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Hepatic and pulmonary microsomal benzene metabolism in CYP2E1 knockout mice

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Abstract

Benzene is an occupational and environmental toxicant. The major health concern for humans is acute myelogenous leukemia. To exert its toxic effects, benzene must be metabolized via cytochrome P450. CYP2E1 has been identified as the most important cytochrome, P450 isozyme in hepatic benzene metabolism in mice, rats, and humans. In pulmonary microsomes CYP2E1 and members of the CYP2F subfamily are both significantly involved. In the current study CYP2E1 knockout mice and wild-type controls were used to further examine the cytochrome P450 isozymes involved in metabolism of 24 μM benzene. The results show that CYP2E1 is the most important isozyme in the liver, accounting for 96% of the total hydroxylated metabolite formation. However, in the lung CYP2E1 was responsible for only 45% of the formation of total hydroxylated metabolite. Chemical inhibitors of CYP2E1 and CYP2F2 were used to further examine the contributions of these isozymes to benzene metabolism. The results confirmed the finding that while CYP2E1 is the most important isozyme in the liver, CYP2F2 and CYP2E1 are both significantly involved in the lung.

Keywords: Benzene; Liver; Lung; CYP2E1; CYP2F2

1. Introduction

The main concern for humans exposed to benzene is acute myelogenous leukemia (Infante et al., 1977; Aksoy, 1980, 1981; Yin et al., 1996). There is a mouse model that develops acute leukemia following benzene exposure (Rithidech et al., 1999), but no animal model fully mimics the leukemogenic process observed in humans. Studies have shown an increase in various types of tumors in mice, including lung tumors, (NTP, 1986; Snyder et al., 1988; Maltoni et al., 1989; Farris et al., 1993) and in rats (NTP, 1986; Maltoni et al., 1989) exposed to benzene. A few studies suggest that lung cancer may also occur in humans following benzene exposure (Aksoy, 1980, 1981; Yin et al., 1996).

For benzene to exert most of its toxic effects, it must be metabolized by the cytochrome P450 enzyme system. Benzene is first metabolized to benzene oxide (Jerina et al., 1968). Phenol is formed from the non-enzymatic rearrangement of

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benzene oxide (Jerina et al., 1968) and is metabolized to hydroquinone and catechol (Sawahata and Neal, 1983). Hydroquinone and catechol are further metabolized by myeloperoxidase in bone marrow to form *p*-benzoquinone and *o*-benzoquinone (Eastmond et al., 1987; Sadler et al., 1988).

Several cytochrome P450 isozymes may be involved in benzene metabolism. Using reconstituted rabbit cytochromes P450, Koop et al. (1989) characterized CYP2E1 as a low K_m, low V_{max} isozyme responsible for metabolizing both benzene and phenol. CYP2E1 formed greater amounts of all metabolites versus other cytochrome P450 isozymes with 0.3 mM benzene. However, with 2 mM benzene, CYP2B1 approached CYP2E1 in phenol formation but not hydroquinone formation. Other researchers have identified CYP2B1 as being capable of catalyzing the hydroxylation of both benzene and phenol in purified isozymes from rat liver (Snyder et al., 1993) and in rat liver microsomes (Gut et al., 1996).

The contribution of CYP2E1 to benzene metabolism has been further evaluated in numerous studies. Using diethyldithiocarbamic acid (DDTC, 300 μM), a known CYP2E1 inhibitor, Chaney and Carlson (1995) found metabolism of 17.5 μM benzene decreased by 96% in liver microsomes and 54% in lung microsomes from rat. Gut et al. (1996) found a significant decrease in benzene metabolism using DDTC (100–300 μM) The CYP2E1 inducers ethanol and acetone increased benzene metabolism in rat liver microsomes, and acetone did so in rabbit liver microsomes (Johansson and Ingelman-Sundberg, 1988). Ethanol also increased benzene metabolism in rats in vivo and in the $10\,000 \times g$ fraction (Nakajima et al., 1985). Another CYP2E1 inducer, isopropanol, increased benzene metabolism in rat hepatocytes (Schrenk et al., 1991). Seaton et al. (1994) examined the metabolism of 3.4 μM benzene in human, mouse, and rat liver microsomes and found that human liver samples with higher CYP2E1 activities formed greater amounts of hydroquinone and catechol than did human liver samples with lower CYP2E1 activities. Nedelcheva et al. (1999) also found that human liver samples with higher CYP2E1 activities formed greater amounts of benzene metabolites.

