The Induction of Hepatic Cytochrome P4501A Subfamily Enzymes by Nitroanisidines: Species Difference among Experimental Rodents

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Species differences in the induction of hepatic cytochrome P4501A (CYP1A) subfamily enzymes by nitroanisidines, such as 2-methoxy-4-nitroaniline (2-MeO-4-NA), 2-methoxy-5-nitroaniline (2-MeO-5-NA), and 4-methoxy-2-nitroaniline (4-MeO-2-NA), were investigated among male F344 rats, C57BL/6 Cr mice, and Hartley guinea pigs. All species of animals were treated with a single intraperitoneal injection of each chemical (0.44 mmol/kg body weight), and changes in hepatic microsomal activities for methoxyresorufin *O*-demethylation (MROD) and ethoxyresorufin *O*-deethylation (EROD), which were mainly catalyzed by CYP1A2 and CYP1A1, respectively, were examined. Gene expression levels of hepatic CYP1A subfamily enzymes were also examined by a reverse transcription-polymerase chain reaction (RT-PCR) method. These results indicated that all the chemicals examined showed abilities to induce hepatic CYP1A subfamily enzymes in rats but not in the other examined animal species. In the rat liver, the abilities of the chemicals to induce microsomal MROD activity and *CYP1A2* gene expression were in the order 2-MeO-4-NA > 2-MeO-5-NA > 4-MeO-2-NA, whereas their abilities to induce microsomal EROD activity and *CYP1A1* gene expression were in the order 4-MeO-2-NA > 2-MeO-5-NA > 2-MeO-4-NA. These findings demonstrate for the first time that there are species differences in the induction of hepatic CYP1A subfamily enzymes by the nitroanisidine isomers among rats and other rodents, mice, and guinea pigs.

Key words —— cytochrome P450 induction, CYP1A, species difference, 2-methoxy-4-nitroaniline

INTRODUCTION

Cytochrome P450 subfamily enzymes are often induced by their own substrates. For example, the cytochrome P4501A (CYP1A) subfamily enzymes CYP1A1 and CYP1A2, which mediate metabolic activations[1–3] including *N*-hydroxylation of carcinogenic aromatic amines and epoxidation of carcinogenic aromatic hydrocarbons, respectively, are induced by treatment of rodents with the carcinogen itself.[3–8] Furthermore, the induction of carcinogen activation enzymes by the carcinogen itself in a target organ is thought to be a determining factor for carcinogenic susceptibility.[8–12] Therefore, to assess the risk posed by chemical carcinogens to animals, it is important to clarify the differences among animals including humans in the carcinogen-mediated induction of carcinogen activation enzymes and in the cellular factor(s) responsible for the induction.

The aryl hydrocarbon receptor (AhR)-dependent signal transduction pathway is generally considered[3,13–15] as a possible mechanism for the induction of CYP1A subfamily enzymes. However, for CYP1A2 induction, an AhR-independent pathway is also considered because CYP1A2-selective inducers such as aromatic amines[4–11,16,17] exist and because constitutive and chemical-induced expression levels of CYP1A2, but not CYP1A1, decrease in aromatic amine-induced liver hyperplastic nodules.[18] Furthermore, species, sex-, and tissue-differences in a chemical-mediated induction of CYP1A2 are often observed.[4,8–11]

We previously reported that 2-methoxy-4-nitroaniline (2-MeO-4-NA) is a selective inducer of CYP1A2 in the rat liver and the lowest molecular weight chemical among known CYP1A2-selective inducers[16] and further demonstrated that other nitroanisidines, such as 2-methoxy-5-nitroaniline (2-MeO-5-NA) and 4-methoxy-2-nitroaniline (4-MeO-2-NA), have also abilities to induce CYP1A subfamily enzymes with different selectivity.[17] More recently, we have reported that the 2-MeO-4-NA-mediated induction of CYP1A subfamily enzymes occurs in an AhR-independent and species-selective

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manner.[19)]

In the present study, we examined comparatively the species-difference in the induction of hepatic CYP1A by the nitroanisidine isomers, such as 2-MeO-4-NA, 2-MeO-5-NA, and 4-MeO-2-NA, among rats, mice, and guinea pigs.

