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Musk Xylene Is a Novel Specific Inducer of Cytochrome P-450IA2

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Summary: The effect of musk xylene on contents of both cytochrome P-450IA1 and cytochrome P-450IA2 in rat liver was investigated using Western blotting analysis. Rats were treated i.p. for five consecutive days with either 50, 100 or 200 mg musk xylene/kg body weight. Musk xylene increased both total cytochrome P-450 and cytochrome b_5 contents in rat liver microsomes. Musk xylene induced cytochrome P-450IA2 (384 pmol/mg protein) strongly and preferentially and the ratio of cytochrome P450IA2/P-450IA1 was about 12 at the lowest dose tested. Musk xylene also induced the cytochrome P-450IA1 dose-dependently, but these extents were very small (32-174 pmol/mg protein). These results suggest that musk xylene may be a more specific inducer for cytochrome P-450IA2 than any other inducers reported.

Cytochrome P-450 (P-450) functions as the terminal oxidases in the hepatic microsomal mixed-function oxidation system (1-6) and has been the object of considerable studies with regard to their abilities to oxidize and sometimes reduce procarcinogens to reactive species that might be implicated in tumorigenesis (1,3,4). P-450IA1 and P-450IA2 in the IA subfamily are responsible for the metabolic activation of polycyclic aromatic hydrocarbons (PAHs) (*eg.* benzo[a]pyrene) and heterocyclic aromatic amines (*eg.* pyrolysates of amino acids and proteins), respectively (1,3,4). Although the levels of the members of the IA subfamily are very low in the untreated animals, they are known to be highly inducible by various PAHs such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), benzo[a]pyrene and 3-methylcholanthrene (3MC) (1-7). However, they induce P-450IA1 much more than P-450IA2 except isosafrole (7).

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Musk xylene (MX) has been widely used as a fragrance ingredient in soaps, detergents, lotions and foods. These compounds have been detected in the aquatic or marine environment as pollutants (8,9). In the present report, we investigated the effects of MX on contents of both P-450IA1 and P-450IA2 in rat liver microsomes, and found that it induced P-450IA2 preferentially.

Materials and Methods

Materials: MX (2,4,6-trinitro-5-*tert*-xylene; CAS No. 81-15-2) in Fig. 1 was obtained from Takasago Perfume Co. Ltd., Tokyo, Japan. Its purity was >96% (10). P-450IA1 and P-450IA2 were purified from rat liver microsomes and the antibodies against these cytochromes were prepared as described previously (11,12).

Animals: Male Wistar rats (7 weeks old) were obtained from Japan SLC Co., Shizuoka, Japan. Animals were fed a laboratory chow and water *ad libitum* and housed in plastic cages at constant temperature (24±1 °C) and humidity (55±5 %) under a 12-hr light/dark cycle (light on: 7 a.m.-7 p.m.). MX was suspended in corn oil (<200mg/2.5 ml) by sonication. Rats were injected i.p. for five consecutive days with either 50, 100 or 200 mg MX/kg body weight and were starved 24 hr prior to killing. Control animals received the vehicle only. 3MC (20 mg/kg body weight) was administered to rats by i.p. injection on two consecutive days.

Enzyme Preparation: Rats were killed by decapitation, and the livers were quickly excised, weighted and perfused with ice-cold 1.15% KCl from portal vein, then homogenized with four volumes (w/v) of 10 mM Tris/HCl (pH 7.4) containing 1.15% KCl, 0.1 mM EDTA, 1.0 mM dithiothreitol and 1.0 μM phenylmethylsulfonyl fluoride in a Potter-Elvehjem homogenizer. The 20% homogenate was centrifugated at 9,000 *xg* for 20 min and further centrifugated at 105,000 *xg* for 1 hr. Microsomes (105,000 *xg* pellet) were washed once with the original homogenization buffer and resuspended.

Enzyme Assay: P-450 and cytochrome b_5 levels were determined after dilution of the microsomes in 20% glycerol, 1.0 mM EDTA, 0.2% Emulgen 913 and 100 mM potassium phosphate (pH 7.25) and reduction with dithionite according to the methods of Omura *et al.* (13,14).

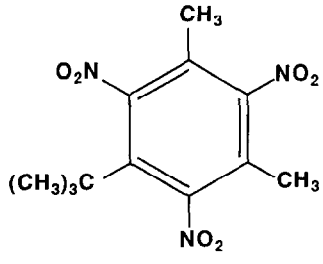

FIG. 1. Chemical structure of musk xylene.

