Detection of Phenolic Metabolites of Styrene in Mouse Liver and Lung Microsomal Incubations^[S]

Shuijie Shen, Fan Zhang, Lingbo Gao, Su Zeng, and Jiang Zheng

Center for Developmental Therapeutics, Seattle Children's Research Institute, Seattle, Washington (S.S., F.Z., J.Z.); College of Pharmaceutical Sciences, Zhejiang University, Hangzhou, China (L.G., S.Z.); and Division of Gastroenterology, Department of Pediatrics, University of Washington, Seattle, Washington (J.Z.)

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ABSTRACT:

Metabolic activation is considered to be a critical step for styrene-induced pulmonary toxicity. Styrene-7,8-oxide is a primary oxidative metabolite generated by vinyl epoxidation of styrene. In addition, urinary 4-vinylphenol (4-VP), a phenolic metabolite formed by aromatic hydroxylation, has been detected in workers and experimental animals after exposure to styrene. In the present study, new oxidative metabolites of styrene, including 2-vinylphenol (2-VP), 3-vinylphenol (3-VP), vinyl-1,4-hydroquinone, and 2-hydroxystyrene glycol were detected in mouse liver microsomal incubations. The production rates of 2-VP, 3-VP, 4-VP, and styrene glycol were 0.0527 ± 0.0045 ,

0.0019 ± 0.0006 , 0.0053 ± 0.0002 , and 4.42 ± 0.33 nmol/(min · mg protein) in mouse liver microsomes, respectively. Both disulfiram (100 μ M) and 5-phenyl-1-pentyne (5 μ M) significantly inhibited the formation of the VPs and styrene glycol. 2-VP, 3-VP, and 4-VP were metabolized in mouse liver microsomes at rates of 2.50 ± 0.30 , 2.63 ± 0.13 , and 3.45 ± 0.11 nmol/(min · mg protein), respectively. The three VPs were further metabolized to vinylcatechols and/or vinyl-1,4-hydroquinone and the corresponding glycols. Pulmonary toxicity of 2-VP, 3-VP, and 4-VP was evaluated in CD-1 mice, and 4-VP was found to be more toxic than 2-VP and 3-VP.

Introduction

Styrene is widely used in the plastic and rubber industries (Miller et al., 1994; IARC, 2002). Human exposure to styrene occurs mainly in manufacturing sites, particularly in developing countries. Animal studies have shown that styrene produced hepatotoxicity and pneumotoxicity in rodents, and styrene is classified as a possible carcinogen (IIB) in humans (Bond, 1989; IARC, 2002). The metabolism of styrene has been extensively investigated. Epoxidation of the vinyl group is the primary oxidation reaction by which styrene is metabolized to styrene-7,8-oxide (SO, 2) (Scheme 1) (Bond, 1989; Sumner and Fennell, 1994). The oxidative metabolite has been reported to be mutagenic and carcinogenic (IARC, 1994; Gadberry et al., 1996).

Aromatic hydroxylation of styrene has been reported, and it is considered to be a minor metabolism pathway of styrene (Bond, 1989; Sumner and Fennell, 1994; Vodicka et al., 2006). Urinary 4-vinyl-

phenol (4-VP) and its glucuronide and sulfate conjugates were detected in urine of rats treated with styrene (Bakke and Scheline, 1970; Pantarotto et al., 1978). These metabolites were also detected in urine of workers in reinforced plastic plants (Pfäffli et al., 1981; Manini et al., 2003). 2-Vinylphenol (2-VP) and 3-vinylphenol (3-VP) have been proposed as aromatic hydroxylation products of styrene (Sumner and Fennell, 1994), but they have never been identified either in experimental animals or in humans exposed to styrene. Most recently, Linhart et al. (2010) reported urinary 3-VP, 4-VP, and possible 2-VP in mice exposed to airborne styrene. Cruzan et al. (2009) stated that differences in styrene oxide blood concentrations did not account for the species selectivity and tumor response to styrene. Styrene is tumorigenic in mouse lung but not in rat lung. These authors also suggested that there were other metabolites generated from styrene by CYP2F2 and that these may be responsible for tumor formation in murine lung (Cruzan et al., 2009). Identification of the phenolic metabolites of styrene can help us to further understand the mechanism of styrene-induced toxicity.

Animal studies showed that 4-VP was more hepatotoxic and pneumotoxic than styrene or SO when these compounds were administered to CD-1 mice intraperitoneally (Carlson, 2002). This observation led us to examine the formation of 2-VP and 3-VP from styrene and their toxicities to the pulmonary system. Our hypothesis is that 2-VP and 3-VP are metabolites of styrene and may be contributors to styrene-induced toxicity.

The formation of 4-VP from styrene has been well documented, but

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ABBREVIATIONS: SO, styrene-7,8-oxide; VP, vinylphenol; 4-VP, 4-vinylphenol; 3-VP, 3-vinylphenol; 2-VP, 2-vinylphenol; P450, cytochrome P450; 5P1P, 5-phenyl-1-pentyne; BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; GC/MS, gas chromatography/mass spectrometry; TLC, thin-layer chromatography; TMS, trimethylsilane; THF, tetrahydrofuran; SG, styrene glycol; SIM, selected ion monitoring; BALF, bronchoalveolar lavage fluid; LDH, lactate dehydrogenase.

