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Metabolism of Styrene to Styrene Oxide and Vinylphenols in Cytochrome P450 2F2- and P450 2E1-Knockout Mouse Liver and Lung Microsomes

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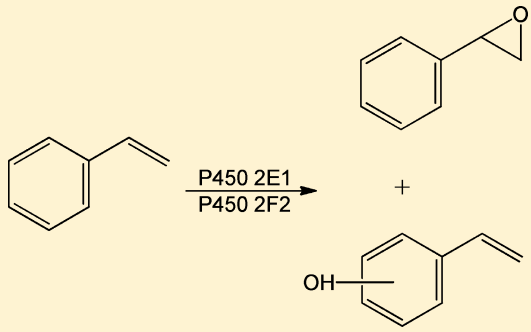
ABSTRACT: Pulmonary toxicity of styrene is initiated by cytochromes P450-dependent metabolic activation. P450 2E1 and P450 2F2 are considered to be two main cytochrome P450 enzymes responsible for styrene metabolism in mice. The objective of the current study was to determine the correlation between the formation of styrene metabolites (i.e., styrene oxide and 4-vinylphenol) and pulmonary toxicity of styrene, using *Cyp2e1*- and *Cyp2f2*-null mouse models. A dramatic decrease in the formation of styrene glycol and 4-vinylphenol was found in *Cyp2f2*-null mouse lung microsomes relative to that in the wild-type mouse lung microsomes; however, no significant difference in the production of the styrene metabolites was observed between lung microsomes obtained from *Cyp2e1*-null and the wild-type mice. The knockout and wild-type mice were treated with styrene (6.0 mmol/kg, ip), and cell counts and LDH activity in bronchoalveolar lavage fluids were monitored to evaluate the pulmonary toxicity induced by styrene. *Cyp2e1*-null mice displayed a susceptibility to lung toxicity of styrene similar to that of the wild-type animals; however, *Cyp2f2*-null mice were resistant to styrene-induced pulmonary toxicity. In conclusion, both P450 2E1 and P450 2F2 are responsible for the metabolic activation of styrene. The latter enzyme plays an important role in styrene-induced pulmonary toxicity. Both styrene oxide and 4-vinylphenol are suggested to participate in the development of lung injury induced by styrene.



INTRODUCTION

Styrene (**1**) is an important industrial chemical widely used in the manufacture of plastics, resins, and synthetic rubbers.[1] Styrene has been detected in cigarette smoke, engine exhausts, heating systems, newly installed carpets, painting,[2] and even food and drinking water stored in polystyrene containers.[3,4] The highest levels of human exposure to styrene occur in the reinforced plastic industry.[5−7] Occupational and environmental exposures to styrene take place mainly by inhalation. Styrene is both hepatotoxic and pneumotoxic in rodents, and it is classified as a possible carcinogen (IIB) in humans.[8−10] The National Toxicity Program lists styrene as a reasonably anticipated human carcinogen.[11]

Metabolic activation is considered to be a critical step for styrene-induced pulmonary toxicity.[12] The main pathway to metabolize styrene is the epoxidation of the vinyl group to styrene-7,8-oxide (styrene oxide, SO, **2**, Scheme 1) catalyzed by cytochromes P450. The resulting styrene oxide is further hydrated to styrene glycol (SG, **3**) or conjugated with glutathione to glutathione conjugates.[13,14] Styrene oxide is both mutagenic and carcinogenic.[13,15] It is thought to be one of the major toxic styrene metabolites. Aromatic hydroxylation is another metabolism pathway of styrene, which leads to the formation of 2-vinylphenol (2-VP, **4**), 3-vinylphenol (3-VP, **5**), and 4-vinylphenol (4-VP, **6**) (Scheme 1). Metabolic hydroxylation of styrene to 4-VP has been well studied in vitro and in vivo.[6,16−18] Recently, Linhart et al. and our group characterized 2-VP and 3-VP as styrene metabolites in mice.[19,20] We found 2-VP was the major isomer among the VP metabolites detected in mouse liver and lung microsomes. 4-VP has been reported more toxic than SO,[21,22] and our earlier study also showed that 4-VP was more toxic than 2-VP and 3-VP in mice.[19] The VPs were further metabolized to the corresponding hydroxystyrene oxides, vinylcatechols, and vinylhydroquinone in mouse microsomes,[19] but whether these metabolites cause cytotoxicity remains unclear.