MATERIALS AND METHODS

Chemicals —— 2-MeO-4-NA, 2-MeO-5-NA, and 4-MeO-2-NA were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Methoxyresorufin and ethoxyresorufin were obtained from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.).

Animal Treatment —— The animal procedures complied with the Japanese Government Animal Protection and Management Law. Male F344 rats, C57BL/6 Cr mice, and Hartley guinea pigs were purchased at 6 weeks of age from Japan SLC Inc. (Hamamatsu, Japan) and used at 7 weeks of age. They were housed in stainless steel cages and given a standard laboratory diet and water *ad libitum*. The animals were treated with an intraperitoneal injection of 2-MeO-4-NA, 2-MeO-5-NA, or 4-MeO-2-NA dissolved in corn oil at a dose of 0.44 mmol/kg body weight and sacrificed 12, 24, or 48 hr later. Control animals were treated with a vehicle (corn oil) alone. The livers were quickly removed, immediately frozen in liquid nitrogen, and stored at –80°C until use.

Microsomal Enzyme Activities —— The hepatic microsomal fraction was prepared from the liver homogenates by differential centrifugations as described previously.[6)] The amount of microsomal protein was determined by the methods of Lowry *et al*.[20)] The hepatic microsomal activities for methoxyresorufin *O*-demethylation (MROD) and ethoxyresorufin *O*-deethylation (EROD) were determined as described previously.[17)]

Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis —— Total hepatic RNAs were prepared with TRIzol reagent (Invitrogen Corp., Carlsbad, CA, U.S.A.) and used for the determination of the gene expression levels of CYP1A1 and CYP1A2. In addition, the gene expression levels of rat ribosomal protein L27a (rRPL27a) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were examined as internal controls. RT-PCR was performed using the SuperScript™ One-Step RT-PCR with Platinum® *Taq* System (Invitrogen Corp., Carlsbad, CA, U.S.A.). The primer sets and PCR conditions used are summarized in Table 1. PCR products were separated by electrophoresis on a 6% polyacrylamide gel, and the separated PCR product was visualized by ethidium bromide staining under ultraviolet light.

RESULTS AND DISCUSSION

We have previously reported that treatment of male rats with 2-MeO-4-NA, 2-MeO-5-NA, or 4-MeO-2-NA at a dose of 0.44 mmol/kg body weight resulted in definite increases in the levels of the mRNAs, proteins, and enzyme activities of CYP1A subfamily enzymes in the liver.[17,18)] In the present experiments, therefore, we selected the dose of 0.44 mmol/kg body weight and examined the time-dependent changes in the hepatic microsomal enzyme activities of MROD and EROD, which are mainly catalyzed by CYP1A2 and CYP1A1, respectively,[21,22)] in the animals after each chemical treatment. In rats, hepatic MROD and EROD activities significantly increased in a time-dependent fashion after each chemical treatment (Fig. 1). However, 2-MeO-4-NA, 2-MeO-5-NA, and 4-MeO-2-NA showed different selectivity for increasing MROD and EROD activities; the ability to increase MROD activity was in the order 2-MeO-4-NA > 2-MeO-5-NA > 4-MeO-2-NA, whereas the ability to increase EROD activity was in the order 4-MeO-2-NA > 2-MeO-5-NA > 2-MeO-4-NA. On the other hand, in mice and guinea pigs treated with each chemical, no such increases were observed at any of the time points examined.

We reported previously that gene expression levels of hepatic CYP1A subfamily enzymes clearly increased at 9–24 hr after the treatment of rats with 2-MeO-4-NA, 2-MeO-5-NA, or 4-MeO-2-NA.[17,18)] In the present experiments, therefore, we examined changes in the expression levels of *CYP1A1* and *CYP1A2* genes at 12 and 24 hr after each chemical treatment. Representative expression patterns of the genes are shown in Fig. 2. In addition, no significant changes in the gene expression levels of *rRPL27a* and *GAPDH*, which were used as internal standards, were observed at any of the time points examined. In rats, the expression level of the *CYP1A2* gene increased at 12 and 24 hr after each chemical treatment. A clear increase in the expression level of the *CYP1A1* gene was also observed particularly at 12 hr after each chemical treatment

