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CYP2C11 AND CYP2B1 ARE MAJOR CYTOCHROME P450 FORMS INVOLVED IN STYRENE OXIDATION IN LIVER AND LUNG MICROSOMES FROM UNTREATED RATS, RESPECTIVELY

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Abstract—The contribution of cytochrome P450s (P450s) to the formation of styrene glycol from styrene in rat liver microsomes was investigated using monoclonal antibodies to P450s. Anti-CYP2E1 inhibited the formation to a similar extent in ethanol-treated microsomes and in control microsomes in terms of percentage inhibition, whereas to a greater extent in the former than the latter in terms of net inhibition, and only at low substrate concentration. Anti-CYP2C11/6 also inhibited the formation in control and in ethanol-treated microsomes at both low and high concentrations of styrene, and the net degree of inhibition was greater than that obtained with anti-CYP2E1, even in ethanol-treated microsomes where CYP2E1 was induced. Anti-CYP2B1/2 and anti-CYP1A1/2 inhibited the formation only in phenobarbital (PB)- and 3-methylcholanthrene (MC)-induced microsomes, respectively. These results suggest that (1) at least four P450s, CYP2C11/6, CYP2E1, CYP2B1/2 and CYP1A1/2, contribute to the metabolism of styrene, (2) CYP2C11/6, which probably corresponds to CYP2C11, is the major form of P450 responsible for the metabolism in untreated rat liver microsomes, and also in those treated with ethanol. Anti-CYP2E1 inhibited styrene oxidation more prominently in microsomes from styrene-treated rats than in those from control rats at a low substrate concentration. Although styrene treatment did not influence the total metabolism of styrene in liver microsomes at a high substrate concentration, inhibition of the metabolism by anti-CYP2C11/6 decreased with increasing styrene dose, whereas that by anti-CYP2B1/2 increased, suggesting that styrene treatment increases CYP2B1/2 but decreases CYP2C11/6 in rat liver, and the major form of P450 which mediates styrene oxidation is CYP2B1/2 after the treatment. Only anti-CYP2B1/2, which probably corresponds to CYP2B1, inhibited styrene oxidation in lung microsomes from untreated and even styrene-treated rats. Thus, the major form of P450 responsible for the metabolism of styrene is different in each tissue.

Key words: monoclonal antibody; ethanol; phenobarbital; 3-methylcholanthrene; enzyme induction; metabolic interaction

Styrene is used in the production of plastics and resins, including polystyrene resins, acrylonitrile-butadiene-styrene polymer resins, styrene-acrylonitrile resins, styrene-butadiene copolymer resins, styrene butadiene rubber and unsaturated polyester resins [1]. Exposure to styrene can also occur owing to its presence in automobile exhaust, cigarette smoke and packed foods and water [2, 3]. Large numbers of people may therefore be exposed to styrene.

Styrene is activated metabolically by P450s** to a toxic, mutagenic and potentially carcinogenic form: styrene 7,8-oxide [1]. Hitherto, studies of styrene metabolism have been focused on liver [4]. P450 consists, however, of multiple isozymes, which are inducible and are expressed differently in tissues [5]. In addition, styrene causes lung tumors in rodents [6]. The cytochrome P450 isozymes involved in styrene metabolism and their distribution, including their induction and destruction by chronic treatment, in target tissues must therefore be addressed.

Although some would take exception to the suggestion that MAbs are unique in their ability to define the specificity of P450, we showed previously the unique ability of MAbs to define the P450 responsible for the metabolism of benzene [7, 8], toluene [9] and trichloroethylene [10]. In this paper, we report on the contributions of CYP1A1/2, CYP2B1/2, CYP2C11/6 and CYP2E1 [11] to the metabolism of styrene in rat liver, using MAbs. We also investigated the constitutive profiles of these isozymes in liver and lung from untreated and styrene-treated rats.

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** Abbreviations: P450, cytochrome P450; MAb, monoclonal antibody; PB, phenobarbital; MC, 3-methylcholanthrene.

