

Polymorphisms in Human Soluble Epoxide Hydrolase

BEATA D. PRZYBYLA-ZAWISLAK, PUNIT K. SRIVASTAVA, JOHANA VÁZQUEZ-MATÍAS, HARVEY W. MOHRENWEISER, JOSEPH E. MAXWELL, BRUCE D. HAMMOCK, J. ALYCE BRADBURY, AHMED E. ENAYETALLAH, DARRYL C. ZELDIN, and DAVID F. GRANT

Department of Pharmacology and Toxicology, University of Arkansas for Medical Sciences, Little Rock, Arkansas (B.D.P.-Z.); Department of Pharmaceutical Sciences, University of Connecticut, Storrs, Connecticut (P.K.S., D.F.G., A.E.E.); Biology and Biotechnology Research Program, Lawrence Livermore National Laboratory, Livermore, California (J.V.-M., H.W.M.); Department of Entomology and Cancer Research Center, University of California, Davis, California (J.E.M., B.D.H.); Division of Intramural Research, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina (J.A.B., D.C.Z.)

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ABSTRACT

Human soluble epoxide hydrolase (hsEH) metabolizes a variety of epoxides to the corresponding vicinal diols. Arachidonic and linoleic acid epoxides are thought to be endogenous substrates for hsEH. Enzyme activity in humans shows high interindividual variation (e.g., 500-fold in liver) suggesting the existence of regulatory and/or structural gene polymorphisms. We resequenced each of the 19 exons of the hsEH gene (*EPHX2*) from 72 persons representing black, Asian, and white populations. A variety of polymorphisms was found, six of which result in amino acid substitutions. Amino acid variants were localized on the crystal structure of the mouse sEH, resulting in the prediction that at least two of these (Arg287Gln and Arg103Cys) might significantly affect enzyme function. The six variants of the hsEH cDNA corresponding to each single polymorphism and one corresponding to a double polymorphism were then con-

structed by site-directed mutagenesis and expressed in insect cells. As predicted, Arg287Gln and the double mutant Arg287Gln/Arg103Cys showed decreased enzyme activity using *trans*-stilbene oxide, *trans*-diphenylpropene oxide, and 14,15-epoxyeicosatrienoic acid as substrates. Lys55Arg and Cys154Tyr mutants had elevated activity for all three substrates. Detailed kinetic studies revealed that the double mutant Arg287Gln/Arg103Cys showed significant differences in K_m and V_{max} . In addition, stability studies showed that the double mutant was less stable than wild-type protein when incubated at 37°C. These results suggest that at least six hsEH variants exist in the human population and that at least four of these may influence hsEH-mediated metabolism of exogenous and endogenous epoxide substrates *in vivo*.

Epoxide hydrolases (EC 3.3.2.3) metabolize exogenous and endogenous epoxides by hydrolyzing them to vicinal diols, which are usually less reactive and less mutagenic because of their higher hydrophilicity. sEH is one of five epoxide hydrolases (the others are hepoxilin EH, leukotriene A_4 hydrolase, cholesterol EH, and microsomal EH), which differ in molecular weight, subcellular localization, pI and substrate specificity.

Previous work suggests the existence of one hsEH gene localized to chromosomal region 8p21-p12 (Larsson et al., 1995). The human sEH gene (*EPHX2*) consists of 19 exons encoding 555 amino acids (Sandberg and Meijer, 1996). Be-

cause the human and mouse proteins are 73% identical (Beetham et al., 1995) with 100% identity in residues forming the catalytic triad, the crystal structure of murine sEH (Argiriadi et al., 1999) is a good model for predicting structure-function correlations of the hsEH. Each monomer of the homodimeric mouse sEH has two domains: an N-terminal domain and a C-terminal catalytic domain connected by a proline rich linker (Argiriadi et al., 1999). The catalytic mechanism involves formation of a covalent alkyl-enzyme ester intermediate as a result of nucleophilic attack by Asp333. This is subsequently hydrolyzed with assistance of the general base His523 in a charge relay with Asp495 to yield the vicinal diol product (Pinot et al., 1995a). This general mechanism, characteristic of α , β -hydrolase fold enzymes, is also used by bacterial haloalkane dehalogenase (Verschuere et al., 1993) and haloacid dehalogenase (Liu et al., 1995). Interestingly, the origin of the hsEH gene is thought to be the result of an early fusion of genes encoding these two bacterial enzymes (Argiriadi et al., 1999). The

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The polymorphisms reported herein are listed at: <https://dir-apps.niehs.nih.gov/egsnp/home.htm>.

ABBREVIATIONS: EH, epoxide hydrolase; hsEH, human soluble epoxide hydrolase; EET, *cis*-epoxyeicosatrienoic acids; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism; WT, wild type; *t*-SO, *trans*-[3 H]stilbene oxide; *t*-DPPO, *trans*-[3 H]diphenylpropene oxide; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis.

conserved catalytic mechanism of sEH suggests that there may be an important role of this protein in vivo.

Epoxides formed during microsomal P450-mediated oxidation of polyunsaturated fatty acids are excellent substrates for human sEH (Zeldin et al., 1995). The EETs, are involved in regulation of renal function (Rahman et al., 1997), vascular tone (Su et al., 1998), cardiac function after ischemia (Wu et al., 1997), pulmonary smooth muscle function and ionic transport (Pascual et al., 1998), and inflammation (Node et al., 1999). Because human liver and kidney have high levels of sEH activity (Pacifci et al., 1988), sEH has been suggested to regulate blood pressure via regulation of renal EET levels. This was confirmed in a recent study examining blood pressure and renal arachidonic acid metabolism in sEH null mice (Sinal et al., 2000). The results demonstrated that blood pressure of male sEH null mice was significantly lower than that of male wild-type mice in both the absence and the presence of dietary salt-loading. Interestingly, blood pressure of female sEH null mice was not significantly different from that of female wild-type mice. Because female mice have lower sEH activity (Pinot et al., 1995b) and lower blood pressure than male mice, male sEH null mice were "feminized" with respect to blood pressure. When incubated with arachidonic acid, renal and hepatic S-9 fractions prepared from male or female sEH-null mice produced much greater levels of EETs compared with wild-type mice.

If sEH proves to be important in regulating the levels of fatty acid epoxides in vivo, variation in sEH activity may have significant clinical implications. A previous study found high interindividual variation in hEH activity [e.g., more than 500-fold in liver (Mertes et al., 1985)], suggesting the existence of variation in coding or regulatory sequences of the gene. In addition, a study of human twins revealed a genetic component in hEH enzymatic activity variation (Norris et al., 1989). In the current study, we found six polymorphic amino acid substitutions in a sample of 72 humans and show that several of these result in significant changes in hEH activity in vitro.