Immunochemical Quantitation: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to Laemmli (15) using 10% acrylamide gel. Under the conditions, P-450IA1 and P-450IA2 were clearly separated. Microsomal proteins were electrophoretically transferred to Clear blot membrane-p (Atto Co., Tokyo, Japan) and probed with the anti-P-450 antibodies. The membranes were immunostained using Vectastain™ avidin-biotin-peroxidase complex kit (Vector Lab. Inc., Burlingame, CA), and scanned by a densitometer (Shimadzu CS-9000). Peak areas of each sample were calculated, and the contents of P-450IA1 or P-450IA2 were determined from standard curves of the purified cytochromes. Protein concentrations were determined by the method of Lowry *et al.* (16) using bovine serum albumin as a standard.

Statistical Analysis: All data were expressed as the mean±SE. Comparisons of the means were performed with a one-way analysis of variance followed by Dunnett's multiple range test for significance; $p < 0.05$ was considered significant.

Results

The effects of MX on the total P-450 and cytochrome b_5 levels in hepatic microsomes are shown in Table I. MX increased both the total P-450 content and the cytochrome b_5 content about 1.4- and 1.5-fold, respectively. The effect of MX on the contents of both P-450IA1 and P-450IA2 in rat liver were investigated using Western blotting analysis (Table II). MX induced P-450IA2 strongly and preferentially and the ratio of P450IA2/P-450IA1 was about 12 at the lowest dose tested. MX also induced the P-450IA1 dose-dependently, but these extents were very small and 5-10% of 3MC-treated rat.

TABLE I. Effects of musk xylene on cytochrome P-450 and cytochrome b_5 contents of rat liver microsomes

	control	Musk xylene		
	(vehicle only)	(50 mg/kg)	(100 mg/kg)	(200 mg/kg)
Cytochrome P-450 *(nmol/mg protein)*	1.065 ± 0.040	1.386 ± 0.058** (130 %)#	1.443 ± 0.097** (135 %)	1.535 ± 0.060** (144 %)
Cytochrome b_5 *(nmol/mg protein)*	0.363 ± 0.005	0.518 ± 0.018** (143 %)	0.523 ± 0.020** (144 %)	0.555 ± 0.014** (153 %)

Each value shows the mean ± SE of four individual rats. #Numbers in parentheses indicate the percentage of the control value. **Significantly different from control ($p < 0.01$).

TABLE II. Effects of musk xylene on levels of cytochrome P-450s

	control	Musk xylene		
	(vehicle only)	(50 mg/kg)	(100 mg/kg)	(200 mg/kg)
P-450IA1 (*pmol/mg protein*)	< 0.5	32.1 ± 13.2	93.2 ± 14.2	174.0 ± 16.1
P-450IA2 (*pmol/mg protein*)	39.6 ± 1.8	383.9 ± 29.9**	343.2 ± 12.7**	323.4 ± 28.7**

Each value shows the mean ± SE of four individual rats. **Significantly different from control ($p < 0.01$).

Discussion

The results of the present study demonstrate that MX induce both two cytochromes of the IA subfamily and cytochrome b_5 in rat liver microsomes. Especially, MX caused a remarkable induction of P-450IA2.

P-450IA2 is immunologically related to P-450IA1, but has different catalytic activities and a different primary structure from P450IA1 (1,3,4,6). P-450IA1 and P-450IA2 are the major PAH-inducible species of cytochromes P-450 in rats (1-7). Thomas *et al.* (7) observed that isosafrole preferentially induced P-450IA2 in comparison to either 3MC or β-naphthoflavone. However, isosafrole induced P-450IA2 only 2.4-fold greater than P-450IA1. MX is a much more selective inducer for P-450IA2 than isosafrole.

MX induced P-450IA1 to a small extent, compared to 3MC. Since the induction of cytochrome P450IA1 by PAH is regulated through the *Ah* receptor (1,2,5,6), MX appears to induce the P-450IA1 in microsomes through the *Ah* receptor. As a dose-dependent relationship observed in the induction of P-450IA1 was not observed in that of P-450IA2, the regulation of the induction of these P-450 isozymes may be different. Although P-450IA2 also seems to be regulated via a receptor-mediated mechanism (2,5), Ohyama *et al.* (17) suggested using PAH-nonresponsive mouse strain that factors other than the *Ah* receptor are also important in the regulation of P-450IA2 induction. It seems necessary to elucidate whether MX may regulate P-450IA2 via the *Ah* receptor-mediated mechanism or other factors

may be responsible for cytochrome P450IA2 induction as observed with isosafrole.

As shown in Table I, another characteristic of MX is inducibility of cytochrome b_5. The ability of P-450IA2 to N-oxidize the mutagenic amino acid pyrolysates such as Trp-P-2 and Glu-P-1 is especially noteworthy. Kamataki *et al.* (18) have shown that the reactions catalyzed by this enzyme were enhanced by addition of cytochrome b_5. Thus, S9 fraction prepared by the treatment of MX may be useful to assay mutagenicity of these kinds of proximate mutagens or carcinogens.

In conclusion, we propose that MX is a novel specific inducer for P-450IA2, but not P-450IA1.

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