P450 2F2 and P450 2E1 are considered as the major cytochrome P450 enzymes responsible for styrene metabolism in mice, although other P450 proteins, such as P450 2B,[13,23−25]

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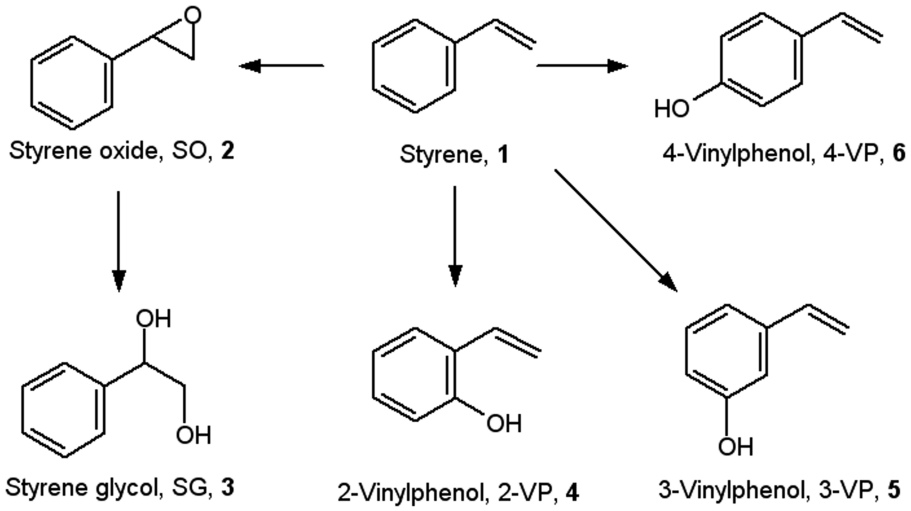
Scheme 1



may be partially involved. P450 2E1 is suggested to dominate the metabolism of styrene in mouse liver as a result of its abundant expression,[26,27] whereas P450 2F2 seems to take effect solely in mouse lung, since P450 2F2 is mainly expressed in mouse lung terminal bronchioles and nasal olfactory epithelium.[25,28] Recent reports emphasized that mouse P450 2F2 is important in the unique toxicity of mouse lung to several lung toxicants.[29]

Transgenic animals have been applied to investigate the metabolism and toxicity of styrene. Carlson found a significant decrease in the metabolism of styrene to styrene oxide in *Cyp2e1*-null mouse liver microsomes but not in the lung microsomes.[23] In addition, *Cyp2e1*-null mice were less susceptible to the hepatotoxicity of styrene than the wild-type animals. However, little change in the susceptibility to the pulmonary toxicity of styrene was observed in *Cyp2e1*-null mice in comparison with that of the wild-type mice.[30] The objectives of the present study included the investigation of the roles of P450 2E1 and P450 2F2 in metabolic transformation of styrene to styrene oxide and vinyl phenols in mouse liver and lung microsomes using *Cyp2e1*-null and *Cyp2f2*-null mouse models. This allows us to better understand the relationship between styrene-induced toxicity and its metabolites styrene oxide and vinyl phenols.

MATERIALS AND METHODS

Chemicals and Apparatus. Styrene (>99%), β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt (NADPH), *N,O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA), and 4-VP were purchased from Sigma-Aldrich (St. Louis, MO). Other chemicals were of analytical grade or higher. Styrene glycol-d_8 (SG-d_8), 2-VP, and 3-VP were synthesized in our lab.[19] An HP 5890 Series II gas chromatograph with a capillary GC column (J&W Scientific BD-5MS, 20 m × 0.18 mm i.d., film thickness 0.18 μm), an HP 5971A mass-selective detector (MSD), and an HP 7673 autosampler were used to analyze metabolite samples. HP Chemstation B.02.05 software was used for GC/MS control and data collection.

Animals. *Cyp2e1*-null mice (129/Sv-*Cyp2e1*^{tm1Gonz}/J) and the wild-type control mice (129S1/SvImJ) were purchased from the Jackson Laboratory (Sacramento, CA). The *Cyp2f2*-null mice were developed by Dr. Xinxin Ding's group in Wadsworth Center, New York State Department of Public Health (Albany, New York). The wild-type control mice of *Cyp2f2*-null mice (C57BL/6J) were purchased from the Jackson Laboratory. The animals were housed in an air conditioned room set to maintain 20−24 °C, 40−60% relative humidity, and 12-h light/dark cycle. The mice were fed standard rodent diets and drinking water ad libitum. The mice were bred in the Animal Laboratory of Seattle Children's Research Institute. Only male mice 2−3 month old were used to prepare microsomes. Liver and lung microsomes were prepared with the same protocol used in our previous work.[19]

Assessment of P450 2E1 and P450 2F2 Expressions. Western blot analysis was carried out to assess the expressions of P450 2E1 and P450 2F2. Protein concentrations of microsomes were measured using bicinchoninic acid protein kit (Pierce Chemical, Rockford, IL). Microsomal proteins (5 μg for liver and 15 μg for lung) were loaded and resolved on SDS−PAGE gel and transferred onto nitrocellulose membranes. The P450 2E1 and P450 2F2 protein bands were probed with rabbit antirat P450 2E1 antibody (StressGen, Victoria, BC, Canada) and rabbit anti-P450 2F antibody (custom-prepared by GenScript, Piscataway, NJ). Calnexin, a marker protein for the endoplasmic reticulum, was detected using a rabbit antihuman calnexin antibody (GenScript). The detected bands were captured with a Bio-Rad GS-710 Imaging Densitometer (Bio-Rad, Hercules, CA).