Materials and Methods

DNA Samples. Genomic DNA was prepared as described previously (Sambrook et al., 1989) from 72 different human lymphoblastoid cell lines (Coriell Institute, Camden, NJ) isolated from healthy individuals with the following ancestries: 24 blacks (16 African American, 8 African Pygmy), 24 Asians (5 Indo-Pakistani, 5 native Taiwanese, 5 mainland Chinese, 3 Cambodian, 3 Japanese, 3 Melanesian) and 24 whites [9 from Utah, 5 Druze (Lebanon), 5 Adygei (eastern Europe), 5 from Moscow].

Genotyping. We applied a resequencing strategy (referred to as resequencing because the same genomic region is sequenced in multiple individuals) that was used previously to identify variation in DNA repair genes (Shen et al., 1998). It involved the direct sequencing of PCR products containing exons plus the adjacent intronic and noncoding regions. The PCR products included the splice sites and 5'- and 3'-regions of the genes.

PCR primers used for isolation of the 19 exons of *EPHX2* gene are listed in Table 1. They were designed so that amplification of the genomic sequence was initiated approximately 75 nucleotides from the intron-exon boundary. This was sufficient distance for high quality sequence data to be obtained before reaching the intron/exon splice site. The PCR primers were positioned so that the PCR products were ~500 bp in length; therefore, the entire fragment could be sequenced in both directions without developing new sequencing primers. The PCR primers were designed using Oligo Primer Analysis Software (National Biosciences, Inc., Plymouth, MN). Appended to the 5'-end of each PCR primer was the primer binding site for the forward or reverse DNA sequencing primer (Amersham Biosciences, Inc., Piscataway, NJ). PCR primers were matched so that the sense and the antisense PCR primers contained different sequencing primer binding sites. PCR reactions were optimized by addition of DMSO. Primers were obtained from Sigma Genosys (The Woodlands, TX). The volume of a PCR reaction for exon amplification was 50 μ l. Typical reaction consisted of 50 ng of genomic DNA, 0.5 μ M of each primer, 0.2 μ M of dNTPs, 10 \times *Taq* Polymerase buffer, and 0.5 μ l of *Taq* Polymerase+Antibody (50:50) (BD Biosciences Clontech, Palo Alto, CA). Exon 2 was amplified using Advantage-CG Genomic PCR Kit, 1.0 M GC Melt (BD Biosciences Clontech). Exon 3 was amplified using the two buffers (Stratagene, La Jolla, CA): Opti-Prime 10 \times buffer 4 (100 mM Tris-HCl, pH 8.3, 35 mM MgCl₂, and 750 mM KCl) and Master Mix 50 \times Buffer (20 mM Tris-HCl, pH 8.0, and 250 mM EDTA). The following cycling conditions were applied to amplify exons: 9 min at 94°C (1 cycle); 30 s at 94°C, 45 s at 63°C, and

TABLE 1
Primers used for amplification of *EPHX2* exons
Sequencing primers are shown in upper cases.

Exon	Size	Forward Primer Sequences	Reverse Primer Sequences
	<i>bp</i>		
1	416	5'-GTTTTCCCAGTCACGACGcggagctcccgtaagggggtg-3'	5'-AGGAAACAGCTATGACCATtcagggccaggtcttcagtttcat-3'
2	463	5'-GTTTTCCCAGTCACGACGgtgaagctgctattaggtggt-3'	5'-AGGAAACAGCTATGACCATatgggtgggcccgaagttcggggagat-3'
3	416	5'-GTTTTCCCAGTCACGACGgaattgcttggtcaccaggaat-3'	5'-AGGAAACAGCTATGACCATtccatctggtgctcagaagatctgt-3'
4	546	5'-GTTTTCCCAGTCACGACGacctgcttgcccacattgg-3'	5'-AGGAAACAGCTATGACCATtgttaagagctatacaatggagcca-3'
5	500	5'-GTTTTCCCAGTCACGACGctgtgtggggctcatatgtgcctt-3'	5'-AGGAAACAGCTATGACCATcgtgtgctgactgcacagtggggtg-3'
6	400	5'-GTTTTCCCAGTCACGACGagaactcagctctggggctccac-3'	5'-AGGAAACAGCTATGACCATctgctggtgctgttttaggtgacctg-3'
7	364	5'-GTTTTCCCAGTCACGACGcatgcacaatccagcaacgggactg-3'	5'-AGGAAACAGCTATGACCATgagacaatgctccactgctcaggtt-3'
8	401	5'-GTTTTCCCAGTCACGACGggtagtgccctgtggtctctgtgt-3'	5'-AGGAAACAGCTATGACCATcagagacacagggcaccgtag-3'
9	457	5'-GTTTTCCCAGTCACGACGcacagatggatagacatcaaaagc-3'	5'-AGGAAACAGCTATGACCATgtcgtatgctgcatacatgtgggc-3'
10	370	5'-GTTTTCCCAGTCACGACGtgggggttaggacttcaacacag-3'	5'-AGGAAACAGCTATGACCATttctccacgaatccttctgtctt-3'
11	489	5'-GTTTTCCCAGTCACGACGcttggtctcctaagcagatgtaa-3'	5'-AGGAAACAGCTATGACCATgtcaccagacactaaagagtgaa-3'
12	636	5'-GTTTTCCCAGTCACGACGcactgctagtgtatgacctgtgt-3'	5'-AGGAAACAGCTATGACCATcttagaccctgggtgccataatc-3'
13	400	5'-GTTTTCCCAGTCACGACGggaactacagtttagcatcttgta-3'	5'-AGGAAACAGCTATGACCATtctctggtcctgcccacacacta-3'
14	382	5'-GTTTTCCCAGTCACGACGcagctctgctggtactcagaggtca-3'	5'-AGGAAACAGCTATGACCATgagctcagccctatgcatgccc-3'
15	450	5'-GTTTTCCCAGTCACGACGggccttgccgaagttcagatg-3'	5'-AGGAAACAGCTATGACCATctgagctatcccaactagagcag-3'
16	408	5'-GTTTTCCCAGTCACGACGcttgctgctagagcttgccaac-3'	5'-AGGAAACAGCTATGACCATgacagatctgtccacaaccagctct-3'
17	329	5'-GTTTTCCCAGTCACGACGgatacaacgtcaggaccacagc-3'	5'-AGGAAACAGCTATGACCATccacatgacaaggtgtatcttct-3'
18,19	685	5'-GTTTTCCCAGTCACGACGgcaagcagcaaccgtgactgta-3'	5'-AGGAAACAGCTATGACCATgcatccatgcaagatgtgtgta-3'

1 min at 72°C (35 cycles); and 7 min at 72°C (1 cycle). Exon 2 required an annealing temperature of 57°C.

