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In vitro metabolism and covalent binding of ethylbenzene to microsomal protein as a possible mechanism of ethylbenzene-induced mouse lung tumorigenesis

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ABSTRACT

This study was conducted to determine species differences in covalent binding of the reactive metabolites of ethylbenzene (EB) formed in the liver and lung microsomes of mouse, rat and human in the presence of NADPH. These data further the understanding of the mechanism by which EB causes mouse specific lung toxicity and a follow-up to our earlier report of the selective elevation, although minor, of the ring-oxidized reactive metabolites in mouse lung microsomes (Saghir et al., 2009). Binding assays were also conducted with or without 5-phenyl-1-pentyne (5P1P), an inhibitor of CYP 2F2, and diethyldithiocarbamate (DDTC), an inhibitor of CYP 2E1 to evaluate their role in the formation of the related reactive metabolites. Liver and lung microsomes were incubated with ^{14}C-EB (0.22 mM) in the presence of 1 mM NADPH under physiological conditions for 60 min. In lung microsomes, binding activity was in the order of mouse (812.4 ± 102.2 pmol/mg protein) >> rat (57.0 ± 3.2 pmol/mg protein). Human lung microsomes had little binding activity (15.7 ± 1.4 pmol/mg protein), which was comparable to the no-NADPH control (9.9–16.7 pmol/mg protein). In liver microsomes, mouse had the highest activity (469.0 ± 38.5 pmol/mg protein) followed by rat (148.3 ± 14.7 pmol/mg protein) and human (89.8 ± 3.0 pmol/mg protein). Presence of 5P1P or DDTC decreased binding across species and tissues. However, much higher inhibition was observed in mouse (86% [DDTC] and 89% [5P1P]) than rat (56% [DDTC] and 59% [5P1P]) lung microsomes. DDTC showed ~2-fold higher inhibition of binding in mouse and human liver microsomes than 5P1P (mouse = 85% vs. 40%; human = 59% vs. 36%). Inhibition in binding by DDTC was much higher (10-fold) than 5P1P (72% vs. 7%) in rat liver microsomes. These results show species, tissue and enzyme differences in the formation of reactive metabolites of EB. In rat and mouse lung microsomes, both CYP2E1 and CYP2F2 appear to contribute in the formation of reactive metabolites of EB. In contrast, CYP2E1 appears to be the primary CYP isozyme responsible for the reactive metabolites of EB in the liver.

1. Introduction

Ethylbenzene (EB)[1] is commonly used as an intermediate in the manufacture of styrene and synthetic rubber. It is also present in agricultural and home insecticide sprays, household degreasers, paints, adhesives, rust preventives and as a major component of mixed xylenes used as a solvent. Occupational exposure to EB may occur during the production of polystyrene as well as during production and use of mixed xylenes (Fishbein, 1985).

EB is not mutagenic in a variety of bacterial or yeast mutagenicity assays, either directly or in the presence of activating enzymes (Dean et al., 1985; Nestmann et al., 1980; NTP, 1999; Zeiger et al., 1992). EB has been reported to not cause an increase in sister chromatid exchanges or chromosomal aberrations in cultured Chinese hamster ovary cells (NTP, 1999). In contrast, EB has reported to be weakly positive in a human lymphocyte sister chromatid exchange assay in the presence of activating enzymes (Norppa and Vainio, 1983) and to increase the incidence of mutations in a mouse lymphoma mutagenicity assay in the absence of activating enzymes (MacGregor et al., 1988). The latter response, however, was obtained only at cytotoxic concentrations in which growth was 13–34% that of controls (MacGregor et al., 1988). EB was negative in an *in vivo* mouse micronucleus assay (NTP, 1992, 1999).

No statistically significant increases in tumors were reported in Sprague–Dawley rats gavaged with 500 mg/kg/day EB (4–5 days/week, for 104 weeks) (Maltoni et al., 1985). However, in an inhalation carcinogenicity study in which F344/N rats and B6C3F1 mice were exposed to 0, 75, 250, or 750 ppm EB 6 h/day, 5 days/week, for 104 weeks, carcinogenic activity has been reported (NTP, 1999). Statistically identified neoplastic changes in the NTP (1999) study included renal tubule adenomas in high exposure group male and female rats, lung alveolar/bronchiolar adenomas in high exposure group male mice (and intermediate exposure

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[1] *Abbreviations used:* EB, ethylbenzene; 2EP, 2-ethylphenol; 4EP, 4-ethylphenol; 5P1P, 5-phenyl-1-pentyne; DDTC, diethyldithiocarbamate.

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group when combined with carcinomas), and liver adenomas in high exposure group female mice.

The mechanism(s) of target-tissue specific tumorigenic activity of EB was evaluated by Stott et al. (1999, 2001) by exposing rats and mice through inhalation of up to 750 ppm EB, 6 h/day, for 5 or 28 consecutive days. Exposure of EB caused an increase in kidney weights in rats accompanied with an induction of CYP2B, CYP2E1 and UGT enzyme activities in male rats and inhibition of Phase I enzyme activities in female rats. Exposure to EB caused an increase in mouse liver weight and CYP1A and/or CYP2B enzyme activity in both sexes. In mouse lungs, CYP1A and CYP2B enzyme activities were inhibited in EB exposed animals.

Engstrom (1984) reported that the major metabolic pathways of EB in rats are its metabolism to 1-phenylethanol, 2-phenylethanol, 2-ethylphenol or 4-ethylphenol. Midorikawa et al. (2004) further reported that 2-ethylphenol and 4-ethylphenol are metabolically transformed to ring-dihydroxylated ethylhydroquinone and 4-ethylcatechol in phenobarbital-induced, Sprague–Dawley rat liver microsomes at 5 mM substrate concentration. Both of these dihydroxylated metabolites were shown to cause DNA damage *in vitro* in the presence of Cu(II). They concluded that these active dihydroxylated metabolites might be involved in the carcinogenesis induced by ethylbenzene.

