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ETHYLBENZENE-MEDIATED INDUCTION OF CYTOCHROME P450 ISOZYMES IN MALE AND FEMALE RATS

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Abstract—Male and female Holtzman rats were exposed to ethylbenzene, and the effect on liver microsomal activities was studied. Hydrocarbon- and sex-dependent effects on P450-dependent metabolism of drugs and aromatic hydrocarbons were investigated. Hydrocarbon treatment produced two patterns of induction in cytochrome P450-dependent activities: (1) induction common to both sexes; and (2) induction exclusively in females. Benzphetamine N-demethylation, 7-ethoxycoumarin O-deethylation, *p*-nitroanisole O-demethylation and aromatic hydroxylation of toluene were induced in both sexes after rats were exposed to ethylbenzene. The rate of benzphetamine N-demethylation increased 4-fold in females and nearly doubled in males. The increase in O-deethylation of 7-ethoxycoumarin was 3-fold in females and doubled in males, while *p*-nitroanisole O-demethylation increased 4-fold in both sexes after exposure to ethylbenzene. Ethylbenzene had its greatest effect upon the formation of aromatic hydroxylated metabolites of toluene. Ethylbenzene exposure increased the rate of *o*-cresol formation by 4- and 9-fold in female and male rats, respectively. The formation rate of *p*-cresol was undetectable in either sex prior to hydrocarbon exposure; however, after the rats were given ethylbenzene, rates increased to 0.4 nmol/min/mg protein in females and to 0.9 nmol/min/mg protein in the males. Ethylbenzene exposure selectively induced aminopyrine demethylation, aniline hydroxylation, *N,N*-dimethylnitrosamine N-demethylation (DMNA) and aliphatic hydroxylation of toluene in females. Rates for aminopyrine, aniline, and DMNA were increased 50% over controls, while formation of benzyl alcohol from toluene was enhanced to 260% of control. Western immunoblotting indicated that ethylbenzene treatment induced cytochrome P450 2B1/2B2 to a greater extent in male rats and cytochrome P450 2E1 only in females. Ethylbenzene exposure did not affect significantly the level of cytochrome P450 1A1.

Aromatic hydrocarbons are major components of a number of industrial and consumer products such as solvents, varnishes, and gasoline, and the common use of these items provides a variety of opportunities for exposure to them. Of the aromatic hydrocarbons that are found frequently in these products, benzene, toluene, and xylenes are found in the highest concentrations, while ethylbenzene and larger alkylbenzenes are found at lower levels. These compounds are encountered frequently in mixtures such as gasoline, but in many cases a consumer product can be composed of a single compound.

Occupational exposure to aromatic hydrocarbons can produce toxic events in cellular systems. Benzene has been determined to be an extremely toxic aromatic hydrocarbon capable of promoting blood abnormalities, certain types of leukemia, and genotoxicity [1]. Toluene, xylenes, and ethylbenzene are considered to be safer, less toxic agents than benzene; however, a limited amount of information suggests that their exposure can result in behavioral disturbances, genotoxicity, and possible changes in the central nervous system, liver, and kidneys depending on the hydrocarbon in question [2–6].

The initial steps of aromatic hydrocarbon metabolism are catalyzed by the hepatic cytochrome P450 (P450§)-dependent monooxygenases [1–4]. The general reaction catalyzed by the P450 isozymes involves hydroxylation of the substrate molecule, converting a lipophilic substrate to a more polar product. Hydroxylation of the aromatic ring converting benzene to phenol is an example of such a reaction [7, 8]. These reactions, however, are not limited to hydroxylation at one location on a molecule. When the substrate is an alkylbenzene, such as toluene, reactions can occur at different positions on the molecule. Toluene has been shown to be metabolized *in vivo* by both aliphatic and aromatic hydroxylation [3]. Hydroxylation of the aliphatic methyl group results in the formation of the primary metabolite benzyl alcohol, while ring hydroxylation could produce *o*-, *m*-, or *p*-cresol. Aromatic oxidation to form cresols has been reported to occur through the formation of epoxides and accounts for less than 1% of an absorbed dose of toluene [9]. Additionally, there is evidence for direct hydroxylation of the aromatic ring which also contributes to the amount of cresol produced [10].

Induction of cytochrome P450 isozymes by various

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§ Abbreviations: P450, cytochrome P450; DMNA, *N,N*-dimethylnitrosamine N-demethylation; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; and BSA, bovine serum albumin.

compounds ranging from polycyclic hydrocarbons to drugs such as phenobarbital has been the subject of continual investigation [11–13]. Exposure to aromatic hydrocarbons has also been implicated in the induction of cytochrome P450 [14, 15]. Certain cytochrome P450-dependent activities were increased in rats exposed to benzene, toluene, or xylenes. Exposure to toluene and xylenes was shown to increase cytochrome P450 levels, and the P450-dependent activities of aminopyrine demethylation and aniline hydroxylation, while exposure to benzene had little effect on these activities [14]. The inductive effect of toluene has also been demonstrated to be associated with the sex and age of the animal [15].

Studies pertaining to alterations in the route of hydroxylation for the alkylbenzenes following hydrocarbon-mediated induction of cytochrome P450 have yet to be addressed. Presently, it is not known if changes in the aliphatic and aromatic pathways of metabolism of an alkylbenzene can selectively be induced following exposure to aromatic hydrocarbons. This study will investigate how exposure to an aromatic hydrocarbon can influence the induction of cytochrome P450 isozymes that hydroxylate toluene, and if this induction is influenced by the gender of the animals. Alterations in the routes of hydroxylation were monitored using toluene as the substrate due to its simplicity as an alkylbenzene, possessing one aliphatic and three possible aromatic hydroxylation sites.

Studies in this laboratory using ethylbenzene as the inducing agent indicated that this compound was one of the more effective alkylbenzenes for the induction of hepatic cytochrome P450-dependent benzphetamine and *p*-nitroanisole demethylase activities. Therefore, this hydrocarbon was chosen as the inducing agent for the experiments described in this manuscript.

