Enzymes involved in benzene metabolism are likely genetic determinants of benzene-induced toxicity. Polymorphisms in human microsomal epoxide hydrolase (mEH) are associated with an increased risk of developing leukemia, specifically those associated with benzene. This study was designed to investigate the importance of mEH in benzene-induced toxicity. Male and female mEH-deficient (mEH−/−) mice and background mice (129/Sv) were exposed to inhaled benzene (0, 10, 50, or 100 ppm) 5 days/week, 6 h/day, for a two-week duration. Total white blood cell counts and bone marrow cell counts were used to assess hematotoxicity and myelotoxicity. Micronucleated peripheral blood cells were counted to assess genotoxicity, and the p21 mRNA level in bone marrow was used as a determinant of the p53-regulated DNA damage response. Male mEH−/− mice did not have any significant hematotoxicity or myelotoxicity at the highest benzene exposure compared to the male 129/Sv mice. Significant hematotoxicity or myelotoxicity did not occur in the female mEH−/− or 129/Sv mice. Male mEH−/− mice were also unresponsive to benzene-induced genotoxicity compared to a significant induction in the male 129/Sv mice. The female mEH−/− and 129/Sv mice were virtually unresponsive to benzene-induced genotoxicity. While p21 mRNA expression was highly induced in male 129/Sv mice after exposure to 100-ppm benzene, no significant alteration was observed in male mEH−/− mice. Likewise, p21 mRNA expression in female mEH−/− mice was not significantly increased upon benzene exposure whereas a significant induction was observed in female 129/Sv mice. Thus mEH appears to be critical in benzene-induced toxicity in male, but not female, mice.

Key Words: benzene; bone marrow; genotoxicity; hematotoxicity; leukemia; mEH; micronuclei.

Benzene, a commonly used industrial chemical, is a carcinogen, genotoxin, and hematotoxin in humans and rodents (Sanz et al., 1997). The major industries using benzene are those involving rubber, paint, shoes, lubricants, dyes, detergents, drugs, and pesticides (Phillips and Johnson, 2001; Wong, 2002). However, there are nonoccupational exposures to benzene at gasoline stations and from individuals who smoke (Runion and Scott, 1985). Benzene is one of the few known etiological factors for acute myelogenous leukemia (AML) in humans (Sanz et al., 1997). While much is known about the types of benzene-induced toxic insults in humans and rodents, the mechanism for these effects are uncertain, thus supporting the need for studies to identify biomarkers in those individuals who are more genetically susceptible to benzene-induced toxicity.

The metabolism of benzene is complex and consists of many possible pathways (Fig. 1). Cytochrome P4502E1 (CYP2E1) is essential for benzene metabolism; without this enzyme, no hematotoxicity or genotoxicity was observed in mice (Valentine et al., 1996). Many of the metabolites, such as benzene oxide and 1,4-benzoquinone can lead to toxic effects (Ross, 2000; Smith, 1996). The pathway most commonly studied with respect to benzene-induced toxicity is the hydroquinone pathway (Ross, 2000; Smith, 1996). Microsomal epoxide hydrolase (mEH; E.C. 3.3.2.3) converts benzene oxide to benzene dihydrodiol, which is then converted to catechol by a dehydrogenase (Ross, 2000). In an in vitro system, mEH can convert benzene oxide to hydroquinone and phenol metabolites (Snyder et al., 1993). In addition, there is significant controversy regarding the production of trans, trans-muconaldehyde through the dihydrodiol intermediate in the mEH pathway (Witz et al., 1996). Thus the importance of mEH in benzene metabolism is unclear.