MATERIALS AND METHODS

Animals. The experiments were performed

in accordance with the Guidelines for Animal Experiment of the Shinshu University School of Medicine. Male Wistar rats (6 weeks of age) were obtained from Nippon SLC Inc. (Shizuoka, Japan) and were housed individually in an air-conditioned (20 ± 2°) room with artificial lighting on a 12 hr light/dark cycle, provided with pellet feed (Clea CE-2, Tokyo, Japan) and tap water *ad libitum* until they reached the age of 8 weeks. Rats were then divided into four groups: controls, and groups treated with ethanol, PB and MC. All groups except the group given ethanol were fed a well-balanced liquid diet (basal diet) prepared according to the recipe of Lieber *et al.* [12], with a slight modification: the basal diet contained 15.0 g sucrose, 3.66 g casein (sodium salt), 51 mg L-cysteine, 31 mg DL-methionine, 2.5 g oil (olive:corn, 4:1), 265 mg ethyl linoleate, 509 mg vitamin mixture, 1.0 g of mineral mixture, 25 mg DL-α-tocopherol and 265 mg carrageenan in a volume of 100 mL (1 kcal/mL). The ethanol-treated group was given a diet of the same composition as the basal diet, except that 2.5 g ethanol was added to 100 mL basal diet in which the sucrose content had been reduced to 5 g/100 mL, and the oil content increased to 5 g/100 mL, so that the ethanol and basal diets were isocaloric. The rats in the other two groups were administered PB at 80 mg/kg per day and MC at 20 mg/kg per day i.p. for 4 days before killing. Rats were given the diets (80 mL/rat) daily at 16:00 hr for 3 weeks as their only source of food and water.

In the study of induction of P450s by styrene, 15 male Wistar rats (7 weeks of age) were divided into three groups, and given corn oil or 5 or 10 mmol/kg styrene by gavage for 4 days.

All rats were killed by decapitation 18 hr after the last injection of inducer or ethanol diet (10:00 hr). The livers and/or lungs were removed promptly and perfused with cold 1.15% (v/v) KCl solution. In order to obtain the microsomal suspension, 25% liver homogenate (w/v) in the salt solution was centrifuged at 10,000 *g* for 10 min, the supernatant was further centrifuged at 105,000 *g* for 60 min, and the microsomal pellets obtained were suspended in 50 mM potassium phosphate buffer (pH 7.4) containing 10% (v/v) glycerin and frozen at −85° until use.

Metabolism assay. The metabolism of styrene was assessed by measuring the rate of formation of styrene glycol. The reaction mixture contained an NADPH-generating system (1 mM NADP, 20 mM glucose 6-phosphate, 2 IU glucose 6-phosphate dehydrogenase and 50 mM magnesium chloride), 200 μg of liver or 400 μg of lung microsomal protein 50 mM potassium phosphate buffer (pH 7.4) and 0.085 mM or 1.85 mM styrene, to a final volume of 0.5 mL. The reaction was initiated by adding the substrate, and the reaction vials were placed in a thermoregulated, shaking water bath at 37°. The two concentrations of styrene were used in order to determine the contribution of styrene were used in order to determine the contribution of styrene metabolizing enzymes at low and at high concentrations. After 10 min of incubation, 0.1 mL of 15% (w/v) zinc sulfate and saturated barium hydroxide was added to stop the reaction for measurement

Table 1. Immunoinhibition by MAbs of styrene glycol formation from styrene at low and high concentrations

Treatments	Hy-Hel	Anti-CYP2E1	Anti-CYP2C11/6	Anti-CYP2B1/2	Anti-CYP1A1/2
Low concentration (0.085 mM)					
Control	0.81 ± 0.24* (100)†	0.60 ± 0.19‡ (75 ± 7)‡	0.20 ± 0.06‡ (26 ± 5)‡	0.79 ± 0.26 (97 ± 3)	0.78 ± 0.23 (96 ± 4)
Ethanol	3.90 ± 1.36§ (100)	2.99 ± 1.15‡ (74 ± 9)‡	1.77 ± 0.48‡ (46 ± 4)‡	3.82 ± 1.28 (97 ± 6)	4.02 ± 1.23 (103 ± 4)
PB	4.37 ± 0.67§ (100)	4.35 ± 0.63 (100 ± 7)	4.45 ± 0.44 (102 ± 4)	1.93 ± 0.36‡ (44 ± 4)‡	4.35 ± 0.64 (100 ± 2)
MC	1.10 ± 0.22 (100)	1.07 ± 0.16 (98 ± 6)	1.02 ± 0.19 (92 ± 6)	1.09 ± 0.20 (99 ± 3)	0.87 ± 0.33 (92 ± 8)
High concentration (1.85 mM)					
Control	4.03 ± 0.73 (100)	3.90 ± 0.52 (97 ± 3)	0.82 ± 0.14‡ (21 ± 4)‡	3.83 ± 0.48 (96 ± 4)	4.10 ± 0.67 (102 ± 2)
Ethanol	6.00 ± 0.89§ (100)	5.42 ± 0.74 (91 ± 5)	3.21 ± 0.87‡ (52 ± 8)‡	5.73 ± 0.89 (95 ± 4)	5.95 ± 0.86 (99 ± 4)
PB	17.36 ± 1.78§ (100)	17.62 ± 1.58 (102 ± 2)	15.15 ± 1.54‡ (88 ± 4)‡	5.80 ± 0.89‡ (33 ± 3)‡	17.51 ± 1.70 (101 ± 1)
MC	4.69 ± 0.70 (100)	4.51 ± 0.70 (97 ± 6)	3.05 ± 0.78‡ (70 ± 5)‡	4.60 ± 0.85 (98 ± 4)	3.98 ± 0.74‡ (85 ± 3)‡