CYP2E1 knockout mice have been used to examine the significance of CYP2E1 in the metabolism or toxicity of several xenobiotics (Ghanayem et al., 2000), including benzene (Valentine et al., 1996). Following the inhalation of 200 ppm benzene for 6 h, knockout mice that did not express a functional CYP2E1 protein produced only 13% of the total metabolites found in the urine of wild-type mice. Furthermore, genotoxicity or cytotoxicity were not detected in bone marrow, spleen, thymus, or blood of CYP2E1 knockout mice but were detected in the wild-type controls, following inhalation exposure to 200 ppm benzene for 6 h per day for 5 days (Valentine et al., 1996).

All these studies suggest that CYP2E1 is the cytochrome P450 isozyme primarily responsible for metabolizing benzene although CYP2B1 may be involved at high substrate concentrations. Accordingly, physiologically based pharmacokinetic (PBPK) models assume that benzene metabolism is due only to CYP2E1 (Seaton et al., 1994; Lovern et al., 1999; Cole et al., 2001). However, another cytochrome P450 isozyme merits attention, especially in the lung. CYP2F2, found in the mouse, is capable of biotransforming xenobiotics, such as, styrene (Carlson, 1997), naphthalene (Chang et al., 1996; Shultz et al., 1999), ethoxyresorufin, pentoxyresorufin, 1-nitronaphthalene, *p*-nitrophenol (Shultz et al., 1999), 2-methylnaphthalene, anthracene, and benzo[a]pyrene (Shultz et al., 2001). Interestingly, CYP2F2 has approximately the same *p*-nitrophenol metabolizing activity as CYP2E1 (Shultz et al., 1999). This is significant because *p*-nitrophenol is considered a model substrate for examining CYP2E1 activity (Reinke and Moyer, 1985; Koop, 1986). Therefore, *p*-nitrophenol hydroxylation may not be specific to CYP2E1 as was previously thought.

We have earlier demonstrated that CYP2E1 is the cytochrome P450 isozyme most responsible for hepatic microsomal benzene metabolism in the mouse, rat, and human, with 24 and 1000 μM substrate (Powley and Carlson, 2000). Results of this study have also shown that members of the

CYP2F subfamily, as well as CYP2E1 are involved in pulmonary metabolism. Isozymes of the CYP2B subfamily were not notably involved at the low benzene concentrations studied. This is not surprising since other researchers found CYP2B1 to be capable of metabolizing benzene to an extent comparable with CYP2E1 only at concentrations higher than were used in our study (Koop et al., 1989; Snyder et al., 1993).

In the current study we used CYP2E1 knockout mice and wild-type controls to identify the cytochrome P450 isozymes involved in the metabolism of 24 μM benzene. This is the lowest concentration of benzene used in our earlier studies (Powley and Carlson, 1999, 2000), and clearly represents a value that is more environmentally relevant than millimolar concentrations. Microsomes from liver and lung were used to determine if different cytochrome P450 isozymes are involved in the metabolism of benzene in these two tissues. Specific chemical inhibitors of CYP2E1 and CYP2F2 were used to further examine the cytochrome P450 isozymes involved in benzene metabolism. Since α-methylbenzylaminobenzotriazole (MBA, 1 μM final concentration), a CYP2B1 inhibitor (Mathews and Bend, 1986) did not have a significant effect on benzene metabolism in an earlier study (Powley and Carlson, 2000), this inhibitor was not used in the current study. Diethyldithiocarbamic acid (DDTC, 300 μM final concentration) was used as an inhibitor of CYP2E1 (Ono et al., 1996), and 5-phenyl-1-pentyne (5P1P, 5 μM final concentration) was used to inhibit CYP2F2 (Chang et al., 1996) The degree of specificity of these inhibitors is not fully known. Roberts et al. (1998) showed that 5P1P in addition to inhibiting CYP2F2, may also inhibit both CYP2E1 and CYP2B1. Although they used a higher concentration of 5P1P than employed in the present study, 50 μM versus 5 μM, there may be some inhibitory effect on CYP2E1 and CYP2B1 at 5 μM.