MATERIALS AND METHODS

Chemicals

Toluene (HPLC grade) and isooctane (HPLC grade) were purchased from Burdick & Jackson (Muskegon, MI). Dichloromethane (spectrophotometric grade) was purchased from EM Science (Gibbstown, NJ). Isopropanol was purchased from Mallinckrodt (Milwaukee, WI). Ethylbenzene was obtained from the Aldrich Chemical Co. (Milwaukee, WI). $NADP^+$, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, aminopyrine, and 7-ethoxycoumarin were purchased from the Sigma Chemical Co. (St. Louis, MO). Benzphetamine hydrochloride was a gift from the Upjohn Co. (Kalamazoo, MI). *p*-Nitroanisole was purchased from Kodak (Rochester, NY).

Animals

Seven-week-old male and female Holtzman rats weighing between 230–270 and 170–190 g, respectively, were obtained from Harlan Sprague Dawley, Inc. (Madison, WI). The rats were housed by sex in wire-bottomed stainless steel cages in a temperature- and light-controlled room (25°, 12 hr light/dark cycle). Rats were allowed to acclimate for 5 days prior to treatment, and were maintained on an unrestricted diet of rodent chow (Ralston Purina, St. Louis, MO) and water that was deionized and passed through an activated charcoal filter to remove potential contaminating hydrocarbons.

Hydrocarbon treatment of animals and preparation of liver microsomes

Rats were injected i.p. with ethylbenzene (2 M suspension in corn oil) for 3 days at a dose of 10 mmol/kg body weight. Control rats received daily injections of corn oil. Animals were allowed free access to food and water throughout the injection period and their body weights were recorded on the first day of injection and at the time of death. Animals were killed by decapitation 24 hr after the last injection. Livers were quickly removed and placed in cold 0.25 M sucrose. Microsomes were prepared by the method of Remmer *et al.* [16]. The washed microsomes were then suspended in 10 mM potassium phosphate buffer, pH 7.25. Microsomal protein content was determined by the biuret method [17], and cytochrome P450 levels were measured according to the method of Omura and Sato [18]. Cytochrome b_5 was determined from the reduced spectrum at 426 nm using a difference extinction coefficient of 111 $mM^{-1}\,cm^{-1}$ [18]. NADPH-cytochrome *c* reductase activity was determined at 37° in 100 mM potassium phosphate buffer, pH 7.25, by a modification of Yonetani [19]. NADH-ferricyanide reductase activity was measured at 37° in 100 mM phosphate buffer, pH 7.25 [20].

Cytochrome P450-dependent reactions

Rat liver microsomes at a concentration of 2 mg protein/mL (0.2 mg protein/mL for the 7-ethoxycoumarin assay) were used in each catalytic assay. Catalytic activities examined, their substrate concentrations, and reaction times were: aminopyrine N-demethylation, 5 mM, for 0–4 min [21, 22]; benzphetamine N-demethylation, 1 mM, for 0–4 min [21, 22]; *N*,*N*-dimethylnitrosamine N-demethylation (DMNA), 2 mM, for 0–8 min [21, 22]; *p*-nitroanisole O-demethylation, 0.4 mM, continuous spectral assays [23]; 7-ethoxycoumarin O-deethylation, 0.5 mM, for 0–4 min [24, 25]; and aniline *p*-hydroxylation, 5 mM, for 0–11 min [26]. The final concentrations of the assay components were: substrate (described above), 5 mM glucose-6-phosphate, 2 U/mL glucose-6-phosphate dehydrogenase, and 10 mM magnesium chloride in 100 mM potassium phosphate (pH 7.25). Reactions were initiated using $NADP^+$ or NADPH (*p*-nitroanisole demethylation) and terminated with 15% trichloroacetic acid.

Toluene metabolism

A saturated aqueous solution of toluene was prepared by adding excess toluene to deionized water, shaking vigorously, and then allowing the solution to stand for 2 days prior to use. The aqueous solution saturated with toluene was removed using a repipet bottle. The concentration of toluene in the saturated solution was determined to be 5.6 mM by measuring the UV absorbance at 262 nm ($\varepsilon_{262\,nm}$ = 0.237 $mM^{-1}\,cm^{-1}$). Rat liver microsomes at a concentration of 2 mg protein/mL were incubated

in sealed vials containing: 3 mM toluene, an NADPH-generating system (0.5 mM $NADP^+$, 5 mM glucose-6-phosphate, 2 U/mL glucose-6-phosphate dehydrogenase, and 10 mM $MgCl_2$), and 100 mM potassium phosphate buffer, pH 7.25, in a final volume of 2.0 mL. Following preincubation for 5 min at 37°, the reaction was started by adding $NADP^+$ to the mixture in the sealed reaction vials using a Hamilton syringe. The reaction was terminated after 5 min by the addition of 140 μL of 1 M HCl, immediately followed by addition of 100 μL of 18% NaCl. To each vial 36 nmol *p*-methylbenzyl alcohol was added to serve as an internal standard. The organic metabolites were extracted three times by addition of 800 μL of dichloromethane to the aqueous phase, shaking vigorously, and then centrifuging at low speed for 15 min. The organic phase was separated from the aqueous phase and dried with sodium sulfate. Metabolites in the organic phase were separated and quantified by isocratic elution using a mobile phase of 0.30% isopropranol in isooctane and were detected at 200 nm using a Waters HPLC system equipped with a 4.6 mm × 25 cm Dupont Zorbax SIL normal phase column (Mac-Mod Analytical Inc., Chadds Ford, PA). Metabolites were quantified using authentic standards and integration of the HPLC chromatograms was done using a Baseline 810 system (Millipore Corp., Milford, MA). The sample injection volume was 100 μL and the flow rate for the mobile phase was 1.6 mL/min. The extraction efficiencies for each of the products in these studies were 86, 70, 77 and 87% for *o*-cresol, *p*-cresol, benzyl alcohol and *p*-methylbenzyl alcohol (internal standard), respectively.