The hydrolysis of xenobiotic epoxides to form the vicinal dihydrodiol is catalyzed by mEH (Fretland and Omiecinski, 2000). Because mEH is involved in the bioactivation of several carcinogens, such as conversion of the polycyclic aromatic hydrocarbon benzo[a]pyrene-4,5 oxide to benzo[a]pyrene-7,8-diol-9,10-epoxide (Sims et al., 1974), and detoxification of more toxic epoxides (Oesch, 1973), there is significant interest in genetic polymorphisms that may alter the activity of mEH. Two major polymorphisms have been observed in the human mEH gene, one in exon 3 and one in exon 4. The exon 3 polymorphism, a T→C substitution causing a histidine to replace a tyrosine at residue 113, results in a 40–50% decrease
in enzyme activity (slow-activity) (Hassett et al., 1994). In exon 4, an A→G substitution results in an arginine replacing a histidine at residue 139, leading to a 25% increase in activity (fast-activity) (Hassett et al., 1994). The slow-activity phenotype is associated with an increased risk of colon (Harrison et al., 1999) and hepatocellular cancer (McGlynn et al., 1995), whereas the fast-activity phenotype is associated with an increased risk of smoking-related lung cancers (Benhamou et al., 1998; London et al., 2000; Zhao et al., 2002). Recently, the slow-activity phenotype was also associated with a reduction in the risk of adult AML (Lebailly et al., 2002), while men, but not women, with the fast-activity phenotype have an increased risk of adult AML. Men having fast-activity phenotype with no known occupational exposures to benzene were also found to have chromosome abnormalities similar to those seen with benzene exposure (Lebailly et al., 2002), including translocations at chromosomes 8 and 21 (Smith et al., 1998).

Mice deficient in mEH expression and activity have a portion of exon 2 deleted and have no unusual phenotype, suggesting that mEH is not essential for reproduction and physiological homeostasis (Miyata et al., 1999). The mEH-deficient mice did not bioactivate 7,12-dimethylbenz[a]anthracene (DMBA) to the carcinogenic metabolite 3,4-diol-1,2-oxide and are therefore highly resistant to DMBA-induced carcinogenesis (Miyata et al., 1999). This finding supports a role for mEH in bioactivating certain polycyclic aromatic hydrocarbons. We hypothesized that mice deficient in mEH have a reduced response to benzene-induced toxicity due to a decrease in the amount of toxic metabolites produced. In this study, using 129/sv (background) and mEH−/− mice, we demonstrate that mEH is critical in benzene-induced genotoxicity, hematotoxicity, and induction of the p53 DNA damage response in male, but not female, mice.

**MATERIALS AND METHODS**

**Animal husbandry.** We maintained a pathogen-free mEH+ colony and a 129/sv (background strain) colony in the CIIT animal facility after the animals were rederived at Charles River (Wilmington, MA). The mEH+ and 129/sv mice were originally obtained from the National Cancer Institute colony (NCI, Bethesda, MD). All animal use was conducted in facilities accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care and approved by the Animal Care and Use Committee of the CIIT Centers for Health Research (CIIT). Mice were housed in shoebox-type cages in a humidity- and temperature-controlled room and provided water and pelleted open-formula rodent diet NIH-07 (Zeigler Brothers, Gardners, PA) ad libitum. The mice were brother-sister mated, and the offspring then genotyped for mEH according to Miyata et al. (1999).

**Experimental animals.** We used male and female mice that were 10–12 weeks old at the start of the study. The mice were housed as described above until they were moved for acclimation to stainless steel wire mesh cages in a Henners-style, whole-body inhalation chamber 2 weeks prior to benzene exposures. During this 2-week acclimation period, the mice were put on a reversed-light schedule (on at 1 A.M. and off at 1 P.M.), with exposures taking place during the light cycle. Only water was given during the exposures. Tissues were collected within 5 h after termination of exposure. Each exposure group, including air-exposed controls, was housed in a separate inhalation...
chamber. Mice used in the determination of basal CYP2E1 activity were not housed in suspended wire cages but remained in the polycarbonate shoebox caging.

**CYP2E1 activity and protein content.** Liver microsomes were prepared using the method of (Guengerich, 1989). Briefly, after mice were euthanized with 5 mg pentobarbital/mouse, livers were removed from naive male and female mice and then homogenized in 4 volumes of ice-cold buffer A (0.154 M KCl, 0.05 M Tris Base [Sigma, St. Louis, MO], pH 7.4). After centrifugation at 10,000 × g for 20 min at 4°C, the supernatant was transferred to an ultracentrifuge tube and spun at 105,000 × g for 60 min at 4°C. After removal of the supernatant, the pellet was resuspended in 7 ml of buffer B (0.05 M Tris, 0.25 M sucrose, 1 mM EDTA [Sigma], pH 7.4). The microsome solution was then centrifuged at 105,000 × g for 60 min at 4°C and the supernatant discarded. The microsome pellet was resuspended in an amount equivalent to the liver weight in buffer C (0.1 M K HPO4, 0.25 M sucrose, pH 7.4) and frozen at −80°C until further use.