Table 1. The PCR Conditions Used in the Present Experiments

Targets (Accession No.)	Primer sets	($5' \rightarrow 3'$)	Reaction condition		
			Denaturation	Annealing	Elongation
Rat *CYP1A1*	forward	TGACCTCTTTGGAGCT	95°C, 1 min	50°C, 30 sec	72°C, 2 min
	reverse	TTGAGCCTCAGCAGAT			
Rat *CYP1A2*	forward	GATGAGAAGCAGTGGAAAGACC	95°C, 1 min	50°C, 30 sec	72°C, 2 min
	reverse	AAAAAGAAAGGAGGAACAA			
Rat *RPL27a*	forward	ATCGGTAAGCACCGCAAGCA	95°C, 1 min	50°C, 1 min	72°C, 2 min
	reverse	GGGAGCAACTCCATTCTTGT			
Mouse *Cyp1a1*	forward	AGATCCAGGAGGAACTAGAC	91°C, 1 min	48°C, 1 min	72°C, 1 min
	reverse	CTCTCCGATGCACTTTCGCTTGC			
Mouse *Cyp1a2*	forward	AAGATCCATGAGGAGCTGGA	91°C, 1 min	48°C, 1 min	72°C, 1 min
	reverse	TCCCCAATGCACCGGCGCTTTCC			
Mouse *GAPDH*	forward	TGTGAACGGATTTGGCCGTA	91°C, 1 min	48°C, 1 min	72°C, 2 min
	reverse	TCGCTCCTGGAAGATGGTGA			
Guinea pig *CYP1A1*	forward	ATCACAACTGCCATCTCCTG	94°C, 1 min	61°C, 1 min	72°C, 2 min
(D11043)	reverse	TAATCTGCCACTGGTTCACA			
Guinea pig *CYP1A2*	forward	CCAGGACTTTGACAAGAACC	94°C, 1 min	60°C, 1 min	72°C, 2 min
(U23501)	reverse	GACCTCCAAGATGAAGGCTT			
Guinea pig *GAPDH*	forward	ACCACAGTCCATGCCATCAC	94°C, 30 sec	65°C, 30 sec	72°C, 2 min
	reverse	TCCACCACCCTGTTGCTGTA			

Targets (Accession No.)	Primer sets Denaturation	Product size (bp)	PCR cycles	Reference
Rat *CYP1A1*	forward	1050	19	23)
	reverse			
Rat *CYP1A2*	forward	328	17	24)
	reverse			
Rat *RPL27a*	forward	233	16	25)
	reverse			
Mouse *Cyp1a1*	forward	356	27	26)
	reverse			
Mouse *Cyp1a2*	forward	359	19	26)
	reverse			
Mouse *GAPDH*	forward	220	18	26)
	reverse			
Guinea pig *CYP1A1*	forward	280	31	—[a]
(D11043)	reverse			
Guinea pig *CYP1A2*	forward	310	33	—[a]
(U23501)	reverse			
Guinea pig *GAPDH*	forward	452	33	27)
	reverse			

a) The primer sets used were designed according to the DDBJ/EMBL/GenBank database.

(Fig. 2). Thus, the all chemicals used increased the expression levels of the *CYP1A2* and *CYP1A1* genes in rats, although they have different selectivity for the gene activations: For the *CYP1A2* gene, 2-MeO-4-NA ≥ 2-MeO-5-NA > 4-MeO-2-NA; for the *CYP1A1* gene, 4-MeO-2-NA > 2-MeO-5-NA > 2-MeO-4-NA. In addition, magnitudes of the increases of the CYP1A2 and CYP1A1 mRNAs were correlated with those of microsomal MROD and EROD activities, respectively. On the other hand, in mice and guinea pigs, no significant change in the expression level of either the *CYP1A2* (*Cyp1a2* in mice) or *CYP1A1* (*Cyp1a1* in mice) gene was observed after treatment with each chemical (Fig. 2). Furthermore, no significant changes in hepatic microsomal MROD and EROD activities were observed in mice and guinea pigs (Fig. 1).