Measurement of cytochrome P450 protein levels by Western blotting

Rat liver microsomal proteins were separated electrophoretically on sodium dodecyl sulfate (SDS)-polyacrylamide gels (10% acrylamide). Following electrophoresis, the gels were transferred to nitrocellulose membranes (Costar, Cambridge, MA) by a modification of Towbin *et al.* [27]. Gels were first equilibrated for 15 min in a protein transfer buffer consisting of 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol. Microsomal proteins were next electrophoretically transferred from SDS–polyacrylamide gels onto the nitrocellulose sheets using an electrophoretic transfer cell (Bio-Rad, Melville, NY). After completion of the transfer process, non-specific binding sites were blocked by incubating the membranes in phosphate-buffered saline (PBS) containing 2% bovine serum albumin (BSA) for 1 hr at 25°. The blocked membranes were then washed in three successive changes of PBS containing 0.05% Tween 20 for 10 min each. Alterations in the levels of specific cytochrome P450 isozymes were measured by Western immunoblotting techniques using rabbit anti-rat polyclonal antibodies for cytochrome P450 1A1, P450 2B1, and P450 2E1 (Oxygene, Dallas, TX). Membranes were incubated for 2 hr at 25° with the antibody in the presence of PBS containing 5% goat serum, 2% BSA, and 0.05% Tween 20, followed by washing as described above. Detection of bound P450 antibodies to microsomal proteins was performed by incubating the membranes with blotting grade goat anti-rabbit IgG alkaline phosphatase conjugate (Bio-Rad) for 1 hr, followed by colorimetric determination with 5-bromo-4-chloro-3-indolylphosphate and *p*-nitro blue tetrazolium [28]. Blots were analyzed by densitometry using a Microcomputer Imaging Device (Imaging Research Inc., Ontario, Canada).

Statistical analysis

The effect of hydrocarbon exposure on cytochrome P450 content and cytochrome P450-dependent activities was analyzed using a fixed-effect one-way analysis of variance (ANOVA) test. Means and standard error of the mean are presented and specific differences between hydrocarbon-mediated and sex-related effects were determined with Tukey's multiple comparison test at the 0.05 significance level. All data were analyzed using a rank transformation to take account of the non-normality of the data. This transformation was performed because the standard errors were not normally distributed but were proportional to the magnitude of the rates obtained.

RESULTS

Male and female rats either given corn oil or ethylbenzene remained healthy in their appearance and maintained normal behavior throughout the study. Body weights increased to about the same extent for male and female rats receiving injections of corn oil, increasing by 10 and 7%, respectively, over the 3-day treatment period (data not shown). Conversely, animals that received ethylbenzene treatment showed decreased body weights. Body weights of males decreased by 4.5% while the weights of the females decreased to a lesser extent, 0.25% (data not shown).

Effect of ethylbenzene treatment on cytochrome P450 content and microsomal activities

Although liver cytochrome P450 content did not change in male rats that received ethylbenzene, the cytochrome P450 content in female rats doubled following hydrocarbon exposure (Table 1). The cytochrome P450 levels in females, however, were lower than those in the male rats for both control and ethylbenzene treatments. Other mixed-function oxidase components were also examined following ethylbenzene treatment. Cytochrome b_5 levels in females increased 67% over control levels; however, in males a smaller but insignificant increase was observed. Substantial increases were observed for both sexes in the activity of NADPH-cytochrome *c* reductase, increasing by 145% in females and by 68% in the males. NADH-ferricyanide reductase activity was also examined and did not show any differences following hydrocarbon exposure in either sex (data not shown).

Effect of ethylbenzene treatment on cytochrome P450-dependent activities

Table 2 summarizes the effect of ethylbenzene administration on different cytochrome P450-dependent activities. Two patterns of induction

Table 1. Effect of ethylbenzene exposure on cytochrome P450, cytochrome b_5, and NADPH-cytochrome *c* reductase

Treatment	N	Cyt. P450 (nmol P450/mg protein)	Cyt. b_5	Cyt. *c* reductase (nmol/min/mg protein)
Female rats				
Corn oil	6	0.25 ± 0.01	0.21 ± 0.01	126 ± 21
Ethylbenzene	6	0.50 ± 0.06*	0.35 ± 0.02*	308 ± 22*
Male rats				
Corn oil	4	0.58 ± 0.12†	0.29 ± 0.02	231 ± 25†
Ethylbenzene	5	0.69 ± 0.09	0.37 ± 0.01	387 ± 23*

Rats were treated with three daily i.p. injections of ethylbenzene at 10 mmol/kg in corn oil or an equivalent volume of corn oil as a control. Microsomes were then prepared, and cytochrome P450 and cytochrome b_5 levels and NADPH-cytochrome *c* reductase activity were determined as described in Materials and Methods. Data are shown as means ± SEM.

* Significant difference ($P < 0.05$) from corresponding control.

† Significant difference ($P < 0.05$) from corresponding female.

Table 2. Effect of ethylbenzene exposure on cytochrome P450-dependent activities

	P450 activity					
Treatment	Benzphetamine	7-Ethoxycoumarin	*p*-Nitroanisole	Aminopyrine	Aniline	DMNA
Female rats						
Corn oil (N = 6)	1.62 ± 0.18	0.82 ± 0.10	0.58 ± 0.054	1.70 ± 0.11	0.39 ± 0.04	0.50 ± 0.06
Ethylbenzene (N = 6)	7.30 ± 0.80*	2.61 ± 0.37*	2.47 ± 0.30*	4.43 ± 0.23*	0.98 ± 0.15*	1.24 ± 0.18*
Male rats						
Corn oil (N = 4)	6.19 ± 1.08†	1.96 ± 0.23†	0.88 ± 0.06†	5.95 ± 0.92†	0.69 ± 0.08†	0.65 ± 0.07
Ethylbenzene (N = 5)	10.7 ± 1.0*†	3.64 ± 0.49*	3.40 ± 0.54*	6.99 ± 0.65	0.94 ± 0.15	1.10 ± 0.23

Details for reaction conditions are given in Materials and Methods. Units for N-demethylation of aminopyrine, benzphetamine, and *N*,*N*-dimethylnitrosamine are: nmol of HCHO/min/mg protein; O-demethylation of *p*-nitroanisole is expressed as nmol of *p*-nitrophenol/min/mg protein; O-deethylation of 7-ethoxycoumarin is expressed as nmol of 7-hydroxycoumarin/min/mg protein; and the units for aniline *p*-hydroxylation are: nmol of *p*-aminophenol/min/mg protein. Data are shown as means ± SEM.