After amplification, PCR products were diluted and used as substrates in sequencing reactions. Dye primer cycle sequencing reactions are performed according to manufacturer's instructions for the DYEnamic Direct cycle sequencing kit with the DYEnamic ET primers (Amersham Biosciences) and loaded into a stretch DNA sequencer (ABI Prism 377; Applied Biosystems, Foster City, CA). All PCR products were sequenced in both directions.

The initial data analysis (lane tracking and base calling) was performed with the ABI prism DNA sequence analysis software (version 2.1.2). Chromatograms created by the ABI prism DNA sequence analysis software were imported into a SUN Microsystems UNIX workstation (Sun Microsystems Inc., Mountain View, CA). The chromatograms were reanalyzed (bases called and quality of sequence values assigned) with Phred (version 0.961028), assembled with Phrap (version 0.960213), and the resultant data viewed with Consed (version 4.1). Description of and documentation for Phred, Phrap, and Consed may be obtained at <http://genome.washington.edu>. "PolyPhred" (version 2.1), a software package that uses the output from Phred, Phrap, and Consed was employed to identify SNPs in heterozygotes (Nickerson et al., 1997).

The common or wild-type allele was defined as the most common allele in the sample set sequenced rather than by the nucleotide at that position in the reference GenBank sequence.

Plasmid Construction and Site-Directed Mutagenesis. The *EPHX2* cDNA was obtained by amplifying viral DNA from a baculovirus carrying the *EPHX2* cDNA (Beetham et al., 1993) with primers 5'-CATGGGATCCATGACGCTGCGCGCGCCGTC-3' and 5'-CTTACTCGAGTACTCTT-TGAGACCACCG-3', which added a *Bam*HI restriction site at the 5' end, and a *Xho*I site at the 3' end of the cDNA. The following PCR conditions were applied: 4 min at 95°C, 20 s at 70°C (1 cycle); 30 s at 95°C, 45 s at 62°C, 30 s at 73°C (3 cycles); 30 s at 95°C, 30 s at 62°C, 30 s at 73°C (25 cycles), and 5 min at 73°C (1 cycle). A 50- μ l PCR reaction contained 50 ng of viral DNA, 0.3 μ M concentrations of each primer, 0.4 μ M concentrations of dNTPs, 2 mM Mg₂SO₄, 10 \times PCR Vent buffer and 1.0 U of Vent Polymerase (New England Biolabs, Inc., Beverly, MA). Because the N-terminal primer used for cloning the hsEH cDNA was based on the cDNA sequence as published (Beetham et al., 1993), the wild-type hsEH cDNA used in this study contains glycine instead of alanine [as described in the gene sequence (Sandberg and Meijer, 1996)] at the fifth residue of the hsEH protein. The PCR product was subcloned into *Bam*HI/*Xho*I sites of the vector pCR-Script (Stratagene) to generate plasmid pCR-Script/hsEH-WT, which was used for site-directed mutagenesis using the QuikChange mutagenesis system (Stratagene). The primers used to introduce amino acid mutations are listed in Table 2. Primers were obtained from Integrated DNA Technologies, Inc. (Coralville, IA). The mutagenesis introducing two mutations (Arg287Gln and Arg103Cys) into the same cDNA were done by mutagenizing plasmid pCR-Script/WT twice: first with primers Arg287Gln then with primers Arg103Cys.

TABLE 2

Primers used for site-directed mutagenesis of the hsEH
The underlined bases indicate mutagenized nucleotides.

Mutation	Sequences of Forward and Reverse Primers
Lys55Arg	5'-ACTACCCGGCTTATGAGAGGAGATCACACTT-3' 5'-AAGTGTGATCTCTCTCATAGCCGGTAGT-3'
Arg103Cys	5'-GCCAGAAAGATCAAT <u>T</u> GCCCCATGCTCCAGGCA-3' 5'-TGCCCTGGAGCATGGGGCA <u>A</u> TTGATCTTTCTGGC-3'
Cys154Tyr	5'-TTCTTGATAGAGTCGT <u>A</u> CCAGGTGGGAATGGTC-3' 5'-GACCATTCCCACTGGTACGACTCTATCAGGAA-3'
Arg287Gln	5'-GCCAGGCAGGTT <u>A</u> CCAGGTCTTAGCTATGGAC-3' 5'-GTCCATAGCTAGGACCTGGTAACTGCTCCGGC-3'
Val422Ala	5'-TTATCCATGCATAAAGCTTGTGAAGCCGGAGGA-3' 5'-TCCTCCCCTTCAACA <u>G</u> CTTTATGCATGGATAA-3'
Glu470Gly	5'-TGGTACCGAAACATGGGAAGGAACCTGGAAGTGG-3' 5'-CCACTTCCAGTTCTCTTCCATGTTTCGGTACCA-3'

The mutation sequence was incorporated into each mutagenesis primer pair along with a diagnostic restriction site (gain of *Mfe*I site for Arg103Cys, gain of an *Rsa*I site for Cys154Tyr, loss of *Hpa*II site for Arg287Gln, and gain of *Hind*III site for Val422Ala) allowing identification of mutant clones. The Lys55Arg and Glu470Gly mutants were confirmed by sequencing across the mutation. The wild type and all mutants were completely sequenced to verify their identity (ABI Prism 377XL sequencer; Applied Biosystems). The BLAST 2 sequences program (<http://www.ncbi.nlm.nih.gov/gorf/bl2.html>) was used to align sequences.