SCHEME 1. Proposed pathways of metabolic vinyl epoxidation and aromatic hydroxylation of styrene in mouse liver microsomes.

cytochrome P450 (P450) isoforms responsible for styrene ring oxidation are unclear. CYP2F2 and CYP2E1 were found to be the principal isoforms to catalyze the biotransformation of styrene to SO in mice (Hynes et al., 1999; Cruzan et al., 2002; Carlson, 2003). We hypothesized that CYP2F2 and CYP 2E1 also participate in styrene ring oxidation.

Rapid disappearance of 4-VP was observed by Carlson et al. (2001) when this substrate was incubated with mouse liver microsomes, but the metabolic fate of 4-VP remains unknown. They also found that inhibition of 4-VP metabolism by 5-phenyl-1-pentyne (5PIP) significantly inhibited the toxicity of 4-VP in rats and mice (Carlson, 2002), indicating that metabolic activation was involved in 4-VP-induced toxicity. Pantarotto et al. (1978) and Manini et al. (2003) reported 4-VP-derived metabolites 4-hydroxystyrene glycol, 4-hydroxymandelic acid, 4-VP glucuronide, and sulfate in urine of workers after exposure to styrene. This result implied that 4-VP underwent metabolism pathways similar to those of styrene, i.e., vinyl epoxidation and ring epoxidation.

In the present study, we characterized new styrene metabolites, including 2-VP, 3-VP, vinyl-1,4-hydroquinone, and 2-hydroxystyrene glycol in mouse microsomal incubations and identified the possible enzymes responsible for aromatic hydroxylation of styrene. We also evaluated the pulmonary toxicity of 2-VP, 3-VP, and 4-VP along with that of styrene and SO in mice. In addition, we investigated the metabolism of 2-VP, 3-VP, and 4-VP in mouse liver microsomes and identified the main metabolites of the VPs.

Materials and Methods

Chemicals and Apparatus. Styrene (>99%), 2-vinylanisole, 3-vinylanisole, sodium thiomethoxide, 4-ethylphenol, styrene- d_8 (98% atom), β -NADPH, Oxone ($2\text{KHSO}_5 \cdot \text{KHSO}_4 \cdot \text{K}_2\text{SO}_4$), 2,3-dihydroxybenzaldehyde, 3,4-dihydroxybenzaldehyde, 2,5-dihydroxybenzaldehyde, disulfiram (97%), and *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) were purchased from Sigma-Aldrich (St. Louis, MO). 4-Vinylphenol (10 wt. % in propylene glycol) and 5-phenyl-1-pentyne (98%) were from Alfa Aesar (Ward Hill, MA). Other chemicals were of analytical grade or higher.

An HP 5890 Series II gas chromatograph with a capillary gas chromatography column (DB-5 ms, 20 m $\times$ 0.18 mm i.d., film thickness 0.18 μm ; J&W Scientific, Folsom, CA), an HP 5971A mass-selective detector, and an HP 7673 autosampler were used to analyze metabolism samples and characterize synthetic compounds. HP Chemstation B.02.05 software was used for GC/MS control and data collection. An Avance 300 NMR spectrometer (Bruker Daltonics, Billerica, MA) was used for structural characterization of synthetic chemicals.

Synthesis of 2-VP (4) and 3-VP (5). To a solution of 250 mg of 2-vinylanisole dissolved in 10 ml of anhydrous dimethylformamide was added 300 mg of sodium thiomethoxide, and the mixture was refluxed until TLC showed that 2-vinylanisole was consumed. The reaction mixture was diluted with 10

ml of water, acidized to pH 2 with 1 N HCl, and extracted with CH_2Cl_2 (three 15-ml extractions). The resulting extracts were washed with water (three 15-ml extractions) and dried on anhydrous Na_2SO_4 , followed by rotary evaporation. The residue was loaded on a silica gel column and eluted with hexane-chloroform (2:3, v/v). An oily light yellow liquid with a strong phenol odor was obtained and identified as 2-VP. $^1\text{H-NMR}$ (CDCl_3): δ 5.10 (s, 1H, phenolic OH), 5.40 (d, $J = 11.2$ Hz, 1H, *cis* $\text{CH}_2 = \text{CH}$), 5.78 (d, $J = 17.7$ Hz, 1H, *trans* $\text{CH}_2 = \text{CH}$), 6.80 (d, $J = 8.0$ Hz, 1H, aromatic CH-3), 6.83–7.00 (m, 2H, aromatic CH-5 and $\text{CH}_2 = \text{CH}$), 7.18 (t, $J = 7.8$ Hz, 1H, aromatic CH-4), 7.43 (d, $J = 7.5$ Hz, 1H, aromatic CH-6). The synthetic phenol compound was derivatized by BSTFA, followed by GC/MS analysis. GC/MS (2-VP-TMS): m/z 177 and 192. 3-Vinylphenol was synthesized using a similar protocol with 3-vinylanisole as starting material. $^1\text{H-NMR}$ (CDCl_3): δ 4.72 (s, 1H, phenolic OH), 5.25 (d, $J = 10.8$ Hz, 1H, *cis* $\text{CH}_2 = \text{CH}$), 5.78 (d, $J = 17.6$ Hz, 1H, *trans* $\text{CH}_2 = \text{CH}$), 6.63–6.73 (m, 1H, $\text{CH}_2 = \text{CH}$), 6.73–6.77 (m, 1H, aromatic CH-4), 6.90 (m, 1H, aromatic CH-2), 7.00 (d, $J = 7.6$ Hz, 1H, aromatic CH-6), 7.22 (t, $J = 7.8$ Hz, 1H, aromatic CH-5). GC/MS (3-VP-TMS): m/z 177 and 192.