* Significant difference ($P < 0.05$) from corresponding control.

† Significant difference ($P < 0.05$) from corresponding female.

occurred for the activities examined. Benzphetamine N-demethylation, 7-ethoxycoumarin O-deethylation and *p*-nitroanisole O-demethylation were induced in both sexes after treatment with ethylbenzene. In females, benzphetamine N-demethylation was increased more than 4-fold over control levels, whereas a less than 2-fold increase was observed in males. A similar effect was seen with 7-ethoxycoumarin metabolism increasing by 3-fold and 2-fold in females and males, respectively. A slightly different pattern was observed with *p*-nitroanisole O-demethylation, with ethylbenzene producing 4-fold increases for both sexes. The rate for each of these activities was greater in male rats than in female rats prior to hydrocarbon treatment. After hydrocarbon treatment, only the rate of benzphetamine N-demethylation was significantly different from that of females treated with ethylbenzene (Table 2).

Three of the activities were induced exclusively in females after exposure to ethylbenzene. Aminopyrine *N*-demethylase, aniline *p*-hydroxylation, and dimethylnitrosamine N-demethylation were each significantly induced in female rats receiving ethylbenzene, with each of the activities increasing by about 2.5-fold over their respective controls.

Effect of ethylbenzene treatment on formation of toluene metabolites

Determination of the rate of toluene metabolism was performed *in vitro* to examine the ability of the hydrocarbon-inducible cytochrome P450 isozymes to hydroxylate aromatic hydrocarbons. A typical HPLC chromatogram showing the metabolites formed from the *in vitro* metabolism of toluene by microsomes from ethylbenzene-treated male rats is shown in Fig. 1. In the chromatograms from the ethylbenzene-treated rat three metabolites of toluene were identified. The primary metabolite was the result of the aliphatic hydroxylation of toluene to

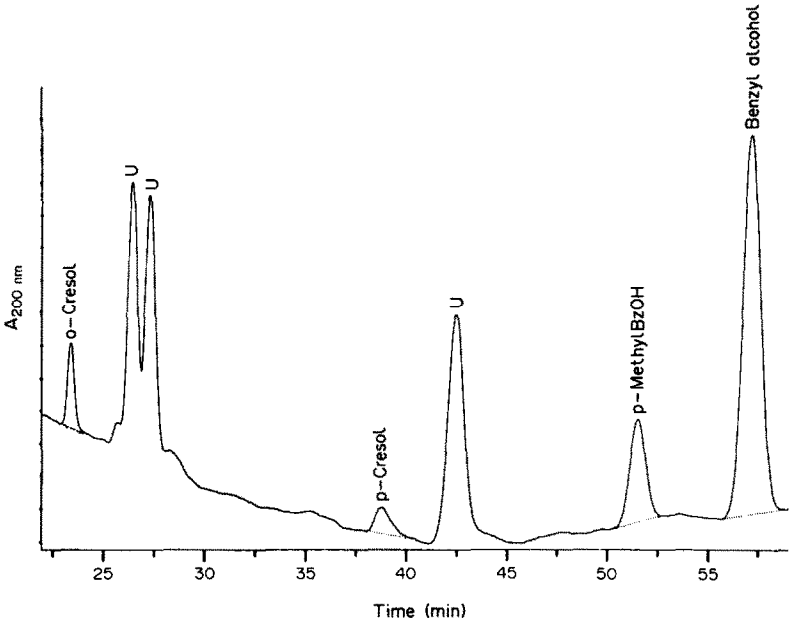

Fig. 1. HPLC chromatograph of toluene metabolites. Microsomes were prepared from rats treated with ethylbenzene as described in Materials and Methods. The chromatograph shows the results from *in vitro* toluene metabolism using microsomes of a male rat treated with ethylbenzene. Metabolites were separated by normal phase chromatography using the conditions described in Materials and Methods, and detected at 200 nm. The chromatograph shows the relative amounts of toluene metabolites formed from a 5-min incubation with 2 mg/mL microsomal protein. *p*-Methylbenzyl alcohol (7 mM) was used as an internal standard. U signifies unknown.

Table 3. Effect of ethylbenzene exposure on *in vitro* metabolism of toluene

Treatment	N	Toluene metabolite (nmol product/min/mg protein)		
		Benzyl alcohol	*o*-Cresol	*p*-Cresol
Female rats				
Corn oil	6	1.94 ± 0.07	0.11 ± 0.02	ND
Ethylbenzene	6	6.94 ± 0.78*	0.48 ± 0.09*	0.44 ± 0.08*
Male rats				
Corn oil	4	9.94 ± 1.50†	0.09 ± 0.03	ND
Ethylbenzene	5	12.0 ± 0.78†	0.83 ± 0.14*†	0.88 ± 0.17*†

Analytical procedures are described in Materials and Methods. Reactions were carried out for 5 min in sealed vials containing 2 mg/mL microsomal protein and 3 mM toluene. Toluene metabolites were detected at 200 nm. ND = not detectable. Data are shown as means ± SEM.

* Significant difference ($P < 0.05$) from corresponding control.

† Significant difference ($P < 0.05$) from corresponding female.

form benzyl alcohol. Smaller concentrations of two aromatic hydroxylation products, *o*- and *p*-cresol, were also observed in ethylbenzene-treated male rats. By contrast, corn oil-treated rats revealed only two metabolites; benzyl alcohol and a very small amount of *o*-cresol (Table 3). No *m*-cresol (retention time =37.5 min) was detected in any of the groups examined. Each of these products co-eluted with authentic standards using both normal phase and reversed phase separations. Changes in the formation of each of these metabolites were quantified and are presented in Table 3.