Expression in Baculovirus. Wild-type and mutated versions of *EPHX2* cDNA were expressed in baculovirus using the Bac-to-Bac system (Invitrogen, Carlsbad, CA). After subcloning cDNAs into *Bam*HI/*Xho*I sites of the pFastBac1 vector, the recombinant baculoviruses were generated and expressed in insect cells according to the manufacturer's instructions with the following modifications. We used *Sf*-21 cells, grown to a density of 2 to 3 \times 10⁶/ml in Ex-Cell 401 with L-glutamine (JRH Biosciences, Lenexa, KS), supplemented with 3% heat inactivated fetal bovine serum (JRH Biosciences) and 1% Pen/Strep antibiotics (Sigma, St. Louis, MO) in a 50 ml spinner flask. One million *Sf*-21 cells were used for the transfection as attached cells in 35-mm² culture dishes. After removal of the transfection mixture, cells were overlaid with 2 ml of 3% agar (made in phosphate-buffered saline, pH 6.2) mixed with growth medium (50:50). After the agar solidified, 1 ml of growth medium was added, and cells were incubated at 28°C for 4 days. A single plaque was picked up from the plate, diluted in 1 ml of medium, vortexed, and incubated for 1 h at room temperature. Viruses from plaques were then amplified twice: 1) by infecting one 1 \times 10⁶ cells with 0.5 ml of plaque solution and growth in 1.5 ml medium and 2) by infecting 5 \times 10⁶ cells with 1.0 ml of virus collected after the first amplification. In each of these amplifications, viruses were collected on the fourth day after infection. Viruses collected after the second amplification were used to determine viral titer using plaque assay as described previously (O'Reilly et al., 1992).

Enzyme Assays. The specific activities of wild-type and mutant enzymes were measured in baculoviruses infected *Sf*-21 cells using 14,15-EET, *t*-DPPO, and *t*-SO as substrates (Zeldin et al., 1993; Borhan et al., 1995; Pinot et al., 1995a, respectively). Cells infected with a recombinant baculovirus carrying the *lacZ* gene were used as a negative control. On the fourth day after infection, cells were washed twice in buffer (100 mM NaH₂PO₄ and 0.1 mg/ml BSA, pH 7.4), centrifuged at 150 g at 4°C, and diluted to the desired protein concentration. The amount of total protein used in the assay was optimized to remain in the linear range of the assay. We used 10 μ g of protein from cells expressing the control LacZ protein (no activity) and 0.15 μ g from cells having the highest enzymatic activity. Specific enzymatic activity was normalized to the same amount of hsEH protein by measuring the hsEH protein content for each mutant in each experiment with a densitometer after SDS gel electrophoresis (GS-710; Bio-Rad Laboratories, Hercules, CA). Specifically, 10 μ g of total *Sf*-21 cell protein content of wild-type and each mutant was loaded on an SDS-PAGE gel and the amount of protein in bands corresponding to hsEH were measured in absorbance units. The absorbance-wild-type/absorbance-mutant was calculated for each mutant and used as a correction factor for specific enzyme activity values. All hsEH-expressing viruses produced a major band at 62 kDa, whereas LacZ recombinant viruses showed no increase in a protein in this molecular weight range. We measured specific enzymatic activity in three or four independent experiments using different enzyme preparations. The normalization was done for each of three or four experiments separately. Enzyme assays within each experiment were performed in triplicate or quadruplicate. Protein concentrations were determined with bicinchoninic acid assay (Pierce, Rockford, IL) using BSA as a standard.

Electrophoresis and Western Blotting. Proteins from *Sf*-21 lysates were resolved on 8% SDS-PAGE gels. Gels were transferred to nylon MagnaGraph (MSI, Inc., Westborough, MA) membrane at

100 V for 1 h. The hSEH was detected with a Rhesus sEH polyclonal antisera (Silva and Hammock, 1987). Antibody was diluted 1:10,000 in buffer containing phosphate-buffered saline, 0.2% Tween 20, 5% milk, and 0.1% BSA. Blots were blocked and washed in the same buffer omitting BSA. Western blot chemiluminescence reagent (PerkinElmer Life Science, Boston, MA) was used to detect antibody-antigen complexes, using a donkey anti-rabbit horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, Piscataway, NY) at dilution 1:10,000.

Kinetic Studies. Michaelis-Menten parameters for wild-type and mutant proteins were determined using *t*-SO as substrate under steady-state conditions using various substrate concentrations (final concentration, 1–10 μ M). K_m and V_{max} values were calculated by the method described by Segel (1975). Assay conditions for kinetic determinations were as specified above and the analysis was performed on two separate enzyme preparations. Appropriate times of incubation were determined that allowed the hydrolysis reaction to be linear during the assay period.

Statistical Analysis. One-way analysis of variance was used for initial comparisons. Student-Newman-Keuls procedure was subsequently used for pair-wise multiple comparisons (SigmaStat; SPSS Science, Chicago, IL). Significance levels were set at $p \leq 0.05$.

Results

Identification of Single Nucleotide Polymorphisms in hSEH Exons. Screening of 72 samples of human genomic DNA revealed the presence of 44 SNPs, and one insertion/deletion in the 19 exons and exon-intron boundaries of the *EPHX2* gene. Of 44 SNPs identified, 31 were in introns and 13 were in exons. Six of the 13 exonic SNPs resulted in amino acid substitutions, whereas seven were silent. None of the SNPs was near splice site sequences (Fig. 1). The distribution of the six exonic SNPs among three ethnic groups is listed in Table 3. Twenty-eight of 72 persons (39%) had at least one amino acid polymorphism in hSEH. Most persons were heterozygous for these polymorphisms. Homozygotes were found in the black population (two persons were homozygous for the Lys55Arg mutation and one person was homozygous for the Arg103Cys mutation) and in the Asian population (one person was homozygous for the Cys154Tyr mutation). Five persons with more than one polymorphism were identified: one Asian with Cys154Tyr and Arg287Gln, one black person with Arg287Gln and Glu470Gly, one black person with Arg103Cys (homozygote) and Arg287Gln, and one black person with Arg103Cys and Val422Ala. One black person had three polymorphisms: Arg103Cys, Arg287Gln, and Val422Ala.

Assuming that the group under study represents the general population, five allelic variants represent true polymorphic loci (frequency of occurrence $\geq 1\%$), whereas the

Glu470Gly frequency was $<1\%$. The Lys55Arg and Arg287Gln mutations are the most common in the group under study (17% and 14% respectively). The distribution of polymorphisms among the three racial groups is nonrandom (χ^2 test, $P = 0.0016$) with a higher than expected frequency of these mutations occurring in black persons and lower than expected occurring in white persons. Given the large degree of genetic diversity and admixture in human populations and the difficulty in defining appropriate populations for sampling, the frequency data reported for any individual group must be considered an approximation.