Identification of the cytochrome P450 isozymes responsible for benzene metabolism has important implications in understanding the effects of benzene exposure on human health. Polymorphism of enzymes involved in xenobiotic metabolism, including cytochromes P450, can alter an individual's disposition to toxicity. Polymorphisms of CYP2E1 are known to exist (Hu et al., 1997). Finding polymorphisms of other cytochrome P450 isozymes involved in benzene metabolism could help identify at risk individuals. Another important use for identifying the cytochrome P450 isozymes involved in benzene metabolism is for use in physiologically based pharmacokinetic modeling.

2. Materials and methods

2.1. Animals

Breeding colonies of CYP2E1 knockout mice (129/Sv-CYP2E1^{tmlGonz}; Jackson Laboratories, Bar Harbor, ME) and wild-type controls (12953/SrImJ; Jackson Laboratories, Bar Harbor, ME) were maintained in an AAALAC accredited animal facility on campus. Mice were housed in group cages kept in environmentally controlled rooms with a 12-h light:12-h dark cycle. Rodent Laboratory Chow (# 5001, Purina Mills, Inc, St. Louis, MO) and water were provided ad libitum.

2.2. Chemicals

Chemicals used included L-ascorbic acid, NADH, and NADPH obtained from Sigma Chemical Co (St. Louis, MO), magnesium chloride and potassium phosphate (monobasic) from Mallinckrodt Specialty Chemicals (Paris, KY), and potassium chloride and potassium phosphate (dibasic) from J.T. Baker (Phillipsburg, NJ).

Chemical inhibitors included diethyldithiocarbamic acid (DDTC) from Sigma Chemical Co (St. Louis, MO), and 5-phenyl-1-pentyne (5P1P) from Lancaster Synthesis (Windham, NH).

Bicinchoninic acid (BCA) protein assay kits were obtained from Pierce (Rockford, IL).

The unlabeled standards solution contained butylated hydroxytoluene, catechol, and hydroquinone from Sigma Chemical Co (St. Louis, MO), ethyl acetate and 1,2,4-trihydroxybenzene from Aldrich Chemical Co (Milwaukee, WI) and phenol from Mallinckrodt Specialty Chemicals (Paris, KY).

^{14}C-benzene (specific activity = 52–60 mCi per mmol) was obtained from American Radiolabeled Chemical (St. Louis, MO) or Moravek Biochemicals (Brea, CA).

2.3. Microsomal preparation

The method developed by Powley and Carlson (1999), a modification of the method of Chaney and Carlson (1995), was used for determining the microsomal metabolism of benzene. Mice were sacrificed by cervical dislocation and lung and liver were removed. Tissue was homogenized in potassium phosphate buffer (0.1 M, pH 7.4) containing 1.15% potassium chloride. This homogenate was centrifuged at $9000 \times g$ for 20 min. The supernatant was then centrifuged at $105\,000 \times g$ for 1 h. Finally, the pellet was resuspended in potassium phosphate buffer (0.1 M, pH 7.4). Part of this suspension was used for the microsomal metabolism assay, and an aliquot was used to determine protein concentration using the bicinchoninic acid (BCA) assay.

2.4. Incubation procedure

An incubation mixture containing 0.1 ml microsomes ($\sim$2–3 mg protein for liver and 0.6–1.6 mg protein for lung), 0.7 ml potassium phosphate buffer (0.1 M, pH 7.4) with ascorbate (10 mM), 80 μl magnesium chloride (25 mM), and 20 μl ^{14}C-benzene (52–60 mCi per mmol, 24 μM final concentration) was placed in 3 ml reacti-vials (Pierce, Rockland, IL) fitted with resealable teflon septa and incubated at 37 °C for 10 min. The reaction was initiated by injecting 0.1 ml of a solution containing NADPH (10 mM) and NADH (10 mM), and the resulting mixture was incubated in a metabolic shaker for 45 min at 37 °C.

