Genetic Polymorphism of Microsomal Epoxide Hydrolase Activity in the Mouse*

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Hepatic microsomal epoxide hydrolase activity (EC 3.3.2.3), assayed using styrene oxide as the substrate, has a pH optimum of 9.5 from C57BL/6J mice and a pH optimum of 8.7 from DBA/2J mice. In cross and backcross matings between C57BL/6J and DBA/2J mice, this phenotypic difference in hepatic hydrolase activity is inherited as a single autosomal trait, with co-dominant expression in heterozygotes. Heating liver microsomes from C57BL/6J and DBA/2J mice at 62°C for 30 min produced a slight decrease in enzyme activity, whereas the same treatment of DBA/2J microsomes reduced enzyme activity to less than 3% of its initial value. Twenty-six recombinant inbred strains derived from C57BL/6J and DBA/2J mice, respectively. Using 24 recombinant inbred strains derived from C57BL/6J and DBA/2J mice, Eph-1 is proposed as the locus symbol of the structural gene for microsomal epoxide hydrolase, with superscripts b and d designating the alleles carried by C57BL/6J and DBA/2J mice, respectively. Using 24 recombinant inbred strains derived from C57BL/6J and DBA/2J mice, Eph-1 was found to be linked to two loci on Chromosome 1.

Many synthetic and some naturally occurring alkene and arene compounds are oxidized by the microsomal monoxygenase enzymes to epoxide metabolites (1). Some of these epoxides are reactive electrophiles which can bind covalently to cellular macromolecules and produce toxic, mutagenic, and/or carcinogenic effects (2). Epoxides can be converted enzymatically to dihydrodiols by epoxide hydrolase or conjugated with glutathione either nonenzymatically or catalytically by the glutathione-S-transferases (3). Thus, these enzymes play an important role in controlling the level of potentially hazardous epoxide compounds.

Microsomal epoxide hydrolase activity (EC 3.3.2.3), although present at highest levels in the liver, is found in almost all tissues that have been examined (4). The hepatic enzyme activity is increased by the administration of a variety of compounds: phenobarbital (5), 2-acetylaminofluorene (6), trans-stilbene oxide (7), and 2(3)-t-butyl-4-hydroxyanisole (BHA) (8). In mice, BHA is particularly effective, producing an 11-fold increase in hepatic microsomal epoxide hydrolase activity.

Genetic differences in the ability to metabolize drugs have been noted in humans and rodents (9), and several single locus polymorphisms are known in mice, e.g. the Ah locus (10), and the Coh locus (11). Differences in hepatic epoxide hydrolase activity between inbred strains of mice have been noted, but not further characterized (12, 13). In light of the important role hepatic microsomal epoxide hydrolase plays in drug metabolism, we undertook the present study to look for strain differences in this enzyme. We now report a genetic polymorphism in this enzyme activity.

EXPERIMENTAL PROCEDURES

Materials—Styrene oxide was purchased from Aldrich Chemical Co., Milwaukee, WI; [7-3H]styrene oxide (92% pure by gas-liquid chromatography analysis, specific activity 30 mCi/mmol) was purchased from Amershams Corp., Arlington Heights, IL. Benzo(a)pyrene, 4,5-oxide, uniformly tritium-labeled benzo(a)pyrene-4,5-oxide (purity > 80% by thin layer chromatography, specific activity 356 mCi/mmol), and benzo(a)pyrene-trans-4,5-dihydroidiol were obtained from the National Cancer Institute Chemical Repository, Bethesda, MD.

BHA, Tween 80, and bovine serum albumin were purchased from Sigma Chemical Co., St. Louis, MO. MOPS buffer was purchased from Calbiochem, La Jolla, CA. Thin layer chromatography plates (Whatman LK5DF) were purchased from Kontes Glass Co., Vineland, NJ. A 1% BHA diet was prepared by Teklad Test Diets, Madison, WI, by adding BHA to powdered Wayne Mouse Breeder Blox (Allied Mills, Chicago, IL) and then repelleting the diet. A control diet was prepared by the same method but without BHA.