*p*-Nitrophenol (PNP) hydroxylase activity was measured using a spectro-photometric assay (Reinke and Moyer, 1985). Ninety percent of PNP hydroxylase activity reflects CYP2E1 activity, thus it was used as a measure of CYP2E1 activity (Tierney et al., 1992). Incubation mixtures that contained 0.1 M potassium phosphate buffer, 0.1 mM PNP, 1.0 mM ascorbate, and 0.4 mg/ml liver microsomal protein were preincubated for 3 min at 37°C prior to the addition of 1.0 mM NADPH. In control incubation mixtures, NADPH was replaced with an equivalent amount of deionized H2O. The total incubation volume was 1.0 ml. The incubation was performed for 7.5 min at 37°C in a shaking water bath and terminated by adding 0.2 ml 1.5 N perchloric acid. The sample was diluted 10:1 with 10 N NaOH, and the absorbance was measured at 450 nm on a Beckman DU 650 spectrophotometer (Fullerton, CA). The concentration of 4-nitrocatechol was determined using an extinction coefficient of 10.28 cm2/mol (Reinke and Moyer, 1985).

Laemmli sample buffer (Bio-Rad, Hercules, CA) was added directly to microsomal protein and boiled for 5 min. Eight μg protein per sample was loaded onto a 10% polyacrylamide SDS–PAGE gel. The gel was electrophoresed and then transferred onto Immobilon-P transfer membrane (Millipore, Bedford, MA). The membrane was blocked for 2 h in 5% milk plus 0.1% Tween-20 (Sigma, St. Louis, MO) in PBS (GibcoBRL-Gaithersburg, MD) followed by an overnight incubation at 4°C with the primary polyclonal rabbit anti-cytochrome P450 CYP2E1 antibody (Gentest Corporation, Woburn, MA) at a 1:1000 dilution in 0.5% milk in PBS. After 3 washes in 1% milk plus 0.1% Tween-20, the secondary antibody anti-goat-horseradish peroxidase (Santa Cruz Biotechnologies, Santa Cruz, CA) was used at a 1:20,000 dilution in 0.5% milk in PBS for a 1-h incubation at room temperature, followed by more washes. The signal was detected using West Pico Chemiluminescence (Pierce Endogen, Rockford, IL). Densitometry was done using the BIO-RAD Quantity One Quantitation software (Ver. 4).

**Experimental design.** This study used three benzene exposure groups (10, 50, and 100 ppm benzene) and one control air-exposed group (0 ppm). Exposure levels were chosen based on a pilot study using 0, 10, and 100 ppm benzene exposure levels in older mice (data not shown). Mice were exposed to benzene 5 days/week, for 6 h/day for a 2-week duration, in all experiments based on previous studies (Farris et al., 1997).

A previous time course in male 129/SvJ (Jackson Laboratories) mice demonstrated that the optimal sampling time point for determining peak levels of micromolecule reticulocytes (MN-RET; immature RBC) and malaria-infected cells was 6 h after benzene exposure (Boley et al., 2002). The reaction conditions and data analysis were performed according to the manufacturer’s recommended protocol. All samples were run in triplicate using GAPDH as the calibrator gene, since GAPDH levels do not change across genotypes or with benzene exposure (Boley et al., 2002).

**Statistical analysis.** All data are presented as the mean ± standard error of the mean (SEM). A three-way analysis of variance (ANOVA) was done on each variable with the three main-effect factors of gender, strain, and exposure level and their first-order interactions. If any of the interactions were significant, additional analyses were done so that the nature of the interaction could be understood. Tukey’s multiple comparison procedure was used to determine differences among significant factors with three or more levels; p < 0.05 was used as the level of significance for all statistical tests. A Pearson correlation coefficient was calculated for the two variables MN-RET percentages and p21 mRNA expression.
TABLE 1
P-nitrophenol Hydroxylase Activity in 129/Sv vs. mEH–/– Mice

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Gender</th>
<th>PNP hydroxylase activity (nmol/min/mg ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>129/Sv</td>
<td>Male</td>
<td>2.03 ± 0.27</td>
</tr>
<tr>
<td>129/Sv</td>
<td>Female</td>
<td>1.75 ± 0.16</td>
</tr>
<tr>
<td>mEH+</td>
<td>Male</td>
<td>1.33 ± 0.13*</td>
</tr>
<tr>
<td>mEH+</td>
<td>Female</td>
<td>1.04 ± 0.26</td>
</tr>
<tr>
<td>mEH–/– Female</td>
<td>1.04 ± 0.26</td>
<td></td>
</tr>
</tbody>
</table>

Note. Male 129/Sv, n = 4 mice; female 129/Sv, n = 4 mice; male mEH–/–, n = 5 mice; female mEH–/–, n = 3 mice.