Microsomal experiments using inhibitors included diethyldithiocarbamic acid (DDTC, 300 μM final concentration) and 5-phenyl-1-pentyne (5P1P, 5 μM final concentration) incubated with 24 μM benzene. The inhibitors were dissolved in methanol and added to the incubations in 10 μl aliquots prior to the addition of the NADPH and NADH solution.

The reaction was stopped by adding 1 ml of cold ethyl acetate. Extraction of the radiolabeled metabolites was maximized by vortexing the mixture, placing it on ice for 10 min, and repeating. The mixture was centrifuged at $833 \times g$ at 4 °C for 10 min. A portion of the organic layer was placed in a screw-top vial, and stored at −4 °C until analysis. Ten μl of unlabeled standard containing phenol (70 mM), hydroquinone (0.2 M), catechol (0.1 M), and butylated hydroxytoluene (90 mM) were added to the sample before analysis.

2.5. Quantification of metabolites

The components of the organic phase were separated using a Shimadzu HPLC system with a Beckman octadecyl silane (ODS) column (5 μm, 4.6 mm × 25 cm) and a Beckman Ultrasphere ODS guard column (5 μm, 4.6 mm × 4.5 cm). The mobile phases were deionized water purified by a Milli-Q Reagent Water System (A) and HPLC-grade methanol (B). Both phases were filtered through a 0.2 μM Nylaflo filter and degassed prior to use. The flow composition was as follows, 0–5 min, 0% B; 5–10 min, increase to 20% B; 10–15 min, 20% B; 15–30 min, increase to 35% B; 30–36 min, increase to 100% B; 36–45 min, 100% B; 45–50 min, decrease to 0% B; 50–55 min, 0% B. Throughout the analysis the flow rate was 1 ml/min. Sample injections were 200 or 250 μl.

HPLC samples were analyzed by a UV detector to identify fractions containing metabolites. The fractions were subsequently analyzed using a Packard 505TR Flow Scintillation Analyzer (Packard Instrument Co, Inc, Meriden, CT). Each ml of HPLC eluent mixed with 3 ml of Ultima Flo AP scintillation cocktail (Packard Instrument Co, Inc, Meriden, CT) before analysis by the flow analyzer. The radioactive metabolites were identified by the time of their elution compared with the UV peaks of the unlabeled standard. Metabolites were quantified as pmol metabolite per mg microsomal protein per 45 min. The limit of detection was 1 pmol of metabolite.

2.6. Data analysis

Tissues from three pooled samples from CYP2E1 knockout mice and controls were used. The effectiveness of the inhibitors was determined by comparing control groups with treated groups using one-sided paired Student's t-tests. To compare the difference in metabolism between CYP2E1 knockout mice and wild-type controls, one-sided Student's t-tests were performed. The data were normalized using a logarithmic transformation. Values that were below the detection limits, thereby considered zero for averaging purposes, and control values to which they were compared were transformed by $\log x + 1$. The selected level of significance for all tests was 0.05.