Animals—Mice were obtained from the production and research stocks of the Jackson Laboratory, Bar Harbor, ME. The recombinant inbred (RI) strains of mice (designated BXD) were derived by in-breeding the F2 generation of C57BL/6J and DBA/2J mice (14). The mice used in these studies were 9 to 12 weeks old female mice unless otherwise noted. In experiments involving BHA, the mice were placed on the 1% BHA diet for an 8-day period prior to killing. All mice were given food and water ad libitum.

Preparation of Microsomes—Mice were killed by cervical dislocation. Livers were removed, weighed, and homogenized in 3 volumes of 0.15 M KCl with a glass homogenizer fitted with a Teflon pestle. The homogenate was then centrifuged at 10,000 × g for 20 min at 4°C. The resulting postmitochondrial supernatant was centrifuged for 1 h at 100,000 × g at 4°C. The microsomal pellet was rinsed twice and then resuspended in 0.25 M sucrose to a concentration equivalent to 1 g wet weight liver/ml of sucrose. Microsomes were either assayed immediately for epoxide hydrolase activity or frozen at −70°C for subsequent analysis.

The abbreviations used are: BHA, 2(3)-t-butyl-4-hydroxyanisole; MOPS, morpholinopropanesulfonic acid; RI, recombinant inbred.

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later assaying. No change in enzyme activity was observed on storage for up to 2 months.

Buffers—The following buffers were used: Buffer A, (125 mM glycylglycine, 125 mM glycine) with 0.1% Tween 80 added; Buffer B, (83 mM MOPS, 83 mM glycylglycine, 83 mM glycine) with 0.1% Tween 80 added; Buffer C, (4.17 mM MOPS, 4.17 mM glycylglycine, 4.17 mM glycine) with 0.1% Tween 80 added. These buffers were titrated with 1 N sodium hydroxide to the required pH measured at 37°C.

Assays of Epoxide Hydrolase Activity—Microsomal epoxide hydrolase activity was assayed routinely using [7-3H]styrene oxide as a substrate by the method of Oesch et al. (5) with modifications of Seidegard et al. (15) and Jerina et al. (16). The assay was performed in 16 mm round bottom, stoppered glass test tubes to prevent evaporation of the substrate. The reaction mixture consisted of 25 µl of buffer, 50 µl of a suspension of microsomes (containing 20 to 150 µg of protein), and 5 µl of tetrahydrofuran containing 200 nmoles of [7-3H]-styrene oxide (100,000 dpm/assay) (final concentration 2.5 mM). The reaction was started by the addition of the substrate and the tubes were then incubated in a shaking water bath at 37°C for 15 min. Sample blanks received an additional 25 µl of tetrahydrofuran prior to addition of substrate. The reaction was stopped by the addition of 5 ml of ice-cold petroleum ether (b.p. 40-60°C) followed by rapid blending on a Vortex mixer for 15 s. The product, [7-3H]styrene glycol, is then extracted by the method of Oesch et al. (5) and counted by liquid scintillation spectrometry. Product formation is linear with respect to both time and protein concentration. Values obtained are not corrected for recovery of styrene glycol in the extraction procedures. Enzyme activity is expressed as nanomoles of styrene glycol per mg of protein per min. To assure a low zero time blank, the [7-3H]styrene oxide (stored in petroleum ether) is extracted with water periodically to remove water-soluble degradation products which accumulate during storage (15).

Epoxide hydrolase activity was measured using benzo(a)pyrene-4,5-oxide as the substrate by the thin layer chromatography method of Jerina et al. (16) with the following modifications. The buffers used in this assay were the same ones used in the styrene oxide assay (see “Buffers”). Benzo(a)pyrene-4,5-oxide was dissolved in tetrahydrofuran instead of acetonitrile. The reaction was run for 5 min during which product formation was linear with both time and protein concentration.

Protein Measurements—Proteins were determined by the method of Lowry et al. (17) using bovine serum albumin as a standard.

Heat Stability Experiments—Liver microsomes from BHA-treated mice were diluted with 225 mM sucrose, 10 mM potassium phosphate buffer (pH 7.1) and then put into test tubes. The microsomes were heated in a water bath at 62°C for varying time periods, then placed immediately in an ice water bath. After cooling, the microsomes were added to the glass-stoppered test tubes, the pH was adjusted to pH 8.7 with Buffer A and epoxide hydrolase activity was determined. Control microsomes were treated in the same manner except they were not heated.