Structural Analysis of the Polymorphic Form of Human Enzyme Based on Crystal Structure of Mouse sEH. The location of each of the six polymorphic amino acids is shown on the crystal structure of the mouse sEH (Fig. 2). This crystal structure was determined for baculovirus-expressed mouse sEH at 2.8-Å resolution (Argiriadi et al., 2000). The mouse and human sEH proteins are 73% identical. Five of the six amino acid residues that are polymorphic in human sEH (Lys55, Arg103, Cys154, Arg287, and Glu470) are also found in mouse sEH (Lys55, Arg103, Cys154, Arg285, and Glu469). A visual analysis of the location of the polymorphic residues in the crystal structure of the mouse homodimer allows one to predict possible functional consequences of these amino acid substitutions. The lysine 55 residue in the mouse sEH structure points out into the water interface. The substitution of lysine 55 with arginine is conservative, and it would not be expected to result in significant structural changes. The arginine 103 residue would seem to be in a rather unimportant region of the sEH protein; however, closer inspection shows the existence of a potential intramonomeric salt bridge with glutamic acid 142. These two residues are approximately 3 Å apart in the mouse sEH structure. This salt bridge may be important for protein folding and/or stability by orienting the two α helices relative to each other. The cysteine 103 variant would disrupt this putative salt bridge, possibly leading to significant effects on enzyme structure and function in vitro and in vivo. The substitution of cysteine 154 with tyrosine might require additional space within the folded protein. However, the mouse model suggests that this region of the sEH protein can accommodate the extra size of a tyrosine phenyl ring without structural protein changes. The arginine 285 (287 in human sEH) is located along the dimerization interface between the two subunits. The crystal structure shows that each arginine 285 of one monomer is likely to form a salt bridge with glutamic acid 252 on the other monomer. The distance be-

TABLE 3
Polymorphisms in exons of *EPHX2* found in 72 human subjects

Variant	Number of Persons with Variant Genotypes				Allele Frequency
	Black ($n = 24$)	Asian ($n = 24$)	White ($n = 24$)	All ($n = 72$)	
Lys55Arg	8 ^a	2	2	12	14/144
Arg103Cys	4 ^b	0	1	5	6/144
Cys154Tyr	0	4 ^b	0	4	5/144
Arg287Gln	4	5	1	10	10/144
Val422Ala	2	0	0	2	2/144
Glu470Gly	1	0	0	1	1/144
Total number of variants	19	11	4	34	38/144
Total number of individuals with variants	14	10	4	28	

^a Two homozygotes were identified.

^b One homozygote was identified.

tween the intermonomeric arginine 285 and glutamic acid 252 residues is approximately 4.5 Å. It is also possible to form a salt bridge between arginine 285 and glutamic acid 252 on the same monomer (intramonomeric salt bridge). The intermonomeric salt bridge would be expected to be favored in the dimer, whereas the intramonomeric salt bridge would be expected to be favored in a monomeric conformation. The alanine 421 in the mouse sEH (422 in the human sEH) is located along the 25-Å deep, L-shaped cavity that contains the hydrophobic substrate-binding pocket. Valine 422 in the wild-type human sEH is alanine 421 in the mouse sEH. Because this residue is structurally near the substrate-binding pocket, it may be significant even though it is a very conservative change. The valine side chain is somewhat larger than the alanine side chain. Mutation of valine 422 to an alanine may allow greater access to the active site or more rapid release of product, leading to changes in substrate preferences or an altered catalytic rate. The glutamic acid 469 (470 in the human sEH) residue is found along a linker region of the C terminus. This residue would not be predicted to play a significant role in sEH structure or function.

Expression of hsEH Protein Variants in Baculovirus System. The hsEH cDNA (Beetham et al., 1993) was cloned into pCR-Script-Amp vector and used for site-directed mutagenesis to construct seven mutants of hsEH. Six mutants were in single amino acids at positions 55, 103, 154, 287, 422, and 470. One construct contained two mutations [at positions 287 and 103: mutant (287/103)]. The wild-type and the mutated cDNAs were expressed using a single promoter baculovirus system. Enzyme expressed in the single promoter system would correspond to a person homozygous for the mutation. Expression of hsEH proteins, which have a molecular mass of 62.5 kDa, were verified by SDS-PAGE and by immunoblot analyses using a specific sEH antibody (Fig. 3). A recombinant baculovirus expressing LacZ was used as a

negative control. *Sf*-21 cells infected with the control virus have no detectable sEH activity (<10 nmol/min/mg of protein) for all three substrates examined. None of the mutations prevented expression of hsEH protein. The level of protein expression varied among the different mutants. These differences were quantified with densitometric analyses and used for normalization of specific activity values.

Enzymatic Phenotypes. Enzyme activity phenotypes were determined by measuring the specific enzymatic activity of hsEH in whole insect cells as described previously (Pinot et al., 1995a). The exogenous substrates used were *t*-SO and *t*-DPPO (Borhan et al., 1995). 14,15-EET was used as a putative endogenous substrate. Enzyme activity was measured within the linear range of the assays as defined by Wixtrom and Hammock (1985). Enzyme assay results from three to four independent experiments using different preparations of the recombinant enzyme are summarized in Fig. 4. The Arg287Gln single mutant and the Arg287Gln/Arg103Cys double mutant showed statistically significant decreases in enzyme activity compared with wild type when *t*-SO and *t*-DPPO were used as substrates ($p < 0.05$). Three other single mutants (Lys55Arg, Cys154Tyr, and Glu470Gly) showed statistically significant increases in enzyme activity using these two substrates ($p < 0.05$). The specific activity of the wild-type hsEH was 12 ± 3 nmol/min/mg of total protein for *t*-SO and 329 ± 59 nmol/min/mg of total protein for *t*-DPPO. Enzyme activity from four independent experiments using different preparations of the recombinant enzymes with 14,15-EET as substrate are summarized in Fig. 5. As with the exogenous substrates, Arg287Gln and the double mutant Arg287Gln/Arg103Cys showed significantly reduced activity ($p < 0.05$). The Lys55Arg and the Cys154Tyr mutants tended to have increased activity; however, these differences were not statistically significant ($p = 0.12$ and $p =$