Benzyl alcohol formation. The rate of benzyl alcohol formation in females given ethylbenzene was increased more than 3.5-fold over the rate of those receiving corn oil; however, ethylbenzene treatment did not increase the rate of benzyl alcohol formation in male rats (Table 3). These results indicate that

female rats were more sensitive to hydrocarbon-mediated induction of benzyl alcohol production than were males. Despite the apparent increased hydrocarbon inducibility in female rats, the total rates of benzyl alcohol formation in females remained lower than those in males.

Ortho-*cresol formation.* Untreated rats were capable of producing small amounts of *o*-cresol from toluene, which was increased in both sexes following exposure to ethylbenzene. Rates of *o*-cresol formation increased to a much greater extent than did those of aliphatic hydroxylation following ethylbenzene exposure. For females treated with ethylbenzene, the rate of *o*-cresol formation increased 4-fold (Table 3). Males treated with ethylbenzene produced an even larger effect. In contrast to their lack of enhanced aliphatic hydroxylation, the formation of *o*-cresol from toluene was increased 9-fold over control levels in males (Table 3). This rate was about 2-fold greater than that occurring for females receiving ethylbenzene, and as with the formation of benzyl alcohol, the overall rate of *o*-cresol formation in females was lower than that in males. Control groups, however, did not show significant differences in the rate of *o*-cresol formation from toluene.

Para-*cresol formation.* Prior to hydrocarbon exposure, aromatic hydroxylation to produce *p*-cresol did not contribute to overall toluene metabolism (Table 3). Ethylbenzene exposure increased the rate of *p*-cresol formation over the control levels in both males and females. The rate increased from undetectable levels to a rate of 0.44 nmol/min/mg protein for females, and 0.88 nmol/min/mg protein in males following hydrocarbon exposure (Table 3). As with formation of *o*-cresol, after treatment with ethylbenzene the rate of *p*-cresol formation in male rats was almost 2-fold higher than that found in females.

Effect of ethylbenzene exposure on expression of P450 1A1, P450 2B1, and P450 2E1

The levels of specific isozymes of cytochrome P450 were examined to determine if the induction of catalytic activities by aromatic hydrocarbons was attributable to increased levels of particular P450 isozymes. The microsomal proteins were probed with polyclonal antibodies to some of the more commonly induced forms of cytochrome P450, namely, P450 2B1, P450 2E1, and P450 1A1 (Figs. 2–4). Western immunoblotting revealed that cytochrome P450 2B1 was induced substantially in both male and female rats after exposure to ethylbenzene. The levels of P450 2B1 in the male and female control groups were similar; however, following treatment with ethylbenzene, males showed greater expression of this isozyme (Fig. 2 and Table 4). Intense bands for protein immunoreacting with anti-P450 2B1 appeared after ethylbenzene treatment for both sexes but not with

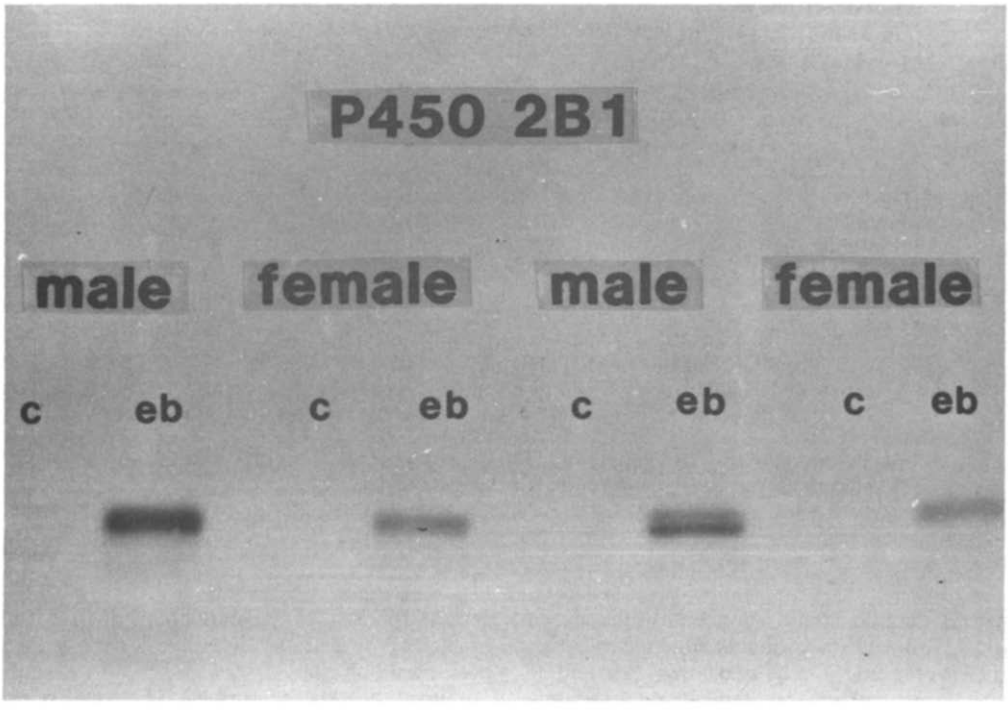

Fig. 2. Effect of ethylbenzene treatment on the expression of P450 2B1. Microsomes from rats given corn oil or ethylbenzene were prepared as described in Materials and Methods, and Western immunoblotting was performed using rabbit anti-rat P450 2B1 polyclonal antibody. Each lane contained 5 μg of hepatic microsomal protein from different male or female rats treated with corn oil or ethylbenzene.