Synthesis of 3-Vinylcatechol (7), 4-Vinylcatechol (9), and Vinyl-1,4-Hydroquinone (8). To a solution of 200 mg of 2,3-dihydroxybenzaldehyde dissolved in 10 ml of anhydrous THF were added 200 mg of sodium hydride (60%) and 400 μl of bromomethyl methyl ether (90%). The mixture was stirred at room temperature until TLC showed that 2,3-dihydroxybenzaldehyde was completely consumed. The reaction mixture was poured into 10 ml of water and neutralized with 1 N HCl and then extracted with chloroform (three 15-ml extractions). The extracts were pooled, dried with anhydrous Na_2SO_4 , and rotary evaporated. The remaining chloroform extract was chromatographed on a silica gel column eluted with ethyl acetate-hexane (20:80), and 2,3-bis(methoxymethoxy)benzaldehyde was obtained as an oily liquid. GC/MS analysis yielded major ions at m/z 226 and 150. The resulting aldehyde was subjected to the Wittig reaction. To 400 mg of Wittig reagent ($\text{Ph}_3\text{PCH}_3\text{I}$) suspended in 10 ml of anhydrous THF was added 100 mg of sodium hydride (60%). After the mixture was stirred at room temperature for 1 h, 100 mg of 2,3-dimethoxymethoxybenzaldehyde was added, and the reaction was refluxed for 2 h. After the resulting mixture was cooled to room temperature, it was poured into 15 ml of water. The mixture was extracted with chloroform (three 15-ml extractions), and the solvent was evaporated under vacuum. An oily liquid characterized as 2,3-bis(methoxymethoxy)styrene was obtained after silica gel flash chromatography (hexane-ethyl acetate, 85:15). GC/MS analysis yielded major signals at m/z 224 and 148. Deprotection of 2,3-bis(methoxymethoxy)styrene was performed in THF-1 N HCl (1:1) with stirring at room temperature overnight under nitrogen. The reaction mixture was extracted with chloroform (three 15-ml extractions). The extracts were dried with anhydrous Na_2SO_4 , followed by rotary evaporation. Flash chromatography of the remaining residue on silica gel eluted with ethyl acetate-hexane (40:60) afforded 3-vinylcatechol. 4-Vinylcatechol and vinyl-1,4-hydroquinone were synthesized with a similar method using 3,4-dihydroxybenzaldehyde and 2,5-dihydroxybenzaldehyde as starting material, respectively.

3-Vinylcatechol (7), 4-vinylcatechol (9), and vinyl-1,4-hydroquinone (8)

showed the same ion fragments (m/z 280 and 265) with different retention times (Supplemental Table S1) in GC/MS after derivatization by BSTFA. 3-Vinylcatechol $^1\text{H-NMR}$ (CDCl_3): δ 5.38 (d, J = 11.2 Hz, 1H, *cis* CH_2 = CH), 5.79 (d, J = 17.7 Hz, 1H, *trans* CH_2 = CH), 6.55 (d, J = 7.7 Hz, 1H, aromatic CH -6), 6.75–6.82 (m, 2H, aromatic CH -4, 5), 7.01 (m, 1H, CH_2 = CH). 4-Vinylcatechol $^1\text{H-NMR}$ (CDCl_3): δ 5.36 (d, J = 11.1 Hz, 1H, *cis* CH_2 = CH), 5.72 (d, J = 17.2 Hz, 1H, *trans* CH_2 = CH), 6.66–6.69 (m, 2H, aromatic CH -3, 6), 6.87–6.96 (m, 2H, CH_2 = CH and aromatic CH -5). Vinyl-1,4-hydroquinone $^1\text{H-NMR}$ (CD_3OD): δ 5.01 (d, J = 11.8 Hz, 1H, *cis* CH_2 = CH), 5.52 (d, J = 17.1 Hz, 1H, *trans* CH_2 = CH), 6.62–6.83 (m, 2H, aromatic CH -3, 4), 6.86–6.98 (m, 2H, CH_2 = CH and aromatic CH -6).

Synthesis of 2-Hydroxystyrene Glycol (10), 3-Hydroxystyrene Glycol (11), and 4-Hydroxystyrene Glycol (12). To a solution of 40 mg of 2-VP dissolved in 10 ml of THF- H_2O (1:1) solvent was added 400 mg of Oxone, followed by stirring at room temperature for 2 days. When TLC showed that 2-VP was completely consumed, the reaction mixture was poured into 15 ml of water and extracted with ethyl acetate (three 10-ml extractions). The extracts were dried on anhydrous Na_2SO_4 , and the solvent was removed by rotary evaporation. Chromatography of the residue on silica gel (chloroform-methanol, 92:8) gave 2-hydroxystyrene glycol. 3-Hydroxystyrene glycol and 4-hydroxystyrene glycol were synthesized with the same method using 3-VP and 4-VP as starting material, respectively.