* Mean ± SD of remaining activity (nmol/mg protein per min) for four samples.
† Mean ± SD of percentage of remaining activity, expressed as activity with MAbs/activity with Hy-Hel × 100.
‡ Significantly different ($P < 0.05$) from Hy-Hel.
§ Significanty different ($P < 0.05$) from control.

of styrene glycol formation. The mixture was centrifuged at 1800 *g* for 15 min, and 20 μL (liver) and 30 μL (lungs) of supernatant were injected into a high-performance liquid chromatograph with an ultraviolet-vis detector and a Chromatointegrator. The analytical conditions were as follows: 4.0 mm diameter × 250 mm stainless steel column packed with Unisil C18; mobile phase, 10% (v/v) acetonitrile solution containing 0.25% (v/v) phosphoric acid at a flow rate of 1.1 mL/min; wavelength 200 nm. The retention time of styrene glycol was 10.2 min; the amount of styrene glycol formed increased linearly with respect to incubation time for at least 30 min and up to 1.0 mg microsomal protein.

Inhibition by monoclonal antibodies. MAbs were raised at the U.S. National Cancer Institute, Laboratory of Molecular Carcinogenesis, using a modification of the method of Koehler and Milstein [13]. In the present study, four MAbs that had been shown to be specific towards different P450s were used: anti-CYP1A1 MAb (clone 1-7-1), which reacts with both CYP1A1 and CYP1A2 (14); anti-CYP2B1 MAb (clone 2-66-3), which reacts with both CYP2B1 and CYP2B2 [15]; anti-CYP2C11 MAb (clone 1-68-11), which reacts with both CYP2C11 and CYP2C6 [16] and anti-CYP2E1 MAb (clone 1-91-3) [17]. As a control MAb, Hy-Hel to chicken lysozyme was used to determine any nonspecific reaction. MAbs were added to microsomes and buffer and incubated at room temperature for 30 min before the metabolism assay was started by addition of an NADPH-generating system and styrene at 37°. In preliminary experiments, maximal inhibition by each MAb was obtained at MAb protein : microsomal protein ratios below 1.0. Microsomal protein was measured by the method of Lowry *et al.* [18].

Statistics. Analysis of variance was performed. When there was significant difference among groups, means were tested by Student's *t*-test or the paired *t*-test. The 0.05 level of probability was the criterion of significance.

RESULTS

Contribution of P450s to styrene metabolism

Ethanol and PB increased the formation of styrene glycol from styrene to almost the same degree at the low substrate concentration. At the high styrene concentration, PB had the greatest effect, increasing formation of styrene glycol by 4.6-fold, followed by ethanol treatment (1.8-fold); MC did not affect the formation of the glycol (Table 1).

With the low concentration of styrene, anti-CYP2E1 and anti-CYP2C11/6 inhibited the formation of styrene glycol only in control and ethanol-treated microsomes. The percentage of remaining