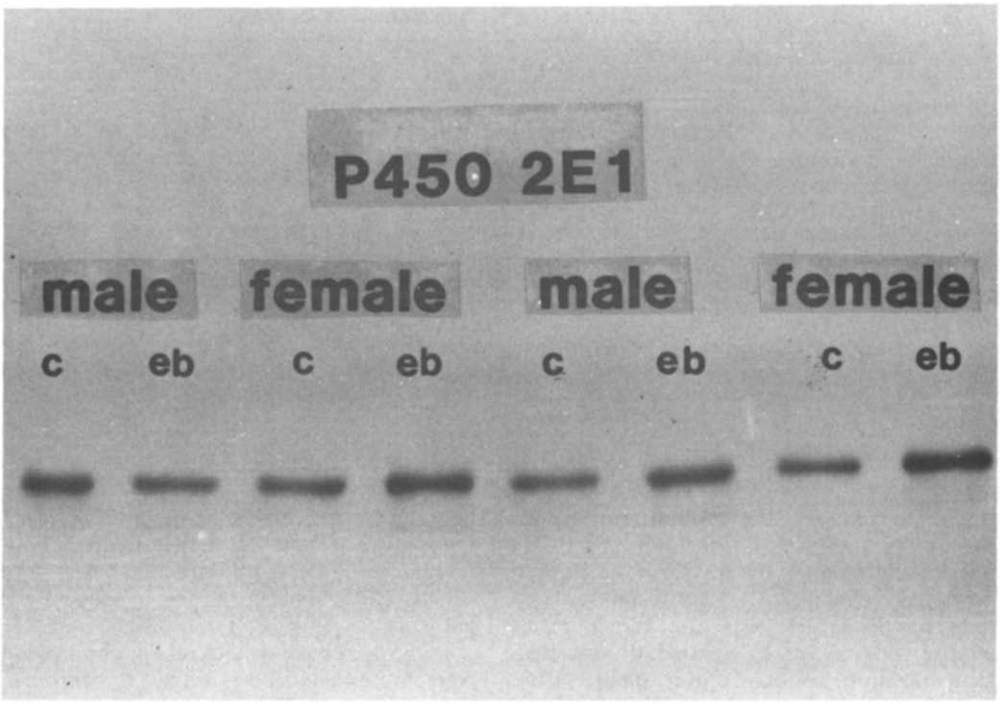

Fig. 3. Effect of ethylbenzene treatment on the expression of P450 2E1. Microsomes from rats given corn oil or ethylbenzene were prepared as described in Materials and Methods, and Western immunoblotting was performed using rabbit anti-rat P450 2E1 polyclonal antibody. Each lane contained 5 μg of hepatic microsomal protein from different male or female rats treated with corn oil or ethylbenzene.

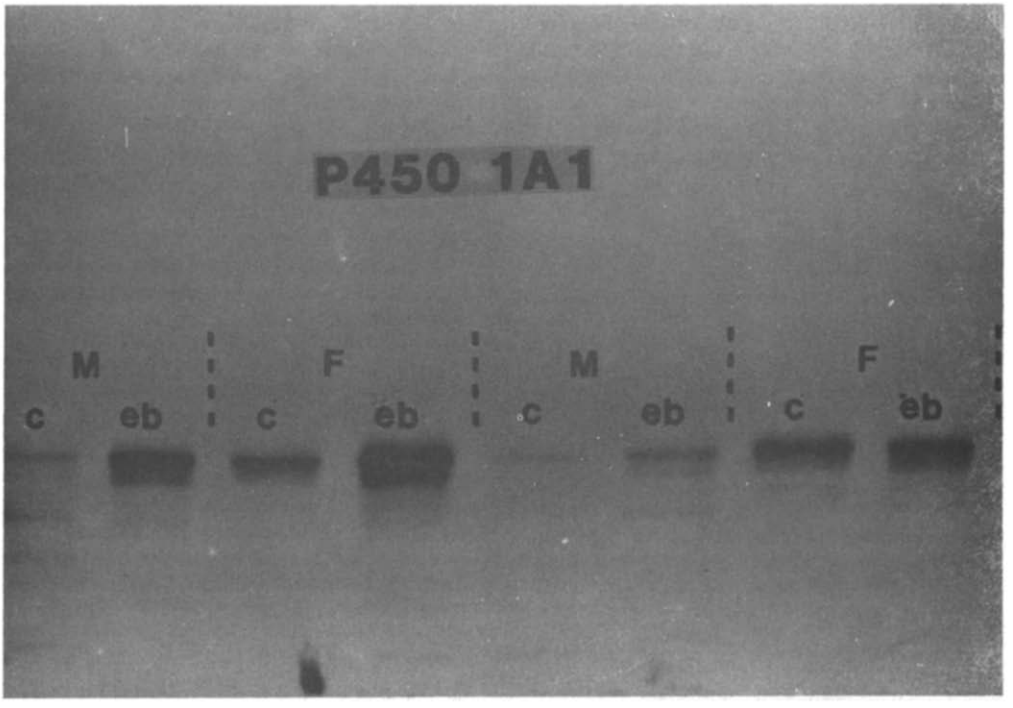

Fig. 4. Effect of ethylbenzene treatment on the expression of P450 1A1. Microsomes from rats given corn oil or ethylbenzene were prepared as described in Materials and Methods, and Western immunoblotting was performed using rabbit anti-rat P450 1A1 polyclonal antibody. Each lane contained 3 μg of hepatic microsomal protein from different male or female rats treated with corn oil or ethylbenzene.

Table 4. Densitometry of Western blotting experiments

Treatment	N	P450 1A1	P450 2B1	P450 2E1
Female rats				
Corn oil	6	1.00 ± 0.29	1.00 ± 0.55	1.00 ± 0.06
Ethylbenzene	6	1.51 ± 0.47	25.9 ± 4.15*	1.42 ± 0.13*
Male rats				
Corn oil	4	0.48 ± 0.34	0.75 ± 0.33	1.11 ± 0.09
Ethylbenzene	5	1.09 ± 0.48	46.8 ± 4.61*†	1.38 ± 0.11

Western blots using microsomes from rats treated with corn oil or ethylbenzene were prepared as described in Materials and Methods. Densitometry was performed, and results have been normalized to control females. Data are shown as means ± SEM.
* Significant difference ($P < 0.05$) from corresponding control.
† Significant difference ($P < 0.05$) from corresponding female.

the untreated groups (Fig. 2). These results demonstrate that the level of cytochrome P450 2B1 was substantially induced by ethylbenzene in both sexes with males exhibiting a larger response.

Cytochrome P450 2E1 was also shown to be induced by ethylbenzene treatment; however, significant induction was found only in females. Blots from male livers did not produce significant increases in the amount of immunoreacting P450 2E1 protein. The levels of immunoreacting P450 2E1 protein were examined by densitometry (Table 4). These results illustrate that P450 2E1 protein levels in females were induced after exposure to ethylbenzene. A representative Western blot for P450 2E1 is shown in Fig. 3.