All hydroxystyrene glycols revealed the same ion fragments (m/z 267 and 147) but with different retention times (Supplemental Table S1) in GC/MS after derivatization with BSTFA. 2-Hydroxystyrene glycol, $^1\text{H-NMR}$ (CD_3OD): δ 3.32 (s, 1H, phenolic OH), δ 3.42 (d, J = 5.7 Hz, 2H, CHCH_2OH), 5.02–5.06 (m, 1H, CHCH_2OH), 6.75–6.85 (m, 2H, aromatic CH -3, 5), 7.09 (t, J = 7.0 Hz, 1H, aromatic CH -4), 7.31 (d, J = 7.6 Hz, 1H, aromatic CH -6). 3-Hydroxystyrene glycol, $^1\text{H-NMR}$ (CD_3OD): δ 3.32–3.34 (m, 1H, phenolic OH), 3.58–3.61 (m, 2H, CHCH_2OH), 4.60–4.64 (m, 1H, CHCH_2OH), 6.68–6.69 (m, 1H, aromatic CH -2), 6.82–6.85 (m, 2H, aromatic CH -4, 6), 7.15 (t, J = 8.0 Hz, 1H, aromatic CH -5). 4-Hydroxystyrene glycol, $^1\text{H-NMR}$ (CD_3OD): δ 3.31–3.34 (m, 1H, phenolic OH), 3.59 (d, J = 6.1 Hz, 2H, CHCH_2OH), 4.60 (t, J = 6.2 Hz, 1H, CHCH_2OH), 6.77 (d, J = 8.5 Hz, 2H, aromatic CH -3, 5), 7.19 (d, J = 8.4 Hz, 2H, aromatic CH -2, 6).

Synthesis of Styrene Glycol- d_8 . To 10 ml of THF-water (1:1) were added 100 mg of styrene- d_8 and 600 mg of Oxone, followed by stirring at room temperature overnight. When TLC showed that styrene- d_8 was completely consumed, the reaction mixture was poured into 30 ml of water and extracted with ethyl acetate. Purification of the resulting mixture on silica gel chromatography (chloroform-methanol, 95:5) offered 80 mg of white crystal. GC/MS (BSTFA derivatized): m/z 185 and 147.

Animals. Adult male CD-1 mice (5 weeks, 19–21 g b.wt.) were purchased from Charles River Laboratories, Inc. (Wilmington, MA). The mice were fed standard rodent diets and allowed drinking water ad libitum. The animals were allowed to adapt to the surroundings for 1 week before the experiment and were housed in an air-conditioned room set to maintain temperature of 20 to 24°C, 40 to 60% relative humidity, and a 12-h light/dark cycle.

Preparation of Microsomes. Male CD-1 mice were terminated by CO_2 . Blood was drawn from the heart with a syringe. Livers and lungs were harvested and rinsed with ice-cold 0.1 M phosphate buffer, pH 7.4. The livers or lungs were pooled and weighed and homogenized in ice-cold 0.1 M phosphate buffer (4 ml/g of tissue). Microsomes were prepared by differential centrifugation. The homogenate was centrifuged at 3000g for 15 min, followed by centrifugation of the resulting supernatant at 12,000g for 30 min. Then, the resultant supernatant was centrifuged at 105,000g for 60 min. The pellets of microsomal preparations were resuspended in ice-cold 0.1 M phosphate buffer. Protein contents were determined by a 660-nm protein assay (Pierce Chemical, Rockford, IL). The microsomes were stored in a freezer at -80°C until use.

Detection of Phenolic Metabolites of Styrene in Mouse Microsomal Incubations. Incubations were performed in glass tubes with polytetrafluoroethylene-faced rubber-lined screw caps (10 $\times$ 1.3 cm i.d.; Kimble Chase, Vineland, NJ). The incubation mixtures contained mouse liver or lung microsomes (1 mg of protein/ml), 5 mM MgCl_2 , 2 mM NADPH, 500 μM styrene, and 50 mM phosphate buffer (pH 7.4) with a total volume of 0.5 ml. After preincubation in a 37°C water bath shaker for 3 min, reactions were initiated by addition of 5 μl of styrene solution (50 mM in dimethyl sulfoxide), and the reaction tubes were capped immediately. Some microsomal incubations were

done with a total volume of 8.0 ml of reaction mixture, and some incubations excluded NADPH as a blank control.

After incubation for 20 min, the reactions were terminated by adding 3 ml of ice-cold ethyl acetate, followed by addition of 5 μl of internal standard solution containing SG- d_8 (100 μM) and 4-ethylphenol (5.0 μM) dissolved in acetonitrile. The mixture was vortexed for 3 min and centrifuged at 5000 rpm for 10 min. The organic supernatant was collected 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 1 h, and subjected to GC/MS analysis. A coelution test was performed to confirm the formation of VPs by spiking with a mixture of authentic 2-VP, 3-VP, and 4-VPs to the samples that have been analyzed.

To directly detect metabolites of VPs as secondary metabolites of styrene, large-scale mouse microsomal incubations were performed. The incubation volume was 8 ml, and the protein concentration was 1 mg/ml for mouse liver microsomes and 0.5 mg/ml for mouse lung microsomes. The concentrations of other components remained the same as those for the small-scale (0.5-ml) incubations.

Inhibition of Styrene Metabolism in Mouse Microsomal Incubations. To investigate whether CYP2E1 or CYP2F2 was involved in the production of VPs from styrene, 100 μM disulfiram (inhibitor of CYP2E1) (Emery et al., 1999; Frye and Branch, 2002) or 5 μM 5P1P (inhibitor of CYP2F2 and CYP2E1) (Roberts et al., 1998; Green et al., 2001; Carlson, 2004) was incorporated into mouse liver or lung microsomes. The other components were the same as those for the incubations without the inhibitors described above. After all components except styrene were mixed in test tubes, samples were preincubated at 37°C for 15 min, followed by addition of styrene and incubation for another 20 min. Samples were processed in the same way as described above.