Determination of 4-Nitrophenol Hydroxylation Activity. 4-Nitrophenol was used as a substrate to assess P450 2E1 and P450 2F2 activities.[31,32] The incubation mixture contained 1.0 mg protein/mL (mouse liver microsomes) or 0.5 mg protein/mL (lung microsomes), 5.0 mM $MgCl_2$, 1.0 mM NADPH, 500 μM 4-nitrophenol, and 100 mM phosphate buffer (pH 7.4) with a total volume of 0.2 mL. 4-Nitrophenol was added to initiate the microsomal reactions. After incubation for 20 min at 37 °C, 20 μL of 3 M trichloroacetic acid solution was added, followed by vortexing for 1 min. The precipitated proteins were removed by centrifuging at 12 000 rpm for 10 min. Twenty microliters of the resulting supernatant was injected to HPLC for analysis. An Agilent 1100 HPLC system was used to quantify the formation of 4-nitrocatechol. The mobile phase was a mixture of acetonitrile (0.1% trifluoroacetic acid) and water (0.1% trifluoroacetic acid) (2:8, v/v), and the column for separation was an Agilent ZORBAX Eclipse XDB C8 (150 × 4.6 mm, 5 μm). UV signals were recorded at 245 nm for 4-nitrocatechol. A 4-nitrocatechol standard curve was prepared with a series of 4-nitrocatechol solutions at concentrations of 2.0, 5.0, 10, 20, and 50 μM in 100 mM phosphate buffer. 4-Nitrocatechol concentrations were calculated by an external standard curve method.

Incubations of Styrene in Mouse Liver and Lung Microsomes. Microsomal incubations were carried out in glass tubes with PTFE-faced, rubber-lined screw caps (10 × 1.3 cm i.d., Kimble Chase, Vineland, NJ). The incubation mixtures contained mouse liver or lung microsomes (1.0 mg protein/mL), 5.0 mM $MgCl_2$, 2.0 mM NADPH, and 500 μM styrene in 100 mM phosphate buffer (pH 7.4) with a total volume of 0.5 mL. After preincubation in a 37 °C water bath shaker for 2 min, reactions were initiated by adding 5.0 μL of styrene stock

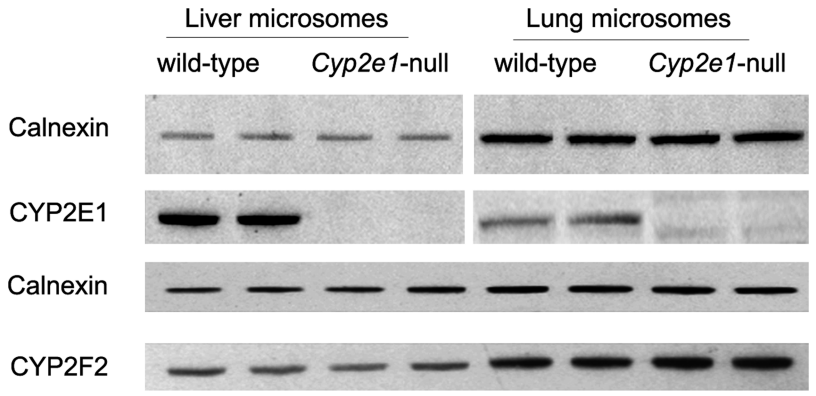


Figure 1. P450 2E1 and P450 2F2 expressions in wild-type and *Cyp2e1*-null mouse liver and lung microsomes.

