0.15 ± 0.02	33,000 ± 5500	48.5 ± 7.4	21.0 ± 1.8	2.3
	PB	0.15 ± 0.01	35,000 ± 2500	47.8 ± 3.4	20.2 ± 0.1	2.4
	MC	0.13 ± 0.00	31,000 ± 1800	44.4 ± 4.3	20.7 ± 0.9	2.1
Rat	None	0.06 ± 0.01	<100	<0.5	<0.5	—
	PB	0.04 ± 0.01	<100	<0.5	<0.5	—
	MC	0.07 ± 0.01	<100	<0.5	<0.5	—
Hamster	None	0.07 ± 0.01	<100	<0.5	<0.5	—
	PB	0.11 ± 0.02	<100	<0.5	<0.5	—
	MC	0.08 ± 0.00	<100	<0.5	<0.5	—
Guinea pig	None	0.07 ± 0.00	<100	<0.5	<0.5	—
	PB	0.07 ± 0.01	<100	<0.5	<0.5	—
	MC	0.08 ± 0.01	11,000 ± 1900*	25.2 ± 4.9*	76.3 ± 10.3*	0.3

All data shown represent the means ± SEM of triplicate samples using one pooled specimen of renal microsomes from five untreated animals or five animals treated with PB or MC, as described in Materials and Methods.
* Statistically significant difference from the corresponding controls; $P < 0.001$.

Microsome preparation. Livers and kidneys from the animals were homogenized with 3 volumes (v/w) of 1.15% KCl using a Polytron homogenizer (Kinematica, Switzerland), as reported previously [8]. Briefly, renal or hepatic microsomes were sedimented from tissue homogenates by differential centrifugation and resuspended in 0.1 M sodium phosphate buffer, pH 7.4. Subcellular fractions were used for experiments immediately after preparation.

Protein and P450 amounts were determined by the methods of Lowry *et al.* [13] and Omura and Sato [14], respectively, using a Beckman 50-DU spectrophotometer.

Microsomal enzyme activity. Activities of the renal and hepatic microsomal enzymes responsible for the N-hydroxylation and mutagenic activation of 3-MeO-AAB were assayed by methods described previously [8]. Briefly, 3-MeO-AAB (200 nmol) in 100 μL of dimethylsulfoxide (DMSO) was added to 3 mL of 0.1 M sodium phosphate buffer solution, pH 7.4, containing NADPH (1 mM), NADH (1 mM), glucose-6-phosphate (20 mM), glucose-6-phosphate dehydrogenase (0.5 unit), $MgCl_2$ (25 mM) and 2–4 mg of renal or hepatic microsomal protein. The mixture was incubated for 15 min at 37° and the metabolites were then extracted from the reaction mixture with 1 mL of ethyl acetate saturated with 1 M sodium ascorbate solution.

For quantitative analysis of 3-MeO-AAB metabolites, an aliquot (100 μL) of an ethyl acetate extract from the reaction mixture was applied in a line to a Merck thin layer chromatography aluminium sheet coated with silica gel 60 (0.2 mm thick), which was developed with dichloromethane as a running solvent. The chromatographs were scanned with a Shimazu Chromatoscanner CS-910 equipped with an integrator. The amounts of metabolites, *N*-OH-3-MeO-AAB (R_f value, 0.27) and 4′-OH-3-MeO-AAB (R_f value, 0.18), were calculated from the integrated absorbance at 360 nm by using calibration curves for authentic azo dyes.

Mutagenicity of the 3-MeO-AAB metabolites was examined by means of the Ames mutation test with *Salmonella typhimurium* TA98. The ethyl acetate extract (100 μL) containing 3-MeO-AAB metabolites was evaporated at room temperature under N_2 gas and then dissolved in 50 μL of DMSO. Samples were added to 0.6 mL of sodium phosphate buffer, pH 7.4, containing 0.1 mL of bacteria suspension (10^8/mL) and were incubated for 20 min at 37°. After incubation, the mixture was mixed with 2 mL of 0.7% agar solution containing histidine (0.05 mM) and biotin (0.05 mM). The resultant agar mixture was poured onto a 1% agar plate containing 25 mL of minimal glucose medium (Vogel Bonner E medium). After incubation for 2 days at 37°, the number of revertant colonies was counted. The spontaneous revertant colony count (with a vehicle alone) ranged from 10 to 30. All data shown in the present experiments represent the values corrected by subtracting the number of spontaneous revertant colonies from the total number of revertant colonies.