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Fig. 1. Effect of pH on hepatic microsomal epoxide hydrolase activity. A, in liver microsomes from control and BHA-treated C57BL/6J and DBA/2J mice. Epoxide hydrolase activity was measured in pooled liver microsomes (three mice/group) using Buffer B as described under “Experimental Procedures.” B, in liver microsomes from several mouse strains. Epoxide hydrolase activity was measured in liver microsomes from individual BHA-treated mice and is expressed as the per cent of the enzyme activity observed at the pH optimum. Specific activities (nanomoles of styrene glycol/mg of protein/min) at the pH optima were: pH 8.7: A/J, 41.4; BUB/BlN, 57.3; CBA/J, 40.8; pH 9.5: AKR/J, 73.7; BALB/cByJ, 38.5; C3H/HeJ, 43.2. Pooled data from two experiments.

Fig. 2. Hepatic microsomal epoxide hydrolase activity in microsomes from BHA-treated C57BL/6J and DBA/2J mice, and in BHA-treated offspring from the genetic crosses of these mice. Epoxide hydrolase activity (measured with Buffer A) is expressed here as the ratio of enzyme activity at pH 9.5/pH 8.7. The numbers shown in the figure are the total number of mice in each group; mice of both sexes were used in this experiment.
RESULTS

The hydration of styrene oxide to styrene glycol, catalyzed by hepatic microsomal epoxide hydrolase, has a pH optimum of 9.5 in C57BL/6J mice and of 8.7 in DBA/2J mice (Fig. 1A). Feeding a 1% BHA diet to these mice increases the level of enzyme activity in both strains 6 to 9-fold, but does not alter the pH optima. B6D2F1/J mice have a pH curve that is a composite of the parent strains with an intermediate pH optimum of 9.1 (data not shown).

The pH curves of hepatic microsomal epoxide hydrolase activity in six other inbred mouse strains are seen in Fig. 1B. AKR/J, BALB/cByJ, and C3H/HeJ mice have pH optima of 9.5 (like C57BL/6J mice) whereas A/J, BUB/BnJ, and CBA/J mice have pH optima of 8.7 (like DBA/2J mice) for this enzyme activity.

To determine whether the low enzyme activity in DBA/2J microsomes at pH 9.5 resulted from denaturation of the enzyme, we incubated the microsomes at pH 9.5 (Buffer C) for 30 min at 37°C, and then adjusted the pH to 8.7 (Buffer A). Epoxide hydrolase activity was the same as that in microsomes maintained at pH 8.7, indicating that the decline in enzyme activity seen when shifting from pH 8.7 to 9.5 is reversible. With benzo[a]pyrene-4,5-oxide as the substrate, the hepatic epoxide hydrolase activity from both C57BL/6J and DBA/2J mice had broad pH optima from pH 7.1 to 8.3, but the activity in DBA/2J microsomes fell off more rapidly at a more alkaline pH range, 9.1 to 9.5, than did the activity from C57BL/6J microsomes (data not shown).

Genetic Segregation of Microsomal Epoxide Hydrolase Activity—The inheritance pattern of hepatic microsomal epoxide hydrolase activity in the BHA-treated offspring of genetic crosses of C57BL/6J, DBA/2J, and B6D2F1/J mice is shown in Fig. 2. Hepatic microsomal epoxide hydrolase activity was measured in each animal at both pH 9.5 and at pH 8.7 and is expressed as the ratio of enzyme activity at pH 9.5/pH 8.7. This ratio value is typically 1.2 to 1.7 for C57BL/6J mice, 0.1 to 0.4 for DBA/2J mice, and intermediate, 0.5 to 1.0, for B6D2F1/J mice. Backcrossing of B6D2F1/J mice to DBA/2J mice produced two classes of offspring (low and intermediate enzyme activity ratios) in an approximately 1:1 distribution. Back-crossing of B6D2F1/J mice to C57BL/6J mice produced an equal number of offspring in the intermediate and high ratio classes. The B6D2F1/J mice fell into three classes (low, intermediate, and high ratios) in an approximately 1:2:1 distribution (p ≥ 0.2). The data are consistent with the hypothesis that there are two alleles of the microsomal epoxide hydrolase gene at one locus with co-dominant expression in heterozygotes.