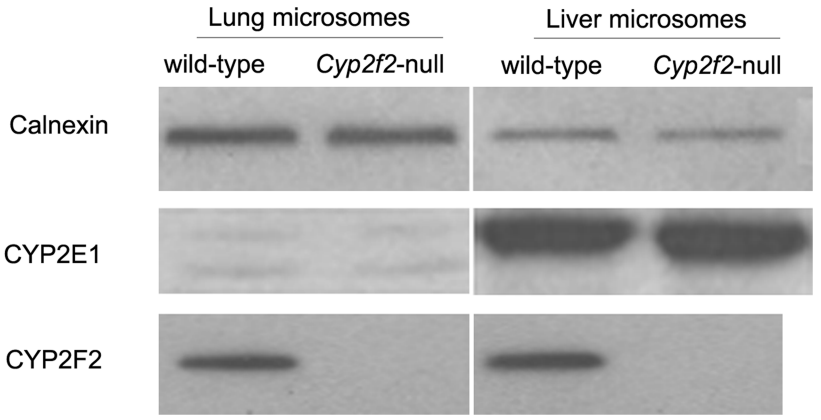


Figure 2. P450 2E1 and P450 2F2 expressions in wild-type and *Cyp2f2*-null mouse liver and lung microsomes.

solution (50 mM in acetonitrile), and the incubation tubes were immediately sealed with caps. Incubations lacking NADPH were treated as blank controls.

After incubation for 20 min, the reactions were terminated by adding 3 mL of ice-cold ethyl acetate, followed by addition of 5.0 μL of internal standard solution containing SG-d_8 (0.1 mM in acetonitrile). The mixture was vortexed for 2 min and centrifuged at 4000 rpm for 10 min. The organic supernatant was transferred to a clean test tube and dried under nitrogen gas flow. The residue was reconstituted with 50 μL of 20% BSTFA−acetonitrile solution (v/v), vortexed, incubated at 60 °C for 30 min, and subjected to GC/MS analysis.

Determination of Kinetics of Styrene Metabolism in Liver and Lung Microsomes. To determine the kinetic parameters of styrene metabolism in various types of microsomes, styrene at 1.0, 2.0, 5.0, 10, 20, 60, or 100 μM was incubated in microsomes. The incubation mixtures contained mouse liver or lung microsomes (1.0 mg protein/mL), 5.0 mM $MgCl_2$, 1.0 mM NADPH, and 100 mM phosphate buffer (pH 7.4) with a total volume of 0.5 mL. The reactions were initiated by addition of styrene. After incubation at 37 °C for 5 min, the reactions were stopped by adding 3 mL of ice-cold ethyl acetate. After adding internal standard SG-d_8 (5.0 μL, 0.1 mM), the samples were processed and derivatized as described previously. The resulting derivatized SG in the samples was analyzed by GC/MS. V_{max} and K_m values were calculated by nonlinear least-squares regression analysis using SigmaPlot software.

Determination of Pulmonary Toxicity of Styrene in Mice. Male *Cyp2e1*-null, *Cyp2f2*-null, and the corresponding wild-type mice (20−26 g) were divided into control and dose groups (6 mice each). The dose groups were treated with styrene (6.0 mmol/kg, i.p.) dissolved in vegetable oil, and the control groups were given vegetable oil (5.0 mL/kg) intraperitoneally. After 18 h, the mice were terminated by CO_2 asphyxiation. The trachea was exposed, where a small nick was made, and an oral feeding needle was inserted and tied in place. The lungs were lavaged twice with 0.9 mL of room-temperature PBS (Mediatech Inc., Manassas, VA). The bronchoalveolar lavage fluid (BALF) was collected (~1.6 mL) and placed on ice. Cells in the BALF were counted using a hemocytometer under a microscope. The remaining BALF from each mouse was centrifuged at 3000 rpm for 5 min. The lactate dehydrogenase (LDH) activity in the BALF supernatant was measured by an In Vitro Toxicology Assay Kit, LDH-based (Sigma-Aldrich, MO).

GC/MS Analysis. The column temperature was held at an initial temperature of 60 °C for 1 min after injection (splitless) of 1 μL of sample, followed by an increase to 170 °C at 10 °C/min, then increased at 20 °C/min to 250 °C and held for 2 min. Injector port temperature was 250 °C, and detector temperature was 280 °C. The GC column was a J&W Scientific BD-5MS (20 m × 0.18 mm id; film thickness: 0.18 μm). The carrier gas was helium with column head pressure set at 10 psi at 50 °C. Dwell time was 50 ms, and EI ionization voltage was 70 eV. Styrene metabolites were derived with BSTFA in 20% BSTFA acetonitrile solution at 60 °C for 30 min. Target ions of the derived styrene metabolites were monitored in SIM mode (m/z 177 and 192 for VP-TMS; m/z 179 for SG-TMS; m/z 185 for SG-d_8-TMS).

Determination of VP and SG contents was achieved using the corresponding standard curves (VPs, 5.0−500 nM; SG, 2.0−200 μM). Standard curves were fitted with a linear equation, where x was the concentration of analytes and y was the peak ratio of analytes vs internal standard. The correlation coefficient of the standard curves was $r^2 > 0.998$.