*mRNA fold-increases. Statistical analyses were done using SAS Statistical Software (SAS Institute, Inc., Cary, NC).

RESULTS

Basal PNP hydroxylase activity and CYP2E1 protein content in liver microsomes of mEH–/– and 129/Sv mice. We determined whether the basal activity of CYP2E1 was altered in the mEH–/– vs. 129/Sv mice to assure that any differences in benzene metabolism were not the result of altered CYP2E1 activity. Male 129/Sv mice had significantly higher PNP hydroxylase activity compared to male mEH–/– mice; however, the fold increase was only 1.5-fold suggesting that this difference is likely not biologically relevant (Bauer et al., in press; Table 1). In addition, the CYP2E1 protein content was not different between the male 129/Sv and mEH–/– mice (Bauer et al., in press; Fig. 2). There were no differences seen in the female 129/Sv and mEH–/– mice with respect to PNP hydroxylase activity or CYP2E1 protein content (Table 1; Fig. 2).

Hematotoxicity and myelotoxicity in mEH–/– and 129/Sv mice exposed to benzene. Benzene causes hematotoxicity, typically reflected in a decrease in WBC, and myelotoxicity in rodents (Farris et al., 1997; Ward et al., 1996). The male 129/Sv mice developed hematotoxicity at the highest benzene exposure level in contrast to the male mEH–/– mice (Bauer et al., in press; Fig. 3A). Significant myelotoxicity was seen in the male 129/Sv mice and was dose responsive (Bauer et al., in press; Fig. 4A) compared to the male mEH–/– mice, which developed no myelotoxicity. Female mEH–/– mice did not differ in response to benzene compared to the 129/Sv female mice (Bauer et al., in press), in that no significant hematotoxicity or myelotoxicity occurred (Figs. 3B and 4B). However, the female mEH–/– mice had a significant increase in total bone marrow cells at the 50 and 100 ppm exposure levels vs. 0 ppm compared to the female 129/Sv mice (Fig. 4B). Interestingly, both the male and female mEH–/– mice had more bone marrow cells in air-exposed mice, and the trend was similar in the WBC counts.

MN analysis as a measure of the genotoxic stress response in the peripheral blood of mice exposed to inhaled benzene. Genotoxicity, measured by MN-RET and MN-NCE, was seen in the male 129/Sv mice at the 100-ppm exposure level (14-fold increase in MN-RET and 2.7-fold increase in MN-NCE) compared to 0 ppm, but barely seen in the male mEH–/– mice (1.7-fold increase in MN-RET and 1.2-fold increase in MN-NCE) (Figs. 5A and 6A). There was a slight yet significant increase in MN-RET at the 100-p.p.m. exposure level in both the female 129/Sv mice (2.7-fold) and mEH–/– mice (1.4-fold) (Bauer et al., in press; Fig. 5B). The female mEH–/– mice had higher basal levels of MN-RET than the female 129/Sv mice. There were no significant changes in the percentage of MN-NCE at any of the exposure levels in the female 129/Sv and mEH–/– mice (Bauer et al., 2003; Fig. 6B). However, both the male and female mEH–/– mice had significantly higher numbers of basal MN-NCE than the 129/Sv mice. There was a clear dose response in MN-RET with both genders in 129/Sv mice and in female mEH–/– mice; however, only the 100-ppm level resulted in increased MN-RET in male mEH–/– mice. Only the male 129/Sv mice had a dose response effect with respect to the MN-NCE parameter.