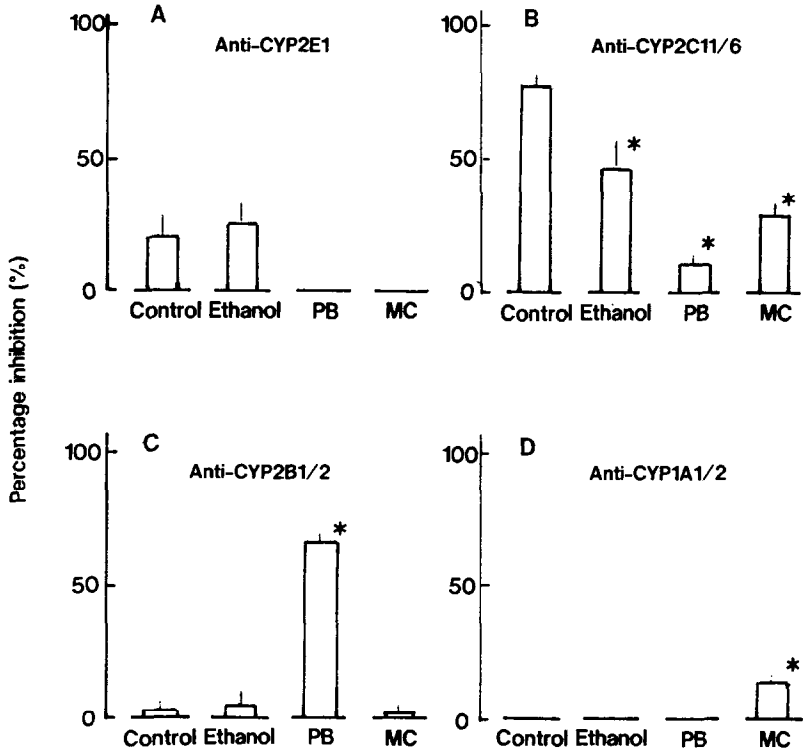

Fig. 1. Contribution of cytochrome P450 isozymes to styrene metabolism in rat liver microsomes using monoclonal antibodies (MAbs). (A) Contribution of CYP2E1 to metabolism at 0.85 mM styrene; (B–D) contribution of CYP2C11/6, CYP2B1/2 and CYP1A1/2 to metabolism at 1.85 mM styrene, respectively. Full activities with Hy-Hel are shown in Table 1. *Significant difference ($P < 0.05$) between control and treated microsomes.

activity in both microsomes exposed to anti-CYP2C11/6 was lower than that exposed to anti-CYP2E1, indicating that CYP2C11/6 contributes to the metabolism of styrene greater than CYP2E1. Anti-CYP2B1/2 inhibited the formation of styrene glycol only in PB-treated microsomes. No difference in the formation was found between microsomes exposed to anti-CYP1A1/2 and Hy-Hel.

At the high styrene concentration, anti-CYP2E1 did not show any inhibition of the formation of styrene glycol, whereas anti-CYP2C11/6 inhibited the formation in all microsomes more prominently than that at the low substrate concentration. The percentage of remaining activity after inhibition by anti-CYP2C11/6 was lowest in control microsomes, followed by ethanol-, MC- and PB-treated microsomes. Anti-CYP2B1/2 and anti-CYP1A1/2 also inhibited the formation of styrene glycol in PB- and MC-treated microsomes, respectively, in preference to the high substrate concentration. Thus, CYP2C11/6, CYP2B1/2 and CYP1A1/2 contribute to the metabolism of styrene to a greater extent at a high substrate concentration than at a low substrate concentration, whereas CYP2E1 contributes more at the latter than at the former. Therefore, a low substrate concentration was used for the measurement of the contribution of CYP2E1 to styrene oxidation, and a high concentration for CYP2C11/6, CYP2B1/2 and CYP1A1/2 in the following experiments.

Figure 1 shows the percentage inhibition [(difference between activity with Hy-Hel and with each MAb)/activity with Hy-Hel × 100] of styrene glycol formation by the MAbs. The inhibition by anti-CYP2E1 at the low substrate concentration in control microsomes was the same degree as that in ethanol-treated microsomes (Fig. 1A), although the net inhibition, difference between activity with Hy-Hel and with each MAb, was greater in ethanol-treated microsomes than in control samples (Table 1). The inhibition by anti-CYP2C11/6 was more prominent in control microsomes than in the ethanol-treated sample without affecting the net inhibition (Fig. 1B). PB and MC treatments clearly decreased the inhibition seen with anti-CYP2C11/6. The inhibition by anti-CYP2B1/2 and anti-CYP1A1/2 was only seen in PB- and MC-treated microsomes, respectively (Fig. 1C, D).

Effects of styrene treatment on the contribution of P450 to its own metabolism

Treatment with styrene did not influence the formation of styrene glycol from styrene in rat lung microsomes. In contrast, styrene enhanced its own metabolism in liver microsomes at the low, but not at the high concentration (Table 2).