Cytochrome P450 1A1 was not induced by ethylbenzene exposure in either sex. The Western blots for P450 1A1 showed a great deal of variability within groups for both sexes, as shown in Fig. 4. Both the control and treatment groups varied in their expression of P450 1A1, and densitometry of blots from each liver did not reveal any substantial induction for either sex (Table 4).

DISCUSSION

The interaction of aromatic hydrocarbons with cytochrome P450 has been shown to produce different patterns of induction for hepatic microsomal electron transport components, P450-dependent activities, and hydrocarbon metabolism. Exposure of rats to ethylbenzene seemed to affect the overall content of a number of microsomal electron transport components differently depending upon the sex of the animal. Ethylbenzene treatment had no significant effect on cytochrome P450 or cytochrome b_5 levels in male rats; however, P450 levels doubled and the level of cytochrome b_5 increased 70% in females (Table 1). A limited number of studies have investigated the effects of aromatic hydrocarbons on cytochrome P450 isozymes. Studies with benzene have not shown induction of hepatic cytochrome P450 levels [14, 29]. Some investigators have reported toluene and xylenes to increase cytochrome P450 content [14, 30, 31]; however, others have not demonstrated this effect [32].

Cytochrome *c* reductase activity following ethylbenzene treatment was increased in both male and female rats (Table 1). Other hydrocarbons (e.g. toluene and xylenes) have been shown to produce similar effects on cytochrome *c* reductase activity [14, 15].

In the present study different patterns of induction were observed with various cytochrome P450-dependent activities following exposure to an aromatic hydrocarbon. Depending on the activity in question, induction following ethylbenzene treatment may be common to both sexes or occur in only one sex. Metabolism of the drugs benzphetamine, 7-ethoxycoumarin, and *p*-nitroanisole, and aromatic hydroxylation of toluene was induced after treatment with ethylbenzene in both male and female rats. On the other hand, aminopyrine N-demethylation, aniline *p*-hydroxylation, dimethylnitrosamine N-demethylation, and aliphatic hydroxylation of toluene to benzyl alcohol were selectively induced in female rats following hydrocarbon exposure.

Induction of certain cytochrome P450-dependent activities has been associated with gender. Studies have been performed on rats examining sex-dependent differences in drug metabolism [15, 33–39]. Evidence has also been presented suggesting that the magnitude of induction of cytochrome P450-dependent activities can be influenced by gender. Kato and Takayanaghi [40] studied the effect of 3-methylcholanthrene on the activity of liver microsomal drug-metabolizing enzymes in male and female rats. Their study indicates that induction of cytochrome P450 by this inducer promotes a different effect in male rats than that occurring in females. Activities that displayed relatively small sex differences in the absence of an inducing agent were increased by 3-methylcholanthrene in both sexes; however, those activities displaying clear sex differences were decreased in male rats but not affected in female rats [40, 41]. Pyykkö [15] has reported sex-dependent induction of microsomal drug-metabolizing activities by toluene treatment. Aryl hydrocarbon hydroxylase activity was greater in male rats but was not induced by toluene. On the other hand, exposure to toluene did promote induction of aryl hydrocarbon hydroxylase activity in the female rats. Thus, the gender of the animal

influences induction of this activity. Aminopyrine *N*-demethylase, aniline hydroxylase and 7-ethoxycoumarin O-deethylation were also examined in this study; however, unlike the gender-selective induction associated with aryl hydrocarbon hydroxylase, these activities were induced equally in both sexes. We have demonstrated that treatment of rats with ethylbenzene also produced a gender selective induction of certain cytochrome P450-dependent activities (Table 2). Like toluene, ethylbenzene produced an equivalent degree of induction of 7-ethoxycoumarin metabolism in both sexes. However, in contrast to the effect of toluene on the metabolism of aminopyrine, ethylbenzene treatment selectively induced this activity in females (Table 2). In addition, toluene metabolism via aliphatic hydroxylation was induced only in females (Table 3). Aromatic hydroxylation was induced in both sexes; however, ethylbenzene treatment more highly influenced the formation of cresols in the male rats (Table 3).

Of the activities that were increased in females, aniline hydroxylation and DMNA are particularly interesting because they are closely associated with cytochrome P450 2E1 [42–44]. Since these activities were enhanced only in females that received ethylbenzene, it is possible that P450 2E1 may be selectively induced. Based upon the results of Western immunoblotting this does appear to occur following exposure to ethylbenzene (Fig. 3). Microsomes from female rats treated with ethylbenzene had higher amounts of immunoreacting protein to anti-rat P450 2E1 than their male counterparts. The results suggest that females are more susceptible to ethylbenzene-mediated induction of cytochrome P450 2E1 isozyme than are males.

Cytochrome P450 2E1 was not the only isozyme induced by ethylbenzene. Cytochrome P450 2B1 was also induced following ethylbenzene treatment. Furthermore, this induction occurred for both sexes. Proteins that immunoreacted with antibody to cytochrome P450 2B1 were increased in both male and female rats following exposure to ethylbenzene (Fig. 2). Cytochrome P450 1A1 was also examined and did not appear to exhibit a sex difference; ethylbenzene treatment did not increase significantly P450 1A1 expression in either male or female rats even though a small increase was observed (Fig. 4).

The most dramatic effects of ethylbenzene exposure occurred for hydrocarbon metabolism. Male and female rats showed induction of hydrocarbon metabolism following exposure to another aromatic hydrocarbon. Pronounced formation of aromatic hydroxylated metabolites of toluene occurred in both sexes following ethylbenzene exposure. Prior to hydrocarbon exposure, cresol formation accounted for less than 1% of toluene metabolites in either sex. However, after exposure to ethylbenzene, formation of cresols increased to approximately 15% of total toluene metabolites in both sexes. Formation of *o*-cresol was enhanced by 9-fold in male rats and by 4-fold in females that received ethylbenzene (Table 3). Even larger effects of hydrocarbon treatment were seen in the formation of *p*-cresol (Table 3).