Analysis of a DNA-damage response in mEH–/– and 129/Sv mice exposed to benzene. The p53 tumor suppressor is typically induced in response to DNA damage, followed by the induction of genes such as p21, which can lead to a cell cycle arrest (Prives and Hall, 1999). p21, a cyclin-dependent kinase inhibitor involved in p53-induced cell cycle arrest, is elevated in response to benzene (Boley et al., 2002; Yoon et al., 2001). Expression of p21 mRNA as a measure of this DNA-damage response revealed a dose-response relationship in the male 129/Sv mice.
129/Sv mice. There were significant 3- and 10-fold increases in p21 mRNA expression at the 50- and 100-ppm levels, respectively, compared to 0-ppm levels in male 129/Sv mice, while the male mEH–/– mice did not undergo any increases in p21 mRNA expression (Fig. 7A). Although the female 129/Sv mice showed a 4-fold increase in expression, and the mEH–/– mice had no significant alterations in p21 expression, the 2 strains were not statistically different at any exposure level (Fig. 7B). p21 mRNA expression was significantly correlated to the genotoxic stress response measured by MN-RET ($r = 0.909; p < 0.0001$) (Figs. 5 and 7) in mice (Bauer et al., 2003).

**DISCUSSION**

Although benzene has been linked to leukemia and lymphoma in humans since the 1970s, studies are still ongoing to determine the health risks of this chemical (Phillips and Johnson, 2001; Hayes et al., 2001). In a recent report submitted to the U.S. Environmental Protection Agency, another subtype of leukemia, chronic lymphocytic leukemia, was also linked to benzene exposure (Phillips and Johnson, 2001). Additional studies are currently ongoing to evaluate the potential health risk of benzene to children because exposure to benzene is not solely occupational (Raaaschou-Nielsen et al., 2001). Benzene is a constituent of gasoline and cigarettes and therefore poses a potential health threat to more than just occupationally exposed individuals, including those exposed to second-hand cigarette smoke (IARC, 2000; Runion and Scott, 1985; Wallace, 1990). The activity of the enzyme systems involved in the activation and detoxification reactions of benzene metabolism are likely key determinants of individual-to-individual variability and risk in response to the toxic effects of benzene. It is unknown exactly what benzene metabolite causes the genotoxicity or hematotoxicity seen with benzene or whether there are several metabolites that synergize to produce the toxic effects
However, several genes involved in benzene metabolism contain known functional polymorphisms that can alter metabolism. A polymorphism in NAD(P)H oxidoreductase-1 (NQO1), the enzyme that can detoxify both 1,2 and 1,4-BQ to less reactive metabolites, leads to NQO1 deficiency if homozygous for this mutation (Ross et al., 2000). Combined NQO1 deficiency and increased CYP2E1 activity have been linked to an increased risk of benzene poisoning in humans (Rothman et al., 1997). Since the aforementioned study demonstrates the need to determine individual genetic susceptibility with respect to those enzymes involved in the metabolism of benzene for risk assessment, and since at least two polymorphisms in the mEH gene are known to exist, we studied the benzene response in mice deficient in mEH.

In the present study, we found that male mice deficient in mEH enzyme activity were unresponsive to benzene in contrast to male 129/Sv mice, which developed significant benzene-induced hematotoxicity, myelotoxicity, and genotoxicity. Female 129/Sv mice exhibited very little response to benzene, as revealed by no significant differences in hematotoxicity, myelotoxicity, and genotoxicity between the female 129/Sv and mEH–/– mice. Expression of p21 mRNA was greatly elevated in the male 129/Sv mice and to a lesser degree in the female 129/Sv mice, while no changes were seen in either gender of the mEH–/– mice.

The gender differences seen in the 129/Sv strain are not unique. Other rodent studies have demonstrated gender differences in MN, sister chromatin exchanges, and benzene metab-
olism (Kenyon et al., 1995; Luke et al., 1988; Meyne and Legator, 1980). The cause of these differences has not been discerned but may involve metabolic differences between the two genders (Kenyon et al., 1995). Whether gender differences occur in humans with respect to benzene-induced toxicity is not clear. One study observed that women have a slightly lower response to benzene. In addition to mEH, we recently studied mice deficient in NQO1 activity and found that female NQO1−/− mice developed benzene-induced genotoxicity and hematotoxicity compared to the NQO1+/− mice, which were unresponsive (Bauer et al., 2003). However, male NQO1−/− mice were only more sensitive to benzene hematotoxicity compared to the NQO1+/− mice. These results further support the need to assess the many different enzymes and gender differences involved in benzene metabolism to identify individuals that are at a higher risk of benzene toxicity and possibly leukemogenesis. Additional studies determining differences in the benzene metabolism pathway will be done using the mEH+/− mice to further understand the responses described here.

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