Heat Stability—Differences in the primary structure of a protein are most likely to be detected as changes in thermo-

![Fig. 3. Heat stability of hepatic microsomal epoxide hydrolase activity. A, in microsomes from C57BL/6J, DBA/2J, and B6D2F1/J mice. Epoxide hydrolase activity was measured in pooled liver microsomes (five to six mice/group) from BHA-treated mice. The “mixture of C57BL/6J + DBA/2J” is a 1:1 mixture (v/v) of microsomes from these two strains. Initial enzyme activities (nanomoles of styrene glycol/mg of protein/min) were: C57BL/6J, 39.6; DBA/2J, 44.4; B6D2F1/J, 49.7; and mixture of C57BL/6J + DBA/2J, 42.3. B, heat stability of epoxide hydrolase activity in microsomes from C57BL/6J, DBA/2J, and B6D2F1/J mice measured with both styrene oxide (—) and benzo[a]pyrene-4,5-oxide (○) as substrates. Enzyme activity was measured in the same microsomes used in 3A. Combined data from two experiments.

### TABLE I

| Strain distribution of microsomal epoxide hydrolase alleles | pH optimum 9.5 sensitive to heat denaturation | pH optimum 8.7 sensitive to heat denaturation |
|-----------------------------------------------------------|---------------------------------------------|---------------------------------------------|
| Strain                                      | Eph-1| Strain                                      | Eph-1|
| AKR/J                                      | MA/MyJ | A/J                                      | A/HeJ |
| A1/SsJ                                      | PL/J   | BUB/BnJ                                    | CBA/J |
| BALB/cByJ                                   | RIIS/J | C3H/HeJ                                    | CBA/J |
| C3H/HeJ                                    | RF/J   | C57BL/6J                                   | CBA/GnJ|
| C57BL/6J                                   | SEA/GnJ | C57BL/KsJ                                  | DBA/2J |
| C57BR/cdJ                                  | SEC/1ReJ | C57BR/cdJ                                  | DBA/1J |
| C57L/J                                     | SJL/J  | C57L/J                                    | DBA/1J |
| HRS/J                                      | 129/J  | HRS/J                                    | 129/J |
| LG/J                                       | 85     | LG/J                                    | 85    |

3. Heat stability of hepatic microsomal epoxide hydrolase activity. A, in microsomes from C57BL/6J, DBA/2J, and B6D2F1/J mice. Epoxide hydrolase activity was measured in pooled liver microsomes (five to six mice/group) from BHA-treated mice. The “mixture of C57BL/6J + DBA/2J” is a 1:1 mixture (v/v) of microsomes from these two strains. Initial enzyme activities (nanomoles of styrene glycol/mg of protein/min) were: C57BL/6J, 39.6; DBA/2J, 44.4; B6D2F1/J, 49.7; and mixture of C57BL/6J + DBA/2J, 42.3. B, heat stability of epoxide hydrolase activity in microsomes from C57BL/6J, DBA/2J, and B6D2F1/J mice measured with both styrene oxide (—) and benzo[a]pyrene-4,5-oxide (○) as substrates. Enzyme activity was measured in the same microsomes used in 3A. Combined data from two experiments.
Mice were phenotyped by determining the ratio of enzyme activity at pH 9.5/pH 8.7 as described in the text. “B” and “D” are used as generic symbols for alleles inherited from C57BL/6J and DBA/2J mice, respectively. The C57BL/6J genotype is Sas-1" Ltw-4' Eph-1' Mls' and the DBA/2J genotype is Sas-1' Ltw-4" Eph-1" Mls". Regions where cross-overs have resulted in recombination of the parental alleles in the BXD strains are denoted by an ×. Animals of both sexes were used in this experiment.

Table I summarizes the distribution of two pheno-

types among the BXD RI strains had previously been determined (23, 24). A partial linkage map of the distal end of mouse Chromosome 1 is shown in Fig. 4. The one strain (BXD-23) in which the Eph-1 genotype is not concordant with the two markers. This result strongly favors the placement of Eph-1 between Ltw-4 and Mls. The one exception is not surprising since double cross-overs are common in RI chromosomes because there are multiple opportunities for recombination in the development of an RI strain.