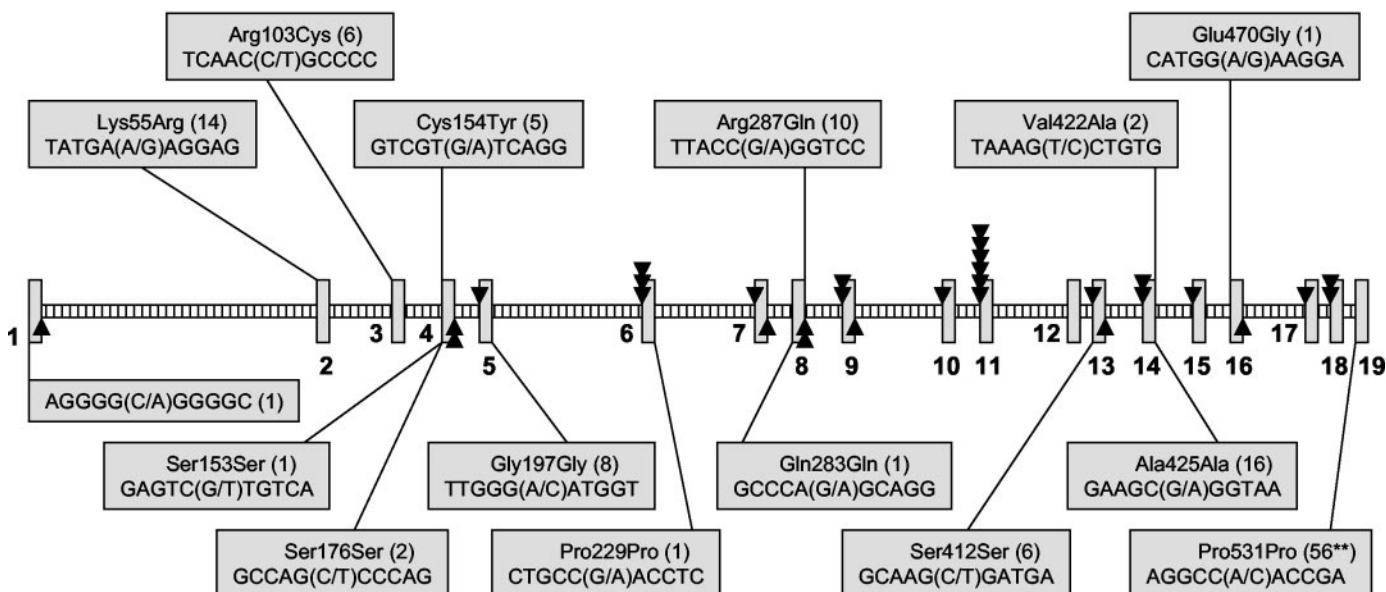


Fig. 1. Single nucleotide polymorphism (SNPs) in the human soluble epoxide hydrolase (*EPHX2*) gene. The *EPHX2* gene is represented by the horizontal bar; vertical bars represent the 19 exons. SNPs occurring in exons are shown in boxes above (SNPs that lead to change in amino acid sequences) and below (SNPs that lead to silent mutations) the gene. The frequency of occurrence of each SNP (per 144 alleles) is given in parentheses. The change in base sequence is given below the amino acid position. ▼, SNPs found in intron-exon regions and their numbers. Relative intron size is drawn approximately to scale; however, exon size is not drawn to scale (Sandberg and Meijer, 1996). **, the frequency data for this SNP are questionable because of sequencing ambiguity.

0.10, respectively). The specific activity of wild-type hsEH was 116 ± 17 nmol/min/mg of total protein for 14,15-EET.

Kinetic Studies. The kinetic parameters (apparent K_m and V_{max}) for wild-type hsEH and those mutants having statistically significant differences in enzyme activity were determined using *t*-SO. With this substrate, the double mutant (Arg287Gln/Arg103Cys) had a statistically significant difference in V_{max} (2.1 ± 1.1 versus 11.3 ± 2.1 nmol/min/mg of total protein for wild type) and K_m (7.1 ± 0.8 versus 3.8 ± 0.6 μ M for wild type). This results in a decrease of approximately 10-fold in the V_{max}/K_m for the double mutant compared with the wild-type enzyme.

Stability Studies. The double mutant Arg287Gln/Arg103Cys had approximately 15 to 20% of wild-type activity when expressed in SF-21 cells after 4 days of infection. hsEH

protein amount was normalized for all of the expressed mutants by densitometric analysis before enzyme assays, and we noticed that the amount of hsEH protein produced in cell suspension by the Arg287Gln/Arg103Cys mutant was always much less than the wild-type hsEH protein. A possible explanation for this observation is that mutations at position 287 and 103 affected the stability of the enzyme. The crystal structure of mouse sEH shows the existence of a potential intramonomeric salt bridges between Arg103 and Glu142 and between Arg287 and Glu252. These may be important for protein folding and/or stability by orienting the α helices relative to each other. The Arg103Cys and the Arg287Gln variants would disrupt these putative salt bridges, potentially having significant effects on enzyme structure and function. To test this hypothesis, we incubated wild-type,