Anti-CYP2E1 inhibited the formation of styrene glycol to a greater extent in styrene-treated liver microsomes than in control microsomes. The remaining activity of the formation after inhibition by anti-CYP2C11/6 increased dependent on styrene dose. In contrast, although anti-CYP2B1/2 did not inhibit the formation of styrene glycol in control microsomes, this MAb did inhibit the formation more prominently in liver microsomes from rats with high-dose styrene than in those with low-dose styrene, suggesting that CYP2B1/2 in the liver increases, but CYP2C11/6 decreases, by styrene treatment. Anti-CYP1A1/2 did not inhibit the metabolism of styrene in any of the microsomes.

Only anti-CYP2B1/2 inhibited the formation of styrene glycol in lung microsomes: the percentage inhibition was more than 50%, and was not affected by styrene treatment, suggesting that the major form of P450 responsible for the formation of styrene glycol in rat lungs is CYP2B1/2.

Table 2. Effect of styrene treatment on the contribution of styrene in rat liver and lungs

MAbs	Control	Styrene (5 mmol/kg)	Styrene (10 mmol/kg)
Liver			
Hy-Hel*	2.04 ± 0.13‡ (100)§	2.85 ± 0.25¶ (100)	3.46 ± 0.32¶ (100)
Anti-CYP2E1*	1.74 ± 0.22‖ (86 ± 2)‖	2.14 ± 0.10‖ (75 ± 4)‖	2.50 ± 0.14‖ (73 ± 3)‖
Hy-Hel†	5.26 ± 0.37 (100)	5.13 ± 0.27 (100)	5.60 ± 0.20 (100)
Anti-CYP2C11/6†	1.47 ± 0.03‖ (28 ± 1)‖	2.58 ± 0.05‖ (50 ± 2)‖	3.48 ± 0.42‖ (63 ± 10)‖
Anti-CYP2B1/2†	5.37 ± 0.61 (103 ± 5)	4.50 ± 0.21‖ (88 ± 1)‖	2.68 ± 0.15‖ (48 ± 1)‖
Anti-CYP1A1/2†	5.00 ± 0.10 (96 ± 6)	4.87 ± 0.17 (96 ± 9)	5.11 ± 0.30 (91 ± 2)
Lungs			
Hy-Hel*	0.22 ± 0.03 (100)	0.19 ± 0.01 (100)	0.20 ± 0.02 (100)
Anti-CYP2E1*	0.22 ± 0.02 (100 ± 4)	0.20 ± 0.02 (107 ± 5)	0.20 ± 0.02 (102 ± 2)
Hy-Hel†	0.51 ± 0.04 (100)	0.49 ± 0.04 (100)	0.52 ± 0.05 (100)
Anti-CYP2C11/6†	0.52 ± 0.04 (101 ± 1)	0.46 ± 0.04 (96 ± 4)	0.53 ± 0.07 (101 ± 2)
Anti-CYP2B1/2†	0.21 ± 0.01‖ (42 ± 3)‖	0.21 ± 0.02‖ (44 ± 5)‖	0.23 ± 0.02‖ (45 ± 1)‖
Anti-CYP1A1/2†	0.52 ± 0.03 (102 ± 8)	0.47 ± 0.02 (99 ± 2)	0.51 ± 0.05 (98 ± 1)

* Substrate concentration, 0.085 mM; †substrate concentration, 1.85 mM.
‡ Mean ± SD of remaining activity (nmol/mg protein per min) for four samples.
§ Mean ± SD of percentage of remaining activity, expressed as activity with MAbs/activity with Hy-Hel × 100.
‖ Significantly different ($P < 0.05$) from Hy-Hel.
¶ Significantly different ($P < 0.05$) from control.

DISCUSSION

Styrene was metabolized not only in liver but also in lung microsomes from rats. The study with MAbs indicates that CYP2C11/6, CYP2E1, CYP2B1/2 and CYP1A1/2 contribute to the formation of styrene glycol from styrene. The major form involved in this metabolism in untreated rat liver microsomes was CYP2C11/6, which may be much indebted to CYP2C11, because in adult male rats, although CYP2C6 is expressed equally to CYP2C11 [19], the specific activity of the latter for styrene metabolism is 5.4-fold that of the former [4]. Evidence for the conclusion that CYP2C11 is the major form in styrene metabolism in the adult male rat liver may also be found in the following result: the expression of CYP2C6 in liver from adult male rats is equal to that from adult females [19], and the net inhibition by anti-CYP2C11/6 in liver microsomes from the former was 7-fold that from the latter (unpublished data), which may be almost completely attributable to CYP2C6 [19]. In addition, it is of great interest that CYP2C11 was also the major form responsible for the formation of styrene glycol even in microsomes treated with ethanol, which greatly induces CYP2E1 [7]. In contrast, the major form in the metabolism of styrene in rat lung was CYP2B1/2, which probably corresponds to CYP2B1 [20]. Thus, the major P450 subform responsible for styrene metabolism is different between these tissues, even in the same animals.