Disappearance of VPs in Mouse Liver Microsomes. To identify the metabolites, VPs were incubated with mouse liver microsomes. The same protocol for styrene microsomal incubations was applied for those incubations of VPs and subsequent sample preparation. The final concentration of VPs in the incubation mixtures was 50.0 μM with 1.0 mM NADPH. The incubation times were 5, 10, 20, 40, and 60 min. 4-Ethylphenol was used as an internal standard.

GC/MS Analysis. The column temperature was held at the initial temperature of 60°C for 1 min after injection of 1 μl of sample, followed by an increase to 170°C at 10°C/min, and 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 carrier gas was helium with column head pressure set at 10 psi at 50°C. Target ions of styrene metabolites were monitored in SIM mode (m/z 177 and 192 for VPs, m/z 179 for SG, m/z 185 for SG- d_8 , m/z 280 for vinylcatechols and vinyl-1,4-hydroquinone, m/z 194 for 4-ethylphenol, and m/z 267 for hydroxystyrene glycols), dwell time was 50 ms, and electrospray ionization voltage was 70 eV.

The contents of VPs and SG were determined using standard curves (VPs: 5.0–500.0 nM for the VP production experiment and 2.0–50.0 μM for the VP disappearance experiment; SG: 2.0–200.0 μM). Standard curves were fitted with a linear equation, where x was the concentration of analytes and y was the peak ratio of analytes versus internal standard. The correlation coefficient r^2 was >0.998 .

Toxicity of Styrene, SO, and VPs in CD-1 Mice. Adult male CD-1 mice were divided into six groups of three mice each. The control group was injected with propylene glycol intraperitoneally at 5 ml/kg. The other five groups were treated with styrene, SO, 2-VP, 3-VP, or 4-VP at a dose of 100 mg/kg i.p. The dose solution was dissolved in propylene glycol and administered at a volume of 5 ml/kg. After 24 h, the mice were sacrificed by CO_2 asphyxiation; and 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 phosphate-buffered saline (Mediatech, Manassas, VA). The bronchoalveolar lavage fluid (BALF) was collected (1.6 ml). Cells in BALF were counted using a hemocytometer with 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).

Data Analysis. The concentrations of metabolites in the samples were calculated using the corresponding standard curves. Three parallel incuba-

tions were conducted in each experiment. Data are expressed as the mean $\pm$ S.D. Student's *t* test was performed to evaluate the difference between control and treated samples in microsomal P450 inhibition and animal toxicity experiments.

Results

Authentic 2-VP (4) and 3-VP (5) were synthesized by demethylation of the corresponding vinylanisols and characterized by GC/MS and $^1\text{H-NMR}$. In GC/MS, the TMS derivatives of 2-VP, 3-VP, and 4-VP yielded ions at m/z 177 (VP-TMS-CH₃) and 192 (VP-TMS) but

with different retention times. Authentic VP metabolites including vinylcatechols, vinyl-1,4-hydroquinone, and hydroxystyrene glycols were also synthesized, and their main ion fragments and retention times are listed in Supplemental Table S1.

Detection of Phenolic Metabolites of Styrene in Microsomal Incubations. In an effort to detect the phenolic metabolites, styrene was incubated in mouse liver or lung microsomes for 20 min. As expected, styrene glycol (3) and 4-VP (6) were observed by GC/MS. In addition, we detected 2-VP (4) and 3-VP (5) in liver microsomal

FIG. 1. GC/MS SIM chromatograms (m/z 177 and 192) of BSTFA-derivatized VPs in mouse liver microsomal incubations with styrene in the absence (A) and presence of NADPH (B), of BSTFA-derivatized sample B spiked with authentic standards 2-VP (4), 3-VP (5), and 4-VP (6) (C), and of BSTFA-derivatized authentic standards 2-VP (4), 3-VP (5), and 4-VP (6) (D).

incubations. Styrene glycol, the hydrated product of SO, was the predominant metabolite of styrene, whereas 2-VP, 3-VP, and 4-VP, the aromatic hydroxylation products, were minor metabolites relative to styrene glycol. As shown in Fig. 1B, the metabolites detected showed the same retention times and same ion fragments (m/z 177 and 192) as those of the corresponding authentic standards (Fig. 1D) in GC/MS. The samples analyzed above were spiked with a mixture of authentic 2-VP, 3-VP, and 4-VP and reanalyzed by GC/MS. As expected, increases in peak areas of those peaks with the same retention times as that of the authentic VPs spiked were observed, but no new peaks were detected (Fig. 1C). This coelution test confirmed

the production of the individual VPs in the microsomal incubations. No such peaks with the same retention times were observed in the incubations without NADPH (Fig. 1A). Metabolites SG, 2-VP, 3-VP, and 4-VP were also detected in mouse lung microsomal incubations, although the amount was less than that in mouse liver microsomes (Supplemental Fig. S1).