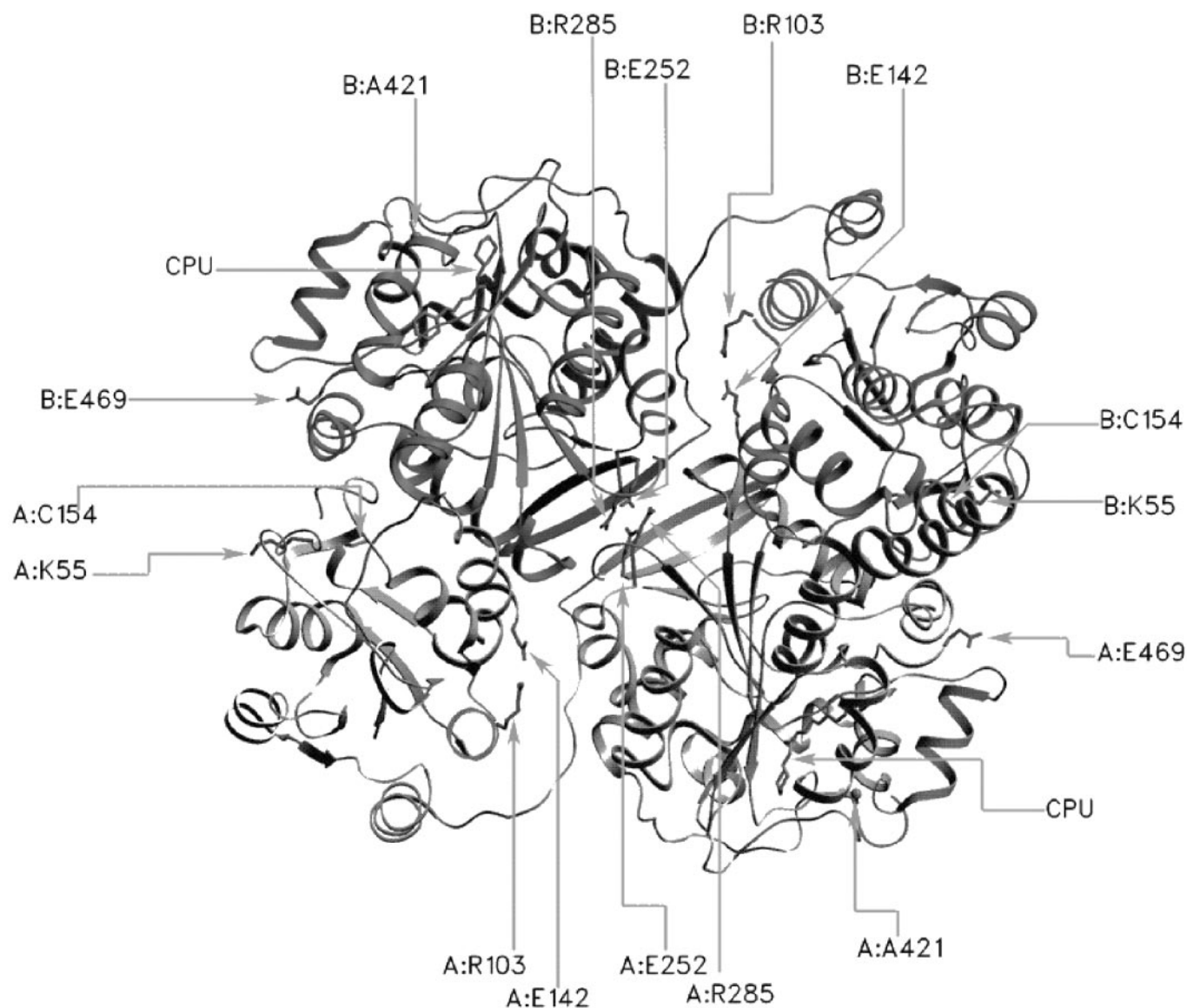


Fig. 2. Crystal structure of the mouse sEH showing the location of the six polymorphic residues found in humans. One subunit of protein (A) is shown in the bottom half, the other in the top half (B). The bound inhibitor is *N*-cyclohexyl-*N'*-(3-phenyl)propylurea (CPU) and indicates the location of the active site. Residues corresponding to human polymorphic sites are marked on both subunits A and B as: K55, R103, C154, R285 (human R287), A421 (human R422), and E469 (human E470). The residues predicted to be involved in formation of salt bridges with polymorphic residues are E142 and E252. The frame to produce this figure was taken from the crystal structure of CPU-bound mouse liver sEH [Protein Data Bank code 1CR6 (Argiriadi et al., 1999)]. K, lysine; R, arginine; C, cysteine; A, alanine; E, glutamine.

Arg103, Arg287, and the Arg287Gln/Arg103Cys double mutant enzymes at 37°C in 100 mM Tris, pH 8.0. After different times of incubation, specific activities were measured. The double mutant lost enzyme activity much more rapidly than

the wild type, Arg103Cys, or Arg287Gln single mutants (Fig. 6).

Discussion

In this article we report the identification of polymorphic variants in the hSEH gene. By genomic sequencing of PCR amplified exons, we found six SNPs resulting in amino acid substitutions in the hSEH peptide sequence. To determine which of these SNPs might be useful markers in predicting individual susceptibility to diseases, we determined enzymatic phenotypes of the polymorphic variants in vitro using a baculovirus expression system.

Two of the mutants were found to be associated with significant changes in enzymatic activity for all substrates tested. Mutations at positions 287 and 287/103 decreased activity of wild-type hSEH to 25 to 58% and 11 to 18%, respectively. As predicted from the structural analysis, mutation of arginine to glutamine at residue 287 could result in the abolition of salt bridges formed by arginine 287 and glutamic acid 252 in both hSEH dimers and monomers. Thus, the Arg287Gln substitution, present in approximately 14% of the general population, could affect the equilibrium existing between monomeric and dimeric forms of hSEH (Argiriadi et al., 2000). In previous studies, there were conflicting reports concerning whether the hSEH monomer is active (Dietze et al., 1990; Gill 1983). Our data suggest the possibility that both monomeric and dimeric forms are active but that they may have different kinetic properties.

The most common polymorphism in the population under study (Lys55Arg, 17%) was predicted based on the mouse sEH crystal model to have no effect on enzyme function. Surprisingly, this polymorphism was found to be associated with a statistically significant increase in specific activity compared with the wild-type hSEH using *t*-SO and *t*-DPPO as substrates and tended to have higher activity toward 14,15-EET. The increase in activity of the Lys55Arg mutation suggests that the N-terminal domain containing the putative

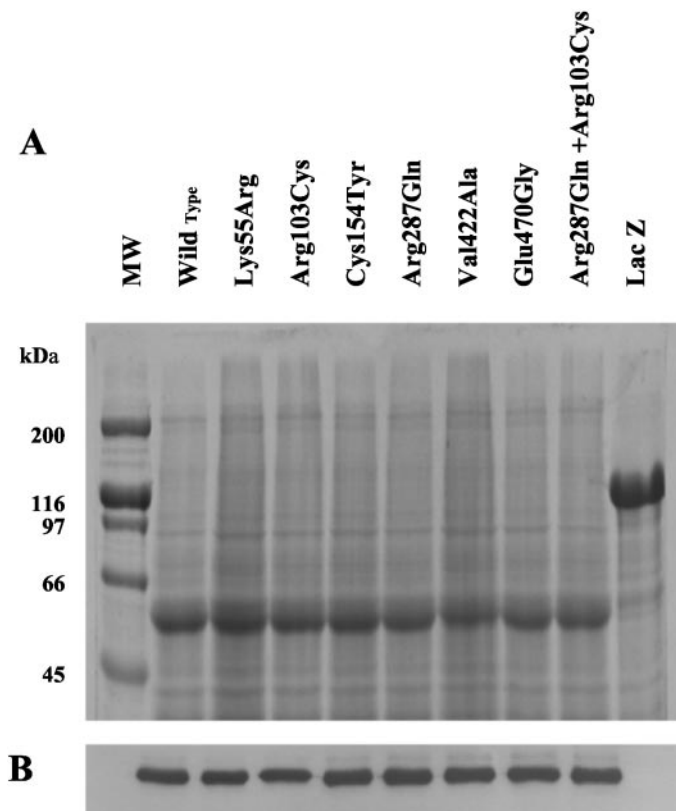


Fig. 3. Expression of wild-type and polymorphic variants of hSEH in *Sf*-21 cells. A, SDS-PAGE of total protein. *Sf*-21 cells were infected with the recombinant viruses, harvested 4 days after infection, washed, lysed with 0.1% SDS, and 20 μ g of total protein was loaded on 8% gel. In the Lys55Arg and Val422Ala lanes, the amounts of loaded proteins were 25 and 40 μ g, respectively, to demonstrate the equal amounts of hSEH used to calculate specific enzymatic activities. B, Western blot of the same proteins. Anti-rhesus sEH antibody was used to identify recombinant hSEH protein.