Statistical analysis. Statistically significant differences in experimental data were analysed by Student's *t*-test.

RESULTS

3-MeO-AAB was incubated with either renal or hepatic microsomes from male mice, rats, hamsters or guinea pigs, and then examined for mutagenic activity and metabolite (*N*-hydroxyl and 4′-hydroxyl derivatives) content. Since N- and 4′-hydroxylations and mutagenic activation of 3-MeO-AAB have been reported to be mediated by microsomal P450 isozymes [8, 15, 16], the renal and hepatic enzyme activities in these reactions were calculated on the basis of P450 content.

Renal enzyme(s) responsible for N- and 4′-hydroxylations of 3-MeO-AAB was detected in mice but not in the other species (Table 1). The mouse renal enzyme(s) showed activity for N-hydroxylation

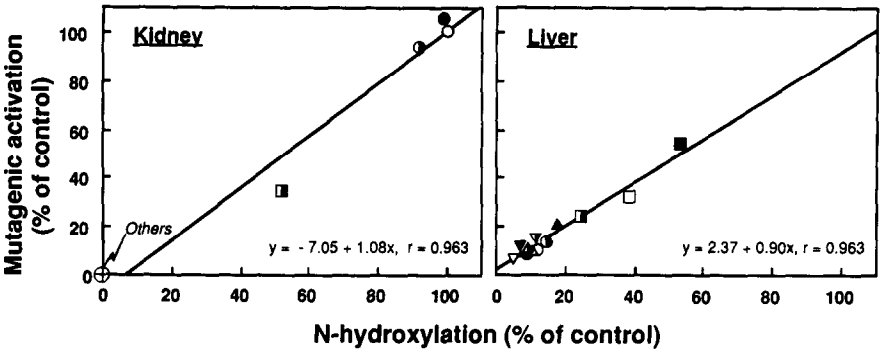

Fig. 1. Relationship between the activities for mutagenic activation and N-hydroxylation of 3-MeO-AAB in rodent renal and hepatic microsomes. Renal and hepatic microsomes were prepared from the pooled organs of untreated animals (○, △, ▽, □) and animals treated with PB (●, ▲, ▼, ■) or MC (◑, ◭, ▽, ◨), as described in Materials and Methods. Their activities for mutagenic activation and N-hydroxylation of 3-MeO-AAB were compared with the corresponding activities of renal microsomes from untreated mice (control). The data shown were calculated using the results described in Tables 1 and 2. (○), (●) and (◑), mice; (△), (▲) and (◭), rats; (▽), (▼) and (▽), hamsters; (□), (■) and (◨), guinea pigs.

two times greater than for 4′-hydroxylation of 3-MeO-AAB.

These enzyme activities were not induced significantly by the treatment of mice with P450 inducers such as PB and MC. These chemicals showed no activity in increasing renal P450 amount in any of the species used. However, MC led to an expression of the enzyme(s) responsible for N- and 4′-hydroxylations of 3-MeO-AAB in guinea pigs but showed no inductive effect on the enzyme in any other species. The MC-induced renal microsomes in guinea pigs were different in character from the mouse microsomes. As opposed to the mouse microsomes, the renal microsomes from MC-treated guinea pigs mediated predominantly 4′-hydroxylation rather than N-hydroxylation of 3-MeO-AAB. In addition, PB showed no inductive effect on the renal enzyme responsible for 3-MeO-AAB metabolism in any of the species.

Mutagenic activation of 3-MeO-AAB was detected with renal microsomes from mice and MC-treated guinea pigs; the renal microsomes from the other species showed no activity. Moreover, the renal activity for 3-MeO-AAB mutagenesis was closely correlated with activity for N-hydroxylation of 3-MeO-AAB (Fig. 1).