3. Results

Benzene metabolism in hepatic and pulmonary microsomes from CYP2E1 knockout mice was compared with that in microsomes from wild-type controls with a substrate concentration of 24 μM. The specific chemical inhibitors used to determine the cytochrome P450 isozymes involved were DDTC (300 μM) and 5P1P (5 μM) Unless otherwise specified, the percent decrease in metabolite formation mentioned in the text was statistically significant at $P < 0.05$. When metabolic inhibition was greater than 50% versus controls, but not statistically significant at $P < 0.05$, it is also included in the text. Benzene metabolism in hepatic microsomes from CYP2E1 knockout mice was significantly less than in the wild-type controls (Table 1). Formation of hydroquinone decreased by 98%, catechol by 97%, phenol by 94%, and total hydroxylated metabolite by 96%. In wild-type mice DDTC inhibited benzene metabolism to a much greater extent than did 5P1P. Hydroquinone formation decreased by 88%, catechol by 88%, phenol by 74%, and total hydroxylated metabolite formation by 80%. 5P1P caused a 22% decrease in phenol production and a 27% decrease in total hydroxylated metabolite production. In CYP2E1 knockout mice DDTC had the greatest effect on benzene metabolism while inhibition due to 5P1P was not as significant. In microsomes incubated with DDTC there were a 70% reduction in hydroquinone, a 69% reduction in phenol, and a 70% reduction in total hydroxylated metabolite. Catechol was not detected in incubations containing DDTC. With 5P1P hydroquinone formation decreased by 29%, phenol by 39%, and total hydroxylated metabolite by 37%. There was a 50% decrease in the amount of catechol, although it was not statistically significant.

Benzene metabolism in pulmonary microsomes from CYP2E1 knockout mice was significantly different from that in wild-type controls (Table 2). However, the percent decreases in metabolite formation were not as dramatic as in the liver. There was a 45% reduction in phenol and a 45% reduction in total hydroxylated metabolite formation.

Table 1
Effect of diethyldithiocarbamic acid (DDTC, 300 μM) and 5-phenyl-1-pentyne (5P1P, 5 μM) on 24 μM benzene metabolism by CYP2E1 knockout and wild-type mouse liver microsomes

Mouse	Inhibitor	n	Hydroquinone	Catechol	Phenol	Total[a]
Wild-type	Control	3	4000 ± 1000	284 ± 65	4600 ± 1000	8800 ± 2100
	DDTC, 300 μM	3	460 ± 110^{c}	35.5 ± 8.0^{c}	1210 ± 300^{c}	1720 ± 420^{c}
	5P1P, 5 μM	3	2600 ± 410	191 ± 28	3610 ± 380^{c}	6450 ± 810^{c}
Knockout	Control	3	84 ± 11^{d}	7.43 ± 0.92^{d}	260 ± 54^{d}	352 ± 66^{d}
	DDTC, 300 μM	3	25.1 ± 4.7^{c}	ND[b,c]	81 ± 10^{c}	107 ± 15^{c}
	5P1P, 5 μM	3	60 ± 21^{c}	3.7 ± 1.6	158 ± 41^{c}	223 ± 60^{c}

[a] Total hydroxylated metabolites.
[b] Below the limit of detection.
[c] Significant decrease ($P < 0.05$) in amount of metabolite formed versus control of same strain.
[d] Significant decrease ($P < 0.05$) in amount of metabolite formed versus wild-type mice.
Data are presented as mean ± S.E. with units of pmols metabolite per mg protein per 45 min.

Table 2
Effect of diethyldithiocarbamic acid (DDTC, 300 μM) and 5-phenyl-1-pentyne (5P1P, 5 μM) on 24 μM benzene metabolism by CYP2E1 knockout and wild-type mouse lung microsomes

Mouse	Inhibitor	*n*	Hydroquinone	Phenol	Total[a]
Wild-type	Control	3	40 ± 12	307 ± 22	348 ± 33
	DDTC, 300 μM	3	17 ± 10	80 ± 25[b]	97 ± 35[b]
	5P1P, 5 μM	3	15.0 ± 3.6	71 ± 13[b]	86 ± 15[b]
Knockout	Control	3	24.5 ± 9.8	168 ± 16[c]	192 ± 24[c]
	DDTC, 300 μM	3	18.9 ± 7.0	61.4 ± 3.0[b]	80.4 ± 9.7[b]
	5P1P, 5 μM	3	17.6 ± 5.8	36.7 ± 8.1[b]	54 ± 13[b]

[a] Total hydroxylated metabolites.
[b] Significant decrease ($P<0.05$) in amount of metabolite formed versus control of same strain.
[c] Significant decrease ($P<0.05$) in amount of metabolite formed versus wild-type mice.
Data are presented as mean ± S.E. with units of pmols metabolite per mg protein per 45 min.