In large-scale microsomal incubations (final volume 8 ml) with styrene, SG (3) and three VPs (4-6) were detected as expected. In addition, vinyl-1,4-hydroquinone (8), 2-hydroxystyrene glycol (10), and 4-hydroxystyrene glycol (12) were identified in mouse liver microsomal incubations (Fig. 2), whereas only 2-hydroxystyrene gly-

FIG. 2. GC/MS SIM chromatograms (m/z 267 and 280) of BSTFA-derivatized metabolites in large scale mouse liver microsomal incubations with styrene in the absence (A) and presence of NADPH (B), of BSTFA-derivatized sample B spiked with authentic standards 2-hydroxystyrene glycol (10), 4-hydroxystyrene glycol (12), and vinyl-1,4-hydroquinone (8) (C), and of BSTFA-derivatized authentic standards 2-hydroxystyrene glycol (10), 4-hydroxystyrene glycol (12), and vinyl-1,4-hydroquinone (8) (D).

TABLE 1

*Inhibition of styrene metabolite formation with P450 inhibitor disulfiram or 5PIP in mouse liver microsomes*Values represent mean $\pm$ S.D. ($n = 3$).

	2-VP	3-VP	4-VP	SG	VPs/SG
	<i>nmol/(min $\cdot$ mg protein)</i>				
Vehicle	0.0527 $\pm$ 0.0045	0.0019 $\pm$ 0.0006	0.0053 $\pm$ 0.0002	4.42 $\pm$ 0.33	0.0136 $\pm$ 0.0017
Disulfiram (100 μ M)	0.0184 $\pm$ 0.0034**	0.0007 $\pm$ 0.0002*	0.0033 $\pm$ 0.0003**	1.92 $\pm$ 0.24**	0.0117 $\pm$ 0.0024
5PIP (5 μ M)	0.0025 $\pm$ 0.0006**	0.0011 $\pm$ 0.0002*	0.0035 $\pm$ 0.0004**	1.87 $\pm$ 0.19**	0.0038 $\pm$ 0.0002

* $P < 0.05$.** $P < 0.01$.

col along with three VPs and SG were observed in mouse lung microsomal incubations (data not shown).

P450 Inhibition of Styrene Metabolism in Mouse Microsomes.

The rates of VP and SG production in mouse liver and lung microsomal incubations with or without inhibitor are listed in Tables 1 and 2. The ratios of VPs/SG production were calculated to determine the attribution of vinyl and aromatic oxidation in styrene metabolism. Larger ratios of VPs/SG were observed in mouse lung microsomes than in mouse liver microsomes. Among the phenolic metabolites generated, 2-VP was formed at a rate 10-fold faster than that of 3-VP and 4-VP in both mouse liver and lung microsomal incubations. However, metabolism of styrene was slower in mouse lung microsomes than in mouse liver microsomes. In particular, the rate of SG formation in mouse lung microsomes was 5.7-fold lower than that in mouse liver microsomes, whereas the rates for the formation of VPs were only 2-fold lower.

To investigate the involvement of CYP2E1 and CYP2F2 in the production of VPs, disulfiram (inhibitor of CYP2E1) or 5PIP (inhibitor of CYP2F2 and CYP2E1) was incorporated into the mouse liver and lung microsomal incubations. Both disulfiram (100.0 μ M) and 5PIP (5.0 μ M) inhibited VPs and SG production in mouse liver and lung microsomes (Tables 1 and 2). Interestingly, the formation of VPs and SG was almost completely inhibited by 5PIP in lung microsomal incubations.

Metabolism of VPs in Mouse Microsomes. To investigate the metabolism of VPs, individual VPs were incubated with mouse liver microsomes. Supplemental Fig. S1 shows the time course of VP disappearance in liver microsomal incubations. It appears that a substantial disappearance of the three VPs took place in 20 min and 4-VP was lost faster than 2-VP and 3-VP. The disappearance rates of 2-VP, 3-VP, and 4-VP were 2.50 ± 0.30 , 2.63 ± 0.13 , and 3.45 ± 0.11 nmol/(min $\cdot$ mg protein), respectively, after a 10-min incubation. Metabolites arising via aromatic hydroxylation were characterized by GC/MS. As expected, vinylcatechols and/or vinyl-1,4-hydroquinone was detected accordingly in the individual incubation mixtures. In particular, 3-vinylcatechol (**7**) and vinyl-1,4-hydroquinone (**8**) were detected in the microsomes incubated with 2-VP (Fig. 3A); 3-vinylcatechol (**7**), vinyl-1,4-hydroquinone (**8**), and 4-vinylcatechol (**9**) were detected in the microsomal incubations with 3-VP (Fig. 3B), and

4-vinylcatechol (**9**) was detected in the microsomes after exposure to 4-VP (Fig. 3C). In addition, the three VPs were metabolized to the corresponding glycols (Fig. 4). All metabolites were characterized by comparing their retention times and ion fragments with those of the corresponding authentic standards in GC/MS.

Our results indicate that, like styrene, VPs undergo two metabolism pathways. One is vinyl epoxidation and the other involves aromatic hydroxylation. Vinyl epoxidation produces epoxides that are further transformed to glycols by spontaneous or enzymatic hydration. The aromatic hydroxylation affords catechols and hydroquinone.

Supplemental Table S2 shows the peak area ratios of VPs metabolites versus internal standard SG-d₈, and the ratios were used to estimate the production of metabolites. Based on this semiquantitative analysis, 3-VP was preferentially metabolized by aromatic hydroxylation, resulting in vinyl-1,4-hydroquinone. However, 4-VP was mostly metabolized by vinyl of the oxidation, thus generating glycol; 2-VP was metabolized to both catechols/hydroquinone and glycol at similar rates.