In conclusion, we confirm herein that 2-MeO-

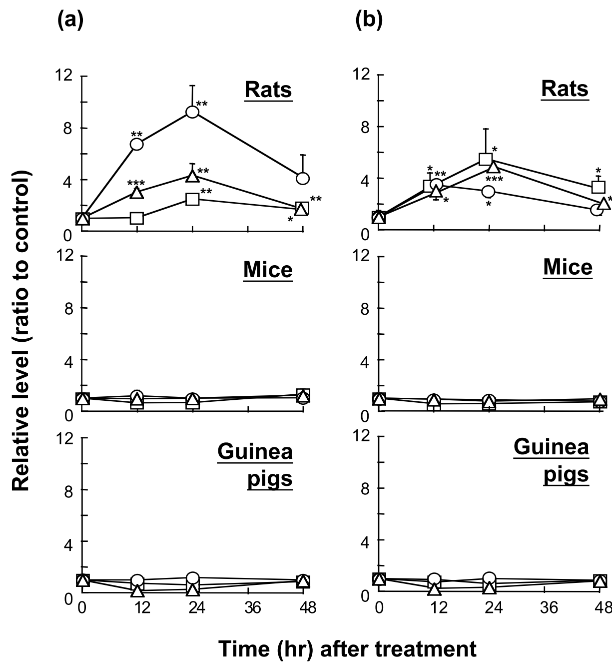

Fig. 1. Time-Dependent Changes of the Microsomal MROD (a) and EROD (b) Activities after Treatment of Animals with Isomeric Nitroanisidines

The values shown represent the ratios to the corresponding control levels, and the bars show their standard deviations ($n = 3$). Significant differences from the corresponding controls assessed by Student's t-test: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Open circles: 2-MeO-4-NA-treatment; Open triangles: 2-MeO-5-NA-treatment; Open squares: 4-MeO-2-NA-treatment.

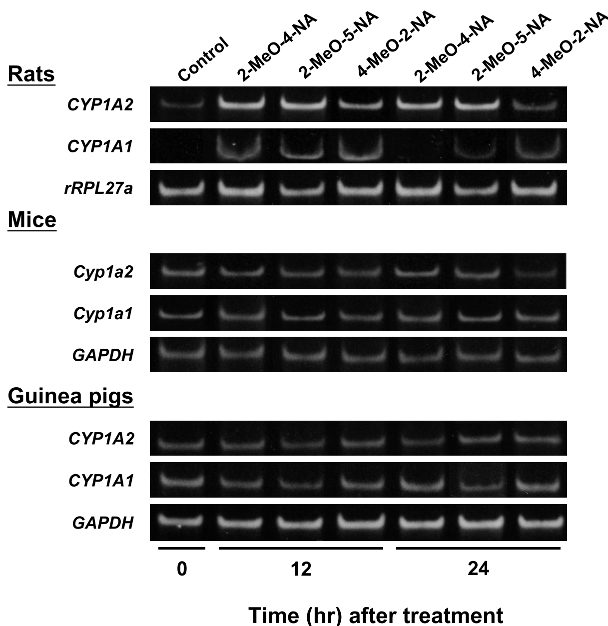

Fig. 2. Representative Gene Expression Patterns of Hepatic CYP1A and Cyp1a Subfamily Enzymes after Treatment of Animals with Isomeric Nitroanisidines

Number of PCR cycles used for amplification of each CYP cDNA is shown in Table 1.

4-NA, 2-MeO-5-NA, and 4-MeO-2-NA have definite capacities for inducing CYP1A subfamily enzymes with different selectivity and demonstrate the species difference among rats, mice, and guinea pigs in the induction of hepatic CYP1A subfamily enzymes by these nitroanisidines. In addition, our preliminary results from the luciferase assay for screening AhR ligands, which was established in our previous study,[28] suggested that the any nitroanisidines used herein can not be AhR ligands. Mechanisms for the AhR-independent and species-selective induction of CYP1A subfamily enzymes by the nitroanisidines remain unclear.

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