Data Analysis. All values were represented as mean ± SD. The two-sided, unpaired Student t-test was used to compare the difference between the wild-type and knockout mouse microsomes. The styrene metabolism kinetic parameters were fitted with the enzyme kinetic equation using Sigmaplot 9.0 software (Systat Software, CA).

RESULTS

P450 2E1 and P450 2F2 Protein Expressions. As an initial step, we determined the expressions of P450 2E1 and P450 2F2 in the liver and lung of the knockout and wild-type mice. The P450 proteins were assessed by immunoblot using the corresponding antibodies. As expected, little P450 2E1 protein was found in *Cyp2e1*-null mouse liver as well as in the lung (Figure 1). In addition, the immunoblot results demonstrated that much more P450 2E1 protein was expressed in the liver than in the lung in the wild-type mice (Figure 1). P450 2F2 protein was detected in the wild-type mouse liver and lung but not in those tissues obtained from *Cyp2f2*-null mice (Figure 2). Apparently, levels of P450 2F2 expression found in the lung were similar to that in the liver of the wild-type mice (Figures 1 and 2).

Microsomal 4-Nitrophenol Hydroxylation Activity. In addition to the assessment of P450 2E1 and P450 2F2 protein expressions, we compared their activities in liver and lung microsomes obtained from *Cyp2f2*-null and *Cyp2e1*-null mice and the corresponding wild-type mice. The enzyme activities were measured by assessing hydroxylation of 4-nitrophenol. As shown in Table 1, a significant decrease in 4-nitrophenol hydroxylation activity was observed in both *Cyp2f2*-null and *Cyp2e1*-null mouse liver and lung microsomes, relative to those obtained from the corresponding wild-type animals. Interestingly, the loss of 4-nitrophenol hydroxylation activity took place mainly in the liver of *Cyp2e1*-null mice (46.8% in liver vs 25.2% in lung), whereas the enzyme activity loss occurred almost exclusively in the lung of *Cyp2f2*-null mice (86.0% in lung vs 15.1% in liver), compared with those of the corresponding wild-type mice (Table 1).

Table 1. 4-Nitrophenol Hydroxylation Activity of *Cyp2e1*-null, *Cyp2f2*-null, and the Corresponding Wild-Type Mouse Liver and Lung Microsomes[a]

mouse strain	liver microsomes (nmol/min/mg protein)	lung microsomes (nmol/min/mg protein)
129S1/SvImJ	2.50 ± 0.22	0.583 ± 0.008
Cyp2e1-null	1.33 ± 0.02[b]	0.436 ± 0.003[b]
C57BL/6J	2.84 ± 0.05	0.580 ± 0.029
Cyp2f2-null	2.41 ± 0.08[b]	0.081 ± 0.003[b]

[a]Mean ± SD, $n = 3$. [b]$p < 0.01$ compared with the wild-type mice.

Metabolism of Styrene in *Cyp2e1*-null and Wild-Type Mouse Liver and Lung Microsomes. To understand the role of P450 2E1 in styrene metabolism, we investigated the biotransformation of styrene to styrene glycol and vinyl phenols in liver and lung microsomes of *Cyp2e1*-null and the wild-type mice. The rates of SG and VP production are listed in Table 2 (the unit for SG formation is nmol/min/mg of protein; the unit for VP production is pmol/min/mg of protein). As expected, styrene was metabolized mainly to SG in all four types of microsomes. The formation of SG was significantly slowed down (44.2% drop) in *Cyp2e1*-null mouse liver microsomes compared with the reactions taking place in the wild-type liver microsomes; however, no significant difference in the rate of SG production was observed between the lung microsomes obtained from *Cyp2e1*-null and the wild-type mice. All three vinyl phenols were detected in *Cyp2e1*-null and wild-type mouse liver and lung microsomes at low rates, in comparison with that of SG formation. The order of VP formation was 2-VP > 4-VP > 3-VP in wild-type mouse liver and lung microsomes. A similar order of the VP production was found in the *Cyp2e1*-null microsomes, but at lower rates relative to that observed in the wild-type mouse liver and lung microsomes. Interestingly, lung microsomes generated more 2-VP than liver microsomes of both *Cyp2e1*-null and the wild-type mice.

Metabolism of Styrene in *Cyp2f2*-null and Wild-Type Mouse Liver and Lung Microsomes. The role of P450 2F2 in styrene metabolism was also studied, using *Cyp2f2*-null and wild-type mouse liver and lung microsomes. A dramatic decrease in the rate of SG generation was observed in *Cyp2f2*-null mouse liver and lung microsomes, compared with that found in wild-type mouse liver and lung microsomes. Specifically, as much as a 64% drop in the formation of SG from styrene was found in *Cyp2f2*-null mouse lung microsomes, and a 25% drop in liver microsomes, relative to that observed in wild-type mouse lung or liver microsomes (Table 3). The three VPs were all found in wild-type mouse liver and lung microsomes; however, only 2-VP was observed in *Cyp2f2*-null mouse liver microsomes, and little VPs was detected in *Cyp2f2*-null mouse lung microsomes after incubation with styrene.

Kinetics of Styrene Metabolism in *Cyp2e1*-null, *Cyp2f2*-null, and Wild-Type Mouse Liver and Lung Microsomes. The kinetic parameters of vinyl epoxidation of styrene in *Cyp2e1*-null, *Cyp2f2*-null, and the corresponding wild-type mouse liver and lung microsomes were determined by incubation of styrene at a series of concentrations in the individual microsomes. V_{max} and K_m values were calculated on the basis of the production of the resulting SG. No statistical difference in V_{max} and K_m values was observed in both liver microsomal or lung microsomes between *Cyp2e1*-null mice and the wild-type animals (Table 4); however, reduced V_{max} values were observed in liver and lung microsomes of *Cyp2f2*-null mice (30% and 63% decreases in liver and lung microsomes, respectively) relative to those of the wild-type animals. Interestingly, decreased K_m values were also observed in the two types of microsomes (47% and 53% decreases in liver and lung microsomes, respectively). As a result, there is no difference in V_{max}/K_m values between the microsomes obtained from *Cyp2f2*-null and the wild-type mice (Table 4).

Acute Pulmonary Toxicity of Styrene in *Cyp2e1*-null, *Cyp2f2*-null and the Wild-Type Mice. Pulmonary toxicity of styrene was evaluated by monitoring cell counts and LDH

Table 2. Velocity of the Production of Vinyl Phenols and Styrene Glycol in *Cyp2e1*-null and Wild-Type Mouse Liver and Lung Microsomal Incubations with Styrene (500 μM)[a]

	liver microsomes				lung microsomes			
	2-VP	3-VP	4-VP	SG	2-VP	3-VP	4-VP	SG
129S1/SvImJ	1.85 ± 0.15	0.089 ± 0.017	0.877 ± 0.026	2.09 ± 0.04	6.47 ± 0.96	0.155 ± 0.020	0.234 ± 0.047	1.31 ± 0.13
Cyp2e1-null	0.717 ± 0.145[b]	0.063 ± 0.011	0.278 ± 0.015[b]	1.17 ± 0.126[b]	4.35 ± 0.623[c]	0.163 ± 0.020	0.232 ± 0.015	1.37 ± 0.02

[a]Mean ± SD, $n = 3$. VPs, pmol/min/mg of protein; SG, nmol/min/mg of protein. [b]$p < 0.01$. [c]$p < 0.05$ compared with the wild-type mice.

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Table 3. Velocity of the Production of Vinyl Phenols and Styrene Glycol in *Cyp2f2*-null and Wild-Type Mouse Liver and Lung Microsomal Incubations with Styrene (500 μM)[a]

	liver microsomes				lung microsomes			
	2-VP	3-VP	4-VP	SG	2-VP	3-VP	4-VP	SG
C57BL/6J	2.93 ± 0.15	0.370 ± 0.035	0.925 ± 0.082	2.44 ± 0.08	3.72 ± 0.54	0.181 ± 0.007	0.334 ± 0.019	1.13 ± 0.17
Cyp2f2-null	1.90 ± 0.58[c]	N.D.	N.D.	1.83 ± 0.02[b]	N.D.	N.D.	N.D.	0.410 ± 0.007[b]

[a]Mean ± SD, $n = 3$. VPs, pmol/min/mg of protein; SG, nmol/min/mg of protein. [b]$p < 0.01$. [c]$p < 0.05$ compared with the wild-type mice. N.D.: not detected.

Table 4. Kinetic Parameters of Styrene Metabolism to Styrene Glycol in *Cyp2e1*-null, *Cyp2f2*-null, and the Corresponding Wild-Type Mouse Liver and Lung Microsomes[a]

	microsomes					
	liver microsomes			lung microsomes		
	K_m (μM)	V_{max} (nmol/min/mg)	V_{max}/K_m (L/min/g)	K_m (μM)	V_{max} (nmol/min/mg)	V_{max}/K_m (L/min/g)
129S1/SvImJ	7.96 ± 0.85	2.51 ± 0.28	0.32 ± 0.03	12.9 ± 1.3	2.03 ± 0.41	0.16 ± 0.03
Cyp2e1-null	6.20 ± 0.84	1.99 ± 0.32	0.32 ± 0.04	10.9 ± 1.5	1.88 ± 0.36	0.17 ± 0.02
C57BL/6J	25.7 ± 1.9	3.74 ± 0.47	0.14 ± 0.01	14.7 ± 3.0	3.34 ± 0.40	0.23 ± 0.02
Cyp2f2-null	13.5 ± 1.1[b]	2.64 ± 0.25[c]	0.20 ± 0.04	6.87 ± 2.6[c]	1.22 ± 0.25[b]	0.19 ± 0.04

[a]Mean ± SD, $n = 3$. [b]$p < 0.01$. [c]$p < 0.05$ compared with the wild-type mice.

Table 5. Pulmonary toxicity of styrene in *Cyp2e1*-null, *Cyp2f2*-null, and the corresponding wildtype mice[a]

	BALF cell counts (104/mL BALF)		BALF LDH activity (OD490)	
	control	styrene	control	styrene
129S1/SvImJ	2.7 ± 2.6	11.7 ± 3.7[b]	0.167 ± 0.069	1.297 ± 0.220[b]
Cyp2e1-null	1.2 ± 0.4	18.6 ± 9.6[b]	0.161 ± 0.072	0.757 ± 0.094[b]
C57BL/6J	2.8 ± 2.5	18.9 ± 6.8[b]	0.294 ± 0.063	0.944 ± 0.338[b]
Cyp2f2-null	3.1 ± 1.1	3.4 ± 1.3	0.152 ± 0.031	0.145 ± 0.040

[a]Mean ± SD, $n = 3$. [b]$p < 0.01$ compared with the control.

activity in bronchoalveolar lavage fluids. Treatment of styrene induced a significant elevation in BALF cell counts and LDH activity in both *Cyp2e1*-null and the wild-type mice, and *Cyp2e1*-null mice were as susceptible to lung toxicity of styrene as the wild-type animals. In contrast, no changes in cell counts and LDH activity in BALF were observed in *Cyp2f2*-null mice after treatment with styrene, and the knockout animals were not susceptible to the styrene toxicity. However, increased BALF cell counts and LDH activity were observed in the corresponding wild-type mice given the same dose of styrene (Table 5).

DISCUSSION

Multiple cytochromes P450 are involved in styrene metabolism,[13,14] among which P450 2E1 and P450 2F2 have been reported to be the major P450 enzymes responsible for styrene metabolism in mice.[23] It has been suggested that P450 2E1 is more important to styrene metabolism in liver and P450 2F2 plays the dominant role in styrene metabolism in mouse lung, presumably because of the abundance of the individual enzymes expressed in the respective organs.[25,29,33] However, our present immunoblot data did not support the presumption. The expression of P450 2F2 in the liver is at least as much as in the lung in 129S1/SvImJ and C57BL/6J mice (Figures 1 and 2). In the present study, we investigated the sole role of P450s 2E1 and 2F2 in metabolic activation of styrene by use of *Cyp2e1*-null and *Cyp2f2*-null mouse models in comparison with the corresponding wild-type mice.

Vinyl epoxidation of styrene has been suggested to be a key reaction to trigger the toxicity of styrene. We examined the rate of styrene epoxidation in liver and lung microsomes obtained from *Cyp2e1*-null and the wild-type mice. The production of styrene glycol (SG), the hydrated product of styrene oxide, was slowed down by 50% in *Cyp2e1*-null mouse liver microsomes, compared with that in liver microsomes of the wild-type mice. However, lung microsomes of *Cyp2e1*-null mice showed activity similar to that of the wild-type animals. This indicates that lung microsomes of *Cyp2e1*-null mice retained the ability to metabolize styrene to styrene oxide and that the activity to bioactivate styrene left in the lung microsomes of *Cyp2e1*-null mice was contributed by other P450 enzymes. Clearly, P450 2E1 was not critical for the oxidation of styrene in mouse lung, although it does have the activity for metabolic activation of styrene. The present study showed much lower P450 2E1 content in lung microsomes than that in liver microsomes of the wild-type mice. Carlson reported that *Cyp2e1*-null mice were as susceptible to pulmonary toxicity of styrene as the wild-type animals.[30] Our present study showed that treatment of styrene induced a similar magnitude of elevated cell counts and LDH activity in the BALF in *Cyp2e1*-null and the wild-type mice after exposure to styrene (Table 5). The similarity in susceptibility to pulmonary toxicity of styrene observed in *Cyp2e1*-null mice and the wild-type mice may result from the observed no change in the formation of styrene oxide in the lungs of the two types of mice given styrene.

In contrast, *Cyp2f2*-null mouse liver microsomes retained 75% activity to oxidize styrene to styrene oxide, compared with the wild-type mouse liver microsomes. This indicates P450 2F2 was not critical in bioactivation of styrene in mouse liver. It is likely that expression of P450 2F2 is limited in mouse liver

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relative to the expression of P450 2E1 and that the loss of P450 2F2 did not have significant impact on the microsomal activity for the production of styrene oxide. However, *Cyp2f2*-null mouse lung microsomes showed only one-third of the enzyme activity to produce styrene oxide from styrene in comparison with what the wild-type mouse lung microsomes revealed. This implicates that expression of P450 2F2 was essential in vinyl epoxidation of styrene in mouse lung. This situation is very similar to the activity for the hydroxylation of 4-nitrophenol in these microsomes. Dramatically decreased activity for 4-nitrophenol hydroxylation was found in *Cyp2f2*-null mouse lung microsomes, but only a minor decrease in 4-nitrophenol hydroxylation activity was observed in the liver microsomes.

Cruzan et al. reported that *Cyp2f2*-null mice were less susceptible to pulmonary toxicity of styrene than the wild-type animals.[34] Our present study showed that treatment with the same dose of styrene did not cause the elevation in cell counts and LDH activity in the BALF of *Cyp2f2*-null mice. The observed resistance of *Cyp2f2*-null mice to lung toxicity of styrene may be explained by the loss of enzyme activity for the production of styrene oxide in the lung of *Cyp2f2*-null mice.

Vinyl phenols are another group of styrene metabolites resulting from aromatic hydroxylation of styrene. We examined the production of the VPs in *Cyp2e1*- and *Cyp2f2*-null and the corresponding wild-type mouse microsomes. As expected, all three VPs, including 2-, 3-, and 4-VPs, were found in wild-type mouse liver and lung microsomes. However, significant decreases in the formation of 2-VP and 4-VP were observed in *Cyp2e1*-null mouse liver microsomes, but no or a mild decrease in the formation of the VPs was observed in *Cyp2e1*-null mouse lung microsomes. This indicates that P450 2E1 plays an important role in aromatic hydroxylation of styrene in the liver but not in the lung. A dramatic decrease in the production of the VPs was found in *Cyp2f2*-null mouse microsomes. Only 2-VP was found in liver microsomes, and no VPs were detected in lung microsomes. This implies that P450 2F2 is a critical enzyme responsible for metabolism of styrene to the VPs, particularly in the lung.

4-VP has been suggested to be the critical toxic metabolite responsible for styrene-induced pulmonary toxicity.[21,35] However, the results of our present study do not necessarily support this hypothesis. Apparently, the alternations of the levels of 4-VP formed in lung microsomes were consistent with the changes in the susceptibility of mice to the pulmonary toxicity of styrene. However, the changes in the levels of styrene oxide produced in lung microsomal reactions were also consistent with the alternations of the susceptibility of the animals to lung toxicity induced by styrene. Therefore, we cannot exclude the role of styrene oxide in styrene-induced pulmonary toxicity.

Remarkable decreases in the V_{max} of styrene vinyl epoxidation were observed in liver and lung microsomes of both *Cyp2e1*-null and *Cyp2f2*-null mice. Meanwhile, the K_m value of styrene to the microsomes decreased in these microsomes, accordingly. The reason for the decrease in the K_m value is unknown and possibly results from the complexity and multiplicity of microsomal P450 enzyme systems. It is likely that styrene binds to multiple P450s with various catalytic capabilities. Among the P450s, P450 2E1 and P450 2F2 are the major enzymes responsible for the epoxidation of styrene.[23,29,30,34] The observed loss of P450 2E1 or P450 2F2 decreased the vinyl epoxidation V_{max}, but combined with a decrease in the apparent K_m value was possibly due to the high styrene-binding capacities of multiple P450s.

In conclusion, both P450 2E1 and P450 2F2 are greatly involved in the metabolism of styrene to styrene oxide and the VPs, but their importance in styrene metabolism can vary in the liver and lung. The loss of P450 2E1 activity to metabolize styrene can be compensated in the lung but not in the liver. In contrast, the loss of P450 2F2 activity for styrene metabolism can be compensated in the liver but not in the lung. The susceptibility of mice to pulmonary toxicity of styrene is associated with the level of P450 2F2 expression but not that of P450 2E1 expression in the lung. It is likely that both styrene oxide and 4-VP are involved in pulmonary toxicity induced by styrene.

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ABBREVIATIONS

BALF, bronchoalveolar lavage fluid; BSTFA, *N*,*O*-bis-(trimethylsilyl)-trifluoroacetamide; GC/MS, gas chromatography; LDH, lactate dehydrogenase; NADPH, β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SG, styrene glycol; SO, styrene oxide; TMS, trimethylsilyl; VP, vinylphenol

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