Toxicity of Styrene, SO, and VPs in CD-1 Mouse. Male CD-1 mice were treated with styrene, SO, 2-VP, 3-VP, or 4-VP at a dose of 100 mg/kg. After 24 h, BALF samples were collected, and the pulmonary toxicity was evaluated by determination of LDH activities and cell numbers in BALF. Significant elevations in LDH activity and cell numbers in BALF of mice treated with 4-VP were observed, whereas no significant pathophysiological change was found in mice given styrene, SO, 2-VP, or 3-VP at 100 mg/kg relative to that observed in mice treated with vehicle (Table 3).

Discussion

Metabolism of styrene apparently includes two pathways, i.e., vinyl epoxidation and aromatic hydroxylation. The former offers SO (**2**), reported as the primary metabolite of styrene, and the latter produces styrene-derived phenols. Urinary 4-VP (**6**) and its conjugates have been identified in rodents given styrene and in workers exposed to styrene (Bakke and Scheline, 1970; Pantarotto et al., 1978; Pfäffli et al., 1981; Manini et al., 2003). Based on the knowledge of general chemistry, we reasoned that aromatic hydroxylation of styrene would generate three isomers of styrene-derived phenols, i.e., 2-VP, 3-VP, and 4-VP (Scheme 1).

TABLE 2

*Inhibition of styrene metabolite formation with P450 inhibitor disulfiram or 5PIP in mouse lung microsomes*Values represent mean $\pm$ S.D. ($n = 3$).

	2-VP	3-VP	4-VP	SG	VPs/SG
	<i>nmol/(min $\cdot$ mg protein)</i>				
Vehicle	0.0277 $\pm$ 0.0132	0.0010 $\pm$ 0.0002	0.0027 $\pm$ 0.0011	0.77 $\pm$ 0.13	0.0408 $\pm$ 0.0264
Disulfiram (100 μ M)	0.0065 $\pm$ 0.0028*	N.D.	0.0010 $\pm$ 0.0004	0.25 $\pm$ 0.15*	0.0300 $\pm$ 0.0447
5PIP (5 μ M)	N.D.	N.D.	N.D.	N.D.	N.A.

N.D., not detected; N.A., not available.

* $P < 0.05$.** $P < 0.01$.

FIG. 3. SIM chromatograms (m/z 280) of BSTFA-derivatized vinylcatechols and/or vinyl-1,4-hydroquinone from mouse liver microsomal incubations with 2-VP (A), 3-VP (B), and 4-VP (C) and of BSTFA-derivatized authentic standards 3-vinylcatechol (7), vinyl-1,4-hydroquinone (8), and 4-vinylcatechol (9) (D).

In the present studies, we detected all three phenols in styrene microsomal incubations containing styrene. Surprisingly, 2-VP was found to be the dominant metabolite among the three VPs detected. The ratio of the detected 2-VP, 3-VP, and 4-VP was

27.7:1.0:2.8 in mouse liver microsomes. In a recent study, Linhart et al. (2010) reported the detection of urinary 3-VP, 4-VP, possible 2-VP, and mercapturic acids derived from styrene-3,4-oxide and styrene-2,3-oxide in mice given styrene. They found approxi-

FIG. 4. SIM chromatograms (m/z 267) of BSTFA-derivatized hydroxystyrene glycols from mouse liver microsomal incubations with 2-VP (A), 3-VP (B), and 4-VP (C) and of BSTFA-derivatized authentic standards 2-hydroxystyrene glycol (10), 3-hydroxystyrene glycol (11), and 4-hydroxystyrene glycol (12) (D).

mately 3-fold more mercapturic acid derived from styrene-3,4-oxide than that derived from styrene-2,3-oxide in the urine, inferring that styrene-3,4-oxide dominated in the formation of styrene arene oxides. This finding is inconsistent with what we observed in

mouse microsomal incubations (the VPs are presumably derived from the corresponding oxides).

Higher VPs/SG value (3-fold) was observed in lung microsomal incubations than in liver microsomal incubations. This observation

TABLE 3

*Pulmonary toxicity of styrene, SO, 2-VP, 3-VP, and 4-VP in male CD-1 mice*Values represent mean $\pm$ S.D. ($n = 3$).

Treatment	LDH (OD ₄₉₀)	Cell Number (10 ⁴ /ml)
Vehicle	0.337 $\pm$ 0.091	2.5 $\pm$ 1.5
Styrene (100 mg/kg)	0.367 $\pm$ 0.109	8.6 $\pm$ 8.7
SO (100 mg/kg)	0.267 $\pm$ 0.439	5.4 $\pm$ 2.5
2-VP (100 mg/kg)	0.289 $\pm$ 0.064	5.4 $\pm$ 0.8
3-VP (100 mg/kg)	0.229 $\pm$ 0.017	4.5 $\pm$ 2.1
4-VP (100 mg/kg)	1.217 $\pm$ 0.107*	82.8 $\pm$ 10.9*

* $P < 0.05$ compared with the control group.

implies that microsomal enzymes in mouse lung tend to favor the catalysis of aromatic hydroxylation of styrene compared with microsomal enzymes in mouse liver, although styrene oxide was found to be the predominant metabolite of styrene in both liver and lung microsomal incubations, and the absolute amount of SG and

VPs generated in liver microsomes was higher than that formed in lung microsomes (5.7-fold for SG and 1.9-fold for VPs).