Hepatic microsomal activity for N- and 4′-hydroxylations of 3-MeO-AAB also differed between the different species (Table 2). The activity of the enzyme(s) mediating these hydroxylations was greatest in guinea pigs followed by mice/rats and hamsters. The guinea pig 3-MeO-AAB *N*-hydroxylase showed an activity about 9-fold greater, and the mouse or rat enzyme more than 2-fold greater, than that of the hamster enzyme. Hepatic microsomes showed a greater activity for 4′-hydroxylation than N-hydroxylation of 3-MeO-AAB in all the species but especially in hamsters.

PB treatment resulted in a significant increase in the hepatic microsomal P450 content of rats, hamsters and guinea pigs, and as a result, the microsomes in each species showed about 1.5-fold greater activity for N-hydroxylation of 3-MeO-AAB, as compared to the corresponding controls. However, for 4′-hydroxylation of 3-MeO-AAB, the activity of the microsomes was increased significantly in hamsters but decreased in guinea pigs by PB treatment. In rats and guinea pigs, PB-induced microsomes mediated *N*-hydroxylation rather than 4′-hydroxylation of 3-MeO-AAB, as opposed to the corresponding controls. In mice, PB treatment decreased slightly hepatic activity for N-hydroxylation but changed little the activity for 4′-hydroxylation.

MC treatment resulted in a significant increase of the hepatic microsomal P450 content in rats, hamsters and guinea pigs but not in mice. In mice and hamsters, MC-induced microsomes showed greater activity for the hydroxylation of 3-MeO-AAB, especially N-hydroxylation, as compared to the corresponding controls. On the other hand, MC-treatment decreased significantly the hepatic activity for N-hydroxylation of 3-MeO-AAB in guinea pigs and had little affect in rats. In addition, hepatic activity for 4′-hydroxylation of 3-MeO-AAB was increased in rats and affected little in guinea pigs by the treatment.

The hepatic microsomal enzyme for mutagenic activation of 3-MeO-AAB also differed in activity and inducibility between the different species (Table 2) and the enzyme activity in the microsomes of each species was closely correlated with its activity for N-hydroxylation of 3-MeO-AAB (Fig. 1).

DISCUSSION

We demonstrate herein that renal microsomal 3-MeO-AAB *N*-hydroxylase, which mediates mutagenic activation of 3-MeO-AAB, is present selectively in male mice but absent in male rats, hamsters and guinea pigs while the hepatic enzyme is present in all the species of animals tested, and that the renal and hepatic enzymes differ in their activity and inducibility between species. To characterize the

Table 2. Species difference in hepatic microsomal enzymes responsible for bioactivation of 3-MeO-AAB in the rodent

Species	Chemical treatment	P450 contents (nmol/mg protein)	Mutagenic activity (No. of revertants/ nmol cytochrome P450)	Metabolites of 3-MeO-AAB (nmol/nmol cytochrome P450/15 min)		Metabolic Pattern (*N*-OH/4′-OH)
				N-OH	4′-OH	
Mouse	None	0.76 ± 0.03	3600 ± 300	5.9 ± 0.3	9.0 ± 2.1	0.65
	PB	1.07 ± 0.03*	3300 ± 300	4.9 ± 0.1†	7.1 ± 0.3	0.69
	MC	0.73 ± 0.01	4400 ± 300	7.0 ± 0.3†	10.3 ± 0.7	0.68
Rat	None	0.69 ± 0.01	3200 ± 200	5.1 ± 0.5	7.3 ± 0.8	0.70
	PB	0.85 ± 0.01*	6800 ± 1000‡	8.8 ± 0.3*	8.2 ± 0.5	1.08
	MC	0.87 ± 0.04‡	4000 ± 700	5.3 ± 0.0	11.8 ± 0.7‡	0.45
Hamster	None	1.12 ± 0.02	2600 ± 200	2.1 ± 0.1	5.1 ± 0.3	0.42
	PB	1.30 ± 0.01*	3900 ± 400†	3.4 ± 0.6†	6.9 ± 0.7†	0.47
	MC	1.29 ± 0.04‡	4900 ± 300*	5.6 ± 1.1†	8.7 ± 0.7‡	0.65
Guinea pig	None	0.98 ± 0.02	11,000 ± 300	18.7 ± 2.4	24.6 ± 5.9	0.76
	PB	1.31 ± 0.01*	18,000 ± 2000‡	25.8 ± 2.3†	17.3 ± 0.7	1.49
	MC	1.04 ± 0.02†	8100 ± 800‡	11.7 ± 1.8†	28.6 ± 2.0	0.41