Benzene metabolism in wild-type controls was inhibited to a similar degree with both 5P1P and DDTC. DDTC caused 74 and 72% decreases in phenol and total hydroxylated metabolite formation, respectively. 5P1P resulted in a 77% decline in phenol production and a 75% decrease in the production of total hydroxylated metabolite. Although not statistically significant, 5P1P also caused a 62% decrease in hydroquinone formation. In CYP2E1 knockout mice 5P1P had the greatest inhibitory effect, while DDTC also had an effect. DDTC caused a 63% decrease in phenol formation and a 58% decrease in total hydroxylated metabolite formation. Phenol production was inhibited by 78%, and total hydroxylated metabolite was decreased by 72% with 5P1P.

4. Discussion

The knockout mouse has been shown to be a valuable tool in elucidating the contribution of specific cytochrome P450 isozymes (Ghanayem et al., 2000). In this study CYP2E1 knockout mice were used to examine the contributions of other cytochrome P450 isozymes in metabolizing 24 μM benzene. DDTC (300 μM) and 5P1P (5 μM) were used as specific inhibitors of CYP2E1 and CYP2F2.

In hepatic microsomes from the wild-type mouse, CYP2E1 was the major cytochrome P450 isozyme responsible for metabolizing benzene. As would be expected, metabolism decreased significantly in CYP2E1 knockout mice. Total hydroxylated metabolite formation in CYP2E1 knockout mice decreased by 96% versus wild-type mice. In wild-type mice, benzene metabolism was inhibited to the greatest extent using the CYP2E1 inhibitor DDTC. These findings confirm the importance of this isozyme in hepatic benzene metabolism and are in agreement with our results with NSA mice (Powley and Carlson, 2000). Based on inhibition by 5P1P, CYP2F2 may be involved but to a lesser extent. Another result, which describes the inhibitory properties of DDTC, is of interest. With CYP2E1 knockout mice, DDTC greatly affected the benzene metabolizing activity indicating that DDTC is inhibiting another cytochrome P450 isozyme. 5P1P inhibited benzene metabolism in these mice, although to a lesser degree than DDTC. Therefore, it is possible that DDTC may be inhibiting other isozymes including CYP2F2.

Pulmonary benzene metabolism in CYP2E1 knockout mice was significantly lower than in wild-type mice, but the difference was not as marked as in the liver. This indicates that another cytochrome P450 isozyme is involved in benzene metabolism in the lung. This is consistent with results from experiments conducted using pulmonary microsomes from NSA mice which showed that CYP2F2 played as much of a role in benzene metabolism as CYP2E1 (Powley and Carlson, 2000). Benzene metabolism in wild-type controls was inhibited to a similar degree by both

DDTC and 5P1P, further implicating both the isozymes in pulmonary benzene metabolism. As was seen in the liver, DDTC had a significant inhibitory effect on benzene metabolism in pulmonary microsomes from CYP2E1 knockout mice again indicating that DDTC is inhibiting an additional cytochrome P450 isozyme, perhaps CYP2F2.

Results of our study with CYP2E1 knockout mice are fairly consistent with the study of Valentine et al. (1996). These authors showed that benzene metabolism decreased by 87% in CYP2E1 knockout mice versus wild-type controls in vivo as determined by measurement of urinary metabolites. They concluded that this isozyme is mainly responsible for benzene metabolism and that residual activity is due to CYP2B1. Our results suggest that their conclusion is accurate for benzene metabolism in liver. However, in pulmonary microsomes CYP2F2 plays an important role in benzene metabolism and may significantly contribute to the formation of the remaining 13% of benzene metabolites found in the urine of the knockout mice.

In addition to our findings of the importance of CYP2F2 in pulmonary benzene metabolism, we have also demonstrated a problem with using DDTC as an inhibitor of CYP2E1 in the lung. Based on our studies in knockout mice, this compound appears to inhibit other cytochrome P450 isozymes, most notably CYP2F2 in the lung.

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