CYP2F2 and CYP2E1 have been reported to be involved in metabolic activation of styrene in mice (Hynes et al., 1999; Cruzan et al., 2002; Carlson, 2003). CYP2E1 is predominantly expressed in liver with lower levels in other tissue such as lung (Choudhary et al., 2003, 2005), whereas CYP2F2 is expressed both in liver and lung, with a little higher expression in lung (Powley and Carlson, 2001; Baldwin et al., 2004). Our inhibition experiments showed that both disulfiram and 5P1P inhibited VP and SG production in mouse liver microsomes. However, 5P1P caused substantial decrease in VPs/SG value, whereas disulfiram did not (Table 1). Disulfiram is a known CYP2E1 inhibitor and 5P1P has been reported to inhibit both CYP2F2 and CYP2E1. The observation of a substantial drop in VPs/SG value by coincubation with 5P1P indicates that CYP2F2 may be more important for catalyzing the biotransformation of styrene to VPs relative to CYP2E1, especially in mouse lung.

SCHEME 2. Proposed metabolic pathways of VPs in mouse liver microsomes.

Metabolism of VPs was investigated in mouse liver microsomes, and a total of three aromatic hydroxylated products, including 3-vinylcatechol, 4-vinylcatechol, and/or vinyl-1,4-hydroquinone, along with glycols derived from the corresponding VPs, were detected. As shown in Figs. 3 and 4, microsomal incubations with 4-VP gave one hydroxylated product identified as 4-vinylcatechol (9). Two hydroxylated products were detected and characterized as 3-vinylcatechol (7) and vinyl-1,4-hydroquinone (8) in microsomes after incubation with 2-VP. All of the three hydroxylated products (the only possibilities) observed in microsomal incubations with 2-VP or 4-VP were detected in microsomal incubations with 3-VP. These VPs are good substrates for hydroxylation at positions *ortho* or *para* to their phenolic hydroxyl groups. This finding is consistent with the generally electrophilic character of P450 enzymes (Zheng and Hanzlik, 1992). In particular, as shown in Scheme 2, 2-VP undergoes hydroxylation at C-4 and C-6, 3-VP undergoes hydroxylation at C-2, C-4, and C-6, and the symmetrical 4-VP undergoes hydroxylation at C-2(6). Alternatively, all three VPs were metabolized to the corresponding epoxides via oxidation at the vinyl groups and sequentially to the corresponding glycols (10–12) through spontaneous hydration. In parallel, the three VPs were incubated with mouse liver microsomes in the absence of NADPH. As expected, no disappearance of the parent compounds was observed.

We scaled up microsomal incubations of styrene, and this allowed us to obtain sufficient amounts of the secondary metabolites of styrene for GC/MS detection. In up-scaled liver microsomal incubations, SG (3), 2-VP (4), 3-VP (5), 4-VP (6), vinyl-1,4-hydroquinone (8), 2-hydroxystyrene glycol (10), and 4-hydroxystyrene glycol (12) were detected by GC/MS. Lung microsomal incubations with styrene afforded a similar metabolite profile except for vinyl-1,4-hydroquinone and 4-hydroxystyrene glycol. The observation of the glycols infers the formation of the corresponding epoxides (Bond, 1989; Sumner and Fennell, 1994). The observed vinyl-1,4-hydroquinone may be spontaneously oxidized to vinyl-1,4-benzoquinone (Scheme 2) (Shen et al., 1997). Both epoxides and quinones are known toxic species. However, the role of these metabolites in styrene-induced cytotoxicity remains unknown.

The toxicity of 4-VP has been well documented, and 4-VP was found to be more toxic than styrene and styrene oxide. It has been speculated that 4-VP formed in situ is involved in styrene-induced cytotoxicity in mice, which encouraged us to evaluate the pulmonary toxicity of 2-VP and 3-VP. The pulmonary toxicities of styrene, SO, and the three VPs were evaluated in CD-1 mice. 4-VP showed the highest toxicity among these chemicals. Although the three VPs have a similar structure, it appears that 2-VP and 3-VP were less toxic than 4-VP (Table 3). However, 10-fold higher 2-VP production than that of 4-VP was observed both in liver and lung microsomal incubations. The toxicological role of 2-VP in styrene-induced toxicity should not be neglected, because of its high production. The proportion of the chemicals for the pulmonary toxicity studies to reach the lung may not necessarily be the same. We speculate that significant portions of the vinyl phenols are depleted through glucuronidation/sulfation conjugation (first-pass effect) in the liver before they arrive at the target organ, whereas styrene does not undergo the conjugation.

In conclusion, new styrene metabolites 2-VP, 3-VP, vinyl-1,4-hydroquinone, and 2-hydroxystyrene glycol were identified in mouse liver microsomal incubations. CYP2F2 and CYP2E1 were found to catalyze the formation of VPs and SO from styrene in mouse liver and lung microsomes. The detected 2-VP, 3-VP, and 4-VP were further oxidized to the corresponding catechols and/or hydroquinone by aromatic hydroxylation and to the corresponding oxides by vinyl epoxidation. 2-Vinylphenol and 3-VP were not as toxic as 4-VP to the pulmonary system in mice. However, the potential toxicity of 10-fold higher production of

2-VP than that of 4-VP in mouse liver and lung microsomal incubations of styrene should not be neglected.

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Address correspondence to: Dr. Jiang Zheng, Center for Developmental Therapeutics, Seattle Children's Research Institute, Seattle, WA 98101. E-mail: jiang.zheng@seattlechildrens.org