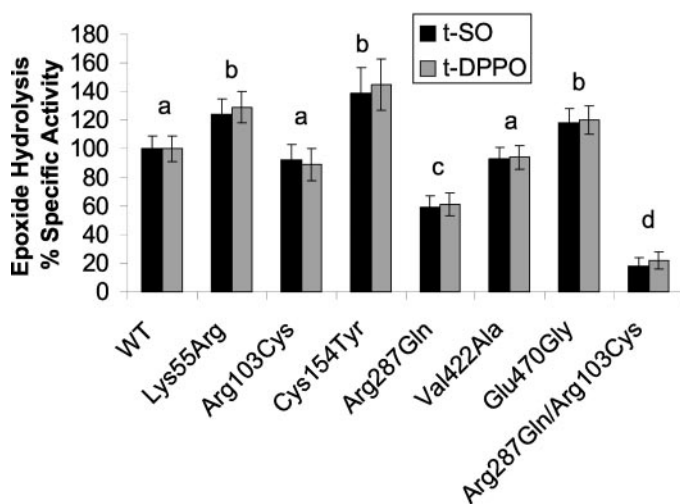


Fig. 4. Enzyme activities of hSEH variants expressed as percentage of wild type. Bars with different letters are significantly different from each other using *t*-SO and *t*-DPPO as substrates ($P < 0.05$). $n = 3$ to 4 independent experiments with three replicates per experiment.

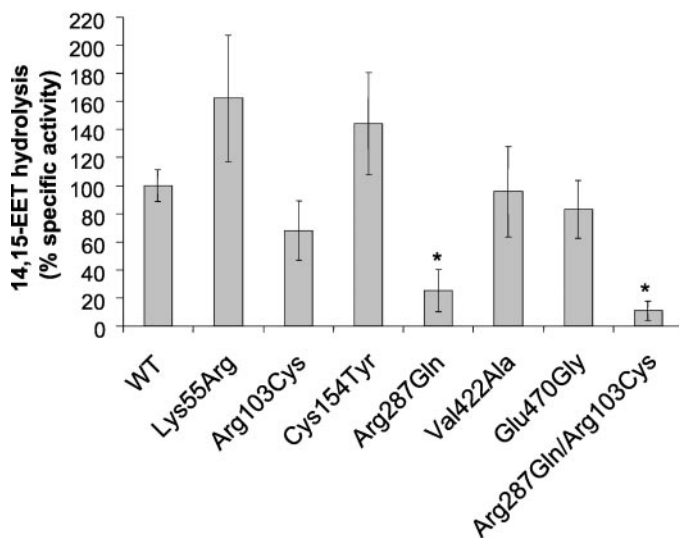


Fig. 5. Enzyme activities of hSEH variants expressed as percentage of wild type using 14,15-EET as substrate. *, significantly different from wild type ($p < 0.05$). $n = 4$ different enzyme preparations with four replicates per experiment.

vestigial active site (Argiriadi et al., 1999) may play a regulatory role in enzyme function.

Of six protein variants of hsEH discovered in this study, one variant (Arg287Gln) was also recently found in EPHX2 cDNAs prepared from 25 human liver samples (Sandberg et al., 2000). The overall frequency of this variant (8%) was similar to that found in the present study [10 of 144 (7%)]. Sandberg and coworkers also found evidence, using *t*-SO as a substrate, for lower activity with the Arg287Gln variant, although the difference was not statistically significant. These authors also found an insertion of an arginine after arginine 403 (Arg402–403ins) in 4% of their 25 human samples. The Arg402–403ins variant exhibited strikingly lower enzymatic activity compared with wild type using *t*-SO.

Ethnic background is an important factor in identification of polymorphisms. In this study, the group of 72 persons used for genotyping is highly heterogeneous and the number of persons representing each ethnic group is too small for any conclusive comparisons. Nevertheless, the statistically significant higher level of polymorphisms in the black population is striking. These racial differences are intriguing in light of the increased incidence of hypertension in black persons (Rywik et al., 2000) and the potential relationship between EET levels and blood pressure (Sinal et al., 2000).

Evidence that EETs play a role in blood pressure regulation in humans comes from studies of pregnancy-induced hypertension (Catella et al., 1990). Women with pregnancy-induced hypertension excreted from 10- to >250-fold higher levels of the 11,12- and 14,15-diols of arachidonic acid compared with age-matched pregnant women who did not have pregnancy-induced hypertension. The response was specific for the 11,12- and 14,15-diols because the levels of the 8,9-diols were not significantly different between the two groups. Because the only known source of these diols is from the corresponding EETs, these data strongly suggest that the EETs (or diols) play a role in blood pressure regulation in humans.

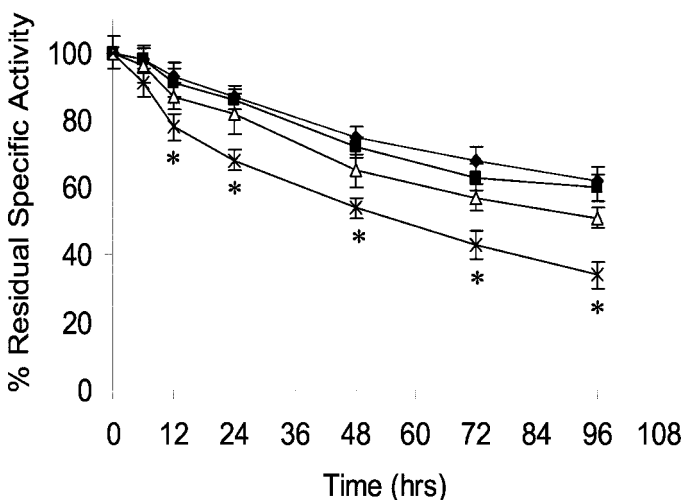