All data shown represent the means ± SEM of triplicate samples using one pooled specimen of liver microsomes from five untreated animals or five animals treated with PB or MC, as described in Materials and Methods.
Statistically significant difference from the corresponding controls; *$P < 0.001$; †$P < 0.05$; ‡$P < 0.01$.

renal and hepatic enzymes responsible for mutagenic activation of 3-MeO-AAB in male mice, rats, hamsters and guinea pigs, we examined the renal or hepatic microsome-mediated metabolites of 3-MeO-AAB *in vitro*. The renal or hepatic microsomes mediating mutagenic activation of 3-MeO-AAB in each species animal gave *N*-OH-3-MeO-AAB, a direct mutagen [11], and 4′-OH-3-MeO-AAB, a non-mutagen [8]. The activity for N-hydroxylation in the renal and hepatic microsomes reflected well their activity for 3-MeO-AAB mutagenesis.

Although we have reported previously [7, 8] that the mouse renal 3-MeO-AAB *N*-hydroxylase, which shows a greater activity than the corresponding hepatic enzyme, is an androgen-dependent P450 isozyme, we demonstrated further in the present experiments that this 3-MeO-AAB *N*-hydroxylase is a mouse-selective enzyme which is not inducible in mice by a P450 inducer such as PB or MC. On the other hand, renal 3-MeO-AAB *N*-hydroxylase was induced selectively in guinea pigs by MC, which showed no inductive effect on the renal enzyme in the other species tested.

Although hepatic 3-MeO-AAB *N*-hydroxylase was found to be present in all the sepcies of animals used, its activity differed markedly between species. Guinea pig activity was about 9-fold greater, and the mouse or rat activity more than 2-fold greater, than the hamster activity. As reported previously [15, 16], rat hepatic microsomal enzyme responsible for mutagenic activation of 3-MeO-AAB was induced by P450 inducers, especially PB. Likewise, PB resulted in about a 1.5-fold increase of the hepatic activity on the basis of P450 amount in guinea pigs and hamsters, while its treatment decreased slightly the mouse activity. On the other hand, MC increased significantly the hepatic activity in both mice and hamsters, whereas its treatment decreased significantly guinea pig activity. Likewise, Thorgeirsson *et al.* [17] had demonstrated that hepatic 2-acetylaminofluorene *N*-hydroxylase was induced in mice, rats and hamsters but hardly at all in guinea pigs by MC. It is notable that although MC-induced hepatic microsomes in each species of animal had been reported [15–20] to show greater activity for metabolic activation of carcinogens such as aromatic amines and polycyclic aromatic hydrocarbons, the MC-induced microsomes in guinea pigs showed a smaller activity for the metabolic activation (N-hydroxylation and mutagenic activation) of 3-MeO-AAB as compared to the corresponding controls.

Renal and hepatic microsomes mediating N-hydroxylation of 3-MeO-AAB were also active for 4′-hydroxylation. The mouse renal and PB-induced guinea pig hepatic microsomes showed greater activity for N-hydroxylation than 4′-hydroxylation of 3-MeO-AAB, whereas the reverse applied for the other hepatic and renal microsomes.

In this study we found species difference between rodents in the activity and inducibility of the renal and hepatic microsomal enzymes responsible for 3-MeO-AAB metabolism (N- and 4′-hydroxylations) and mutagenic activation. These findings suggest that 3-MeO-AAB is useful as a substrate to study species, sex and organ differences in drug metabolizing enzymes.

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