We can begin to identify the isozymes involved in hydrocarbon-mediated induction of P450-dependent drug and hydrocarbon metabolism based on our data as well as recent literature reports [32, 45]. According to the study of Nakajima *et al.* [45], cytochrome P450 isozymes were found to metabolize toluene by different routes of hydroxylation. Using monoclonal antibodies to inhibit the metabolism of toluene, the found that cytochrome P450 2C11/2C6 was responsible for benzyl alcohol formation and, to a smaller degree, *p*-cresol. Cytochrome P450 2E1 was also found to metabolize toluene to benzyl alcohol; however, this was only at low concentrations of toluene. Cytochrome P450 2B1/2B2 metabolized toluene to benzyl alcohol, and to both *o*- and *p*-cresol. In this regard, the larger increase of 2B1 protein in males treated with ethylbenzene would explain their greater rates of cresol formation (Fig. 2, Table 3). The sex difference that occurred for the formation of benzyl alcohol could be related, at least in part, to suppression of P450 2C11 in males exposed to ethylbenzene. This is possible considering that cytochrome P450 2C11 is a male-specific isozyme [46]. This means that untreated male rats would presumably have a least two isozymes contributing to benzyl alcohol formation: P450 2C11 and the low constitutive levels of P450 2B1/2B2 as the major forms contributing to this metabolite. On the other hand, untreated female rats do not have P450 2C11 accounting for lower levels of benzyl alcohol. If 2B1/2B2 was induced by ethylbenzene, then the change in benzyl alcohol formation would be increased in both males and females. However, if ethylbenzene suppressed 2C11 and induced 2B1/2B2, then in male rats only a small change in benzyl alcohol might be expected. This is what appears to occur following treatment with ethylbenzene (Table 3).

Recently, additional evidence has shown that male rats treated with ethylbenzene have decreased levels of cytochrome P450 2C11 [32]. Consistent with this study, ethylbenzene also increased P450 2B1 while having no significant effect on P450 1A1. However, an ethylbenzene-mediated increase in the level of cytochrome P450 2E1 expression was reported [32], which is not consistent with the lack of effect shown herein for male rats (Fig. 3, Table 4). Presently, it is not known why such a difference would occur but it may be due to dietary or strain differences in the expression of this isozyme. In any event, the levels of isozymes as determined by Western immunoblotting and densitometry indicate that in males treated with ethylbenzene no significant increase in cytochrome P450 2E1 was observed.

Ethylbenzene treatment appeared to produce multiple effects on hepatic drug and hydrocarbon metabolism. Some activities were selectively induced in female rats, including cytochrome P450 2E1 metabolism of aniline and DMNA, with the observed increase in P450 2E1 comparable to the change in catalytic activity. A number of studies have investigated the expression of P450 2E1 in the rat. The level of P450 2E1 in female rats has been reported to be twice that of males [47]. However, other investigators have indicated that there is not a significant sex difference in the expression of P450 2E1 [48]. In Fig. 3 we demonstrate that ethylbenzene treatment increased P450 2E1 in female rats but did

not increase significantly the level of this isozyme in male rats.

Induction of P450 2E1 is thought to involve a number of mechanisms. Studies using ethanol as the inducer indicate that P450 2E1 protein is increased without an increase in P450 2E1 mRNA [49–51], an effect proposed to be due to increased translation rate. Other studies have shown that induction of P450 2E1 in diabetic rats is due to mRNA stabilization [52]. Moreover, inducers such as acetone and pyrazole are proposed to induce P450 2E1 by stabilization of protein [53]. The mechanism of ethylbenzene-mediated induction of P450 2E1 remains to be determined.

In summary, of the isozymes examined in this study, P450 2B1/2B2 was induced to the greatest extent by ethylbenzene treatment. This isozyme also appears to be induced to a greater extent in male rats as evidenced by the differential changes in immunoreactive protein levels (Table 4). Changes in aromatic hydroxylation of toluene are also consistent with the greater induction of P450 2B1, where larger increases were observed in male rats for the production of both *o*- and *p*-cresol (Table 3). Literature reports [45] are consistent with P450 2B1/2B2 as isozymes responsible for the aromatic hydroxylation of toluene. However, the effects of ethylbenzene cannot be explained simply by the greater induction of 2B1/2B2 in male rats. Other activities normally attributed to these isozymes were not induced to a greater extent in male rats. Benzphetamine demethylation was increased to a greater extent in female rats (Table 2), whereas aliphatic hydroxylation of toluene was selectively increased in females (Table 3). Since both of these activities have been reported to be catalyzed by P450 2B1/2B2 [45, 46], the changes in these activities are inconsistent with the greater induction of P450 2B1/2B2 (Table 4), unless ethylbenzene also mediates the suppression or inactivation of other forms responsible for the metabolism of these substrates. The results are consistent with the suppression of the male-specific isozyme P450 2C11, in that this isozyme has also been shown to metabolize both benzphetamine and toluene (to benzyl alcohol) and can adequately explain the apparent discrepancy between the greater induction of some 2B1/2B2-dependent activities in males and other 2B1/2B2-dependent activities in females. The induction of P450 2B1 could also be responsible for the increase in 7-ethoxycoumarin deethylation [54], which is generally regarded as a P450 1A1 activity [46].

In addition to the greater induction of P450 2B1/2B2 in male rats, there appears to be a selective induction of P450 2E1 in female rats. Although the effect appears to be small by comparison to the effects on 2B1/2B2 levels, significant differences were observed in female rats both in protein levels (Table 4), as well as some P450 2E1-dependent activities (Table 2).

From a toxicological standpoint, induction of overall toluene metabolism might be expected to increase the rate of elimination of this compound. However, the dramatic increase in the production of *o*-cresol and *p*-cresol after ethylbenzene exposure may point to an increased risk for individuals exposed to aromatic hydrocarbons, particularly if the aromatic hydroxylations are catalyzed via formation of reactive epoxide intermediates. Thus, differences in cytochrome P450 isozyme profiles resulting from aromatic hydrocarbon exposure may have a significant influence on drug and hydrocarbon turnover as well as bioactivation reactions for numerous xenobiotics.

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