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This gene order does not require the postulation of any double cross-overs in the Sas-1-Ltw-4-Eph-1 region and further supports this gene arrangement. The data would not preclude the placement of Eph-1 distal to Mls, but such an arrangement would represent a less likely interpretation of the data.

A partial linkage map of the distal end of mouse Chromosome 1 is shown in Fig. 4. The recombination frequency, estimated by the method of Taylor et al. (25), between Eph-1 and Ltw-4 is 3.9 ± 2.6%; between Eph-1 and Mls this value is also 3.9 ± 2.6%; and between Sas-1 and Ltw-4 this value is 7.6 ± 4.4%. These values are in agreement with previously estimated map distances for this portion of the chromosome.

DISCUSSION

We have presented evidence that there is a genetic polymorphism of hepatic microsomal epoxide hydrolase activity.

1 R. W. Elliott, C. Romejko, and C. Hohman (1980) manuscript submitted for publication.

![FIG. 4. Partial linkage map of mouse Chromosome 1. Locus symbols are explained in the text; the black dot indicates the centromere. Numbers refer to the recombination frequency (in percent) observed between loci.](image)
among inbred mouse strains. Two phenotypes have been distinguished by the criteria of pH optima and thermostability. Our results are consistent with the hypothesis that these phenotypes are a result of allelic variants of a single structural gene for microsomal epoxide hydrolase which is located near the end of Chromosome 1.

Other investigators (12, 13) have noted genetic variation in hepatic epoxide hydrolase activity between inbred strains of mice, but suboptimal assay conditions prevented their identification of this polymorphism.

Hepatic microsomal epoxide hydrolase has been purified from a number of species including mouse (26), but has been studied most extensively in the rat. Rabbit antibody to the purified rat protein gave a single immunoprecipitin band to both solubilized microsomal and nuclear membrane fractions of rat liver (27) and to solubilized hepatic microsomes from C57BL/6J and DBA/2J mice (28). This suggests that there is a single species of this enzyme in rat microsomes, and this enzyme cross-reacts with the mouse enzyme. However, recent evidence (29, 30) suggest that there may be several very closely related species of microsomal epoxide hydrolase in rat liver. At present the problem of whether or not multiple species of epoxide hydrolase exist remains unresolved. Our data indicate that the Eph-1 locus codes for two allelic forms of microsomal epoxide hydrolase in mice, but we cannot state that this is the only structural gene for this enzyme.

A cytosolic epoxide hydrolase enzyme with catalytic properties that are markedly different from the microsomal enzyme has been described recently.3 Styrene oxide, a good substrate for the microsomal enzyme, is not metabolized at all by the cytosolic enzyme. Differences in substrate specificity, pH optimum, and molecular weight (31) indicate that the cytosolic enzyme is not a solubilized form of the microsomal enzyme and therefore is most likely coded for by a separate structural gene.

The only other microsome drug-metabolizing enzyme polymorphism to be mapped in the mouse is coumarin hydroxylase and it appears that microsomal drug-metabolizing enzymes are controlled by genes located at multiple sites in the mouse genome.

In this report we have not examined whether this polymorphism has any consequences in vivo; i.e. if a mouse with one allelic form of epoxide hydrolase is more susceptible to the toxic effects of reactive epoxides. Indirect evidence suggests that it is unlikely that differences in this enzyme will play a critical role in the metabolism of epoxides. Epoxides are inactivated by several other routes, including enzymatic and nonenzymatic conjugation with glutathione (34, 35). We examined the metabolism of styrene oxide in vitro, at pH 6.9 (intracellular pH), and found hepatic microsomal epoxide

hydrolyase activity from C57BL/6J mice had a $V_{\text{max}}$ of 4.9 nmol/mg/min and $K_m$ of 0.13 mm, and from DBA/2J mice a $V_{\text{max}}$ of 8.6 nmol/mg/min, and $K_m$ of 0.32 mm. We think that these small differences in $K_m$ and $V_{\text{max}}$ are unlikely to be significant in vivo.

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