We reported previously that the major P450 form is CYP2E1 for the metabolism of benzene, and trichloroethylene, and toluene at a low substrate concentration, whereas CYP2C11 is responsible for toluene metabolism at a high substrate concentration [21]. Benzene is structurally similar to toluene and styrene, whereas it resembles trichloroethylene in relation to the affinity for CYP2E1 or CYP2C11. The affinity of these chemicals for fat is as follows: styrene > toluene > trichloroethylene > benzene [22, 23]. The affinity of these volatile hydrocarbons for CYP2E1 and CYP2C11 appears to be associated with their solubility in fat rather than their structures.

The expression of P450 forms and their increases or decreases by styrene treatment observed on MAb inhibition study were also confirmed by western blot analysis (data not shown). Styrene treatment influenced some P450 forms in rat liver, but not lungs, by strongly inducing CYP2B1/2 and decreasing CYP2C11/6 without affecting the metabolic activity of styrene measured at a high substrate concentration, suggesting that the major form of P450 is CYP2B1/2 in both rat liver and lungs after treatment with a large amount of styrene. CYP2E1 was also induced in liver by exposure to styrene, which resulted in an increase in its own metabolism only at low substrate concentration. There is some evidence that styrene is carcinogenic in rat lungs [6]. CYP2B1 may play an important role in the activation of styrene to styrene oxide in the rat lungs. Of course, other forms of P450 may be also inserted in the metabolism of styrene in the lung, even at a high concentration in the animals exposed to a high dose.

Many chemicals, similar to styrene, decrease CYP2C11 in liver from adult male rats [24]. One reason may be the competition for available heme with the induction of CYP2B1/2, as reported by Dannan *et al.* [25]. The other may be an indirect effect of styrene via disturbing the pituitary secretion of growth hormone, which regulates CYP2C11 [26]. The mechanism responsible for the decrease of CYP2C11 has yet to be clarified.

Ethanol is a potent inducer of CYP2E1 [7]. However, with anti-CYP2E1, the uninhibited activity was greater in ethanol-treated microsomes than that in control microsomes, suggesting that in addition to CYP2E1, ethanol may induce other P450s involved in styrene metabolism. In our recent experiments, the ethanol dose used also induced CYP3A1, but not CYP2B1/2 (unpublished data). CYP3A1 may be responsible for styrene metabolism as reported by Foureman *et al.* [4]. Thus, although the contribution of other forms of P450 to the metabolism of styrene is of some importance, most of the activity in control and ethanol-treated microsomes can be accounted for in terms of CYP2C11/6 and CYP2E1. In microsomes from PB- and MC-treated animals, the majority of activity at low concentrations of styrene in the liver remains unaccounted for, and at high concentrations much of that in the MC-treated animals remains unexplained.

Styrene is widely used in the production of plastics, resins and synthetic rubber, suggesting that concomitant exposure to other chemicals is common. In rubber synthesis, 1,3-butadiene is used in combination with styrene. 1,3-Butadiene did not influence the metabolism of styrene, but styrene slightly inhibited the metabolism of 1,3-butadiene [27]. The major form of P450 involved in the metabolism of 1,3-butadiene is thought to be CYP2E1 [28] and that of styrene is CYP2C11, the contribution of which was some 80%. Coexposed 1.3-butadiene may hardly influence the metabolic rate of styrene, but coexposed styrene may influence that of 1,3-butadiene in competition with styrene for CYP2E1, which supports a previous report [27].

In conclusion, CYP2C11 is the major cytochrome responsible for styrene metabolism in untreated rat liver, and CYP2B1 for its metabolism in lung microsomes. CYP2B1/2 and CYP1A1/2 also catalysed styrene oxidation in liver microsomes from PB- and MC-treated rats, respectively. CYP2B1/2 was induced by exposure to styrene, but the level of CYP2C11 was decreased. Thus, the major forms of cytochrome P450 involved in the metabolism of styrene are different in each tissue, and differ according to chemical treatment. It is important to clarify the cytochrome P450 forms responsible for the metabolism of styrene in the different target tissues for the toxicity and carcinogenicity of styrene.

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