Ethylphenols (2- and 4-ethylphenol) were found as minor metabolites of EB in microsomal incubations (Saghir et al., 2009), which was above the detection limit only in the mouse liver microsomes and rat and mouse lung microsomes and remained below the limit of detection in human liver and lung microsomes. Since 2- and 4-ethylphenol have no structural alerts indicating cytotoxic potential, these findings point to the subsequent formation of ring-oxidized metabolites of ethylbenzene causing mouse specific lung toxicity. Saghir et al. (2009) have also examined the formation of catechol/hydroquinone, quinones as the downstream metabolites of ethylphenols by trapping the reactive metabolite(s) with excess glutathione (GSH) in the microsomal incubations in the presence of NADPH. Conversion of EB to ring-hydroxylated metabolites (catechols, hydroquinones, quinones) was much lower than conversion to alkyl-hydroxylated metabolites. Lung microsomes were more active in metabolizing EB to ethylphenol-GSH than the liver microsomes, the highest levels of which was found in the mouse lung microsomes (Saghir et al., 2009).

The GSH conjugate formation from microsomal incubation of EB indicated that electrophilic reactive metabolite(s) were formed during the microsomal incubations. Those electrophilic reactive metabolites may also react with nucleophilic microsomal proteins to form covalent adducts of the reactive metabolite(s) of EB. The previous study was not designed to determine covalent binding of the reactive metabolites to microsomal proteins. Therefore, this study was conducted to determine the potential covalent protein binding of mouse, rat and human lung and liver microsomes with reactive EB metabolites (e.g., catechols, hydroquinones, quinones). In this study, covalent binding was determined by conducting similar incubation experiment as described earlier (Saghir et al., 2009) using radiolabeled EB followed by binding assay. Additional experiments were also conducted to characterize the CYP isozymes responsible for EB ring oxidation by using specific cytochrome P450 enzyme inhibitors.

2. Materials and methods

2.1. Chemicals

Non-radiolabeled ethylbenzene (EB) (>99.5% pure) was purchased from Sigma–Aldrich (St. Louis, MO), radiolabeled EB (>99.5% pure) was purchased from Tjaden Bioscience (Burlington, IA). EB was uniformly ^{14}C-labeled on the benzene ring with the specific activity of 24 mCi/mmol. All reagents were purchased from Sigma–Aldrich Chemical (St. Louis, MO). All solvents were purchased from Fisher Scientific (Pittsburg, PA) and were HPLC grade or better.

2.2. Tissue types and source/supplier

The study was conducted using liver and lung microsomes obtained from un-induced male Fischer-344 rats, male B6C3F1 mice, and humans of mixed gender and race. Microsomes were obtained from XenoTech (Lenexa, KS). Rat liver and lung microsomes were prepared from pools of 198 and 30 untreated animals, respectively. Mouse liver and lung microsomes were prepared from pools of 447 and 100 animals, respectively. Human liver microsomes were from a pool of 50 individuals of mixed gender (25 males; 25 females), race (Caucasian, African, Asian, and Hispanic) and age (11–69 years old with most of them below 55 years); 28 of them died from cerebrovascular stroke, 13 from head trauma, 9 from anoxia. Human lung microsomes of non-smokers were prepared from a pool of 4 individuals of mixed gender (2 males; 2 female) and race (Caucasian an Hispanic, 1 died from stroke, 1 from interacranial hemorrhage, 1 from head trauma, and 1 from anoxia. Microsomes were received in small aliquots on dry ice and stored at −80 °C. Each microsomal preparation was reanalyzed for CYP1A1, CYP2B1 and CYP2E1 activities in our laboratory prior to their use in the study (Table 1). Liver microsomes contained 1.21 (mouse), 0.77 (rat) and 0.49 (human) nmol cytochrome P450/mg protein.

2.3. Comparison of binding of ^{14}C-EB with microsomal proteins

Experiments were conducted to determine the difference in the binding of the reactive metabolites of EB to liver and lung microsomes of the three species. Microsomal incubations were conducted for 60 min at 37 °C at a low EB concentration (0.22 mM) at which the maximum velocity of metabolite formation occurred (Saghir et al., 2009). Every incubation contained ~0.23 μCi of radiolabeled EB (~0.01 mM ^{14}C-EB) to ensure enough radioactivity for final radiochemical analysis. Each microsome type was incubated

Table 1
Cytochrome P450 activity of mouse, rat and human liver and lung microsomes used in this study.

	Liver	Lung
	(pmol/min/mg protein)	
CYP1A1 (EROD activity)		
Mouse	25.73	10.91
Rat	10.33	5.64
Human	3.01	0.29
Human (reference value)[a]	–	0.77
Rat (induced)[b]	484.0	–
CYP2B1 (PROD activity)		
Mouse	2.19	2.61
Rat	2.24	9.37
Human	0.41	0.74
Rat (induced)[b]	133.3	–
CYP1E1 (pNP activity)		
Mouse	8.37	32.80
Rat	6.13	14.21
Human	19.93	0.17
Rat (induced)[b]	57.40	–

[a] Activity in non-smoker lung microsomes (n = 24) prepared immediately (within ~15 min) after removal (20–100 g) during clinically indicated lobectomy of lung tissues devoid of macroscopically visible tumors (Smith et al., 2001).

[b] Induced rat liver microsomes were analyzed with study tissues as a reference for comparison. EROD, ethoxyresorufin *O*-dealkylation; PROD, pentoxyresorufin *O*-dealkylation; pNP, *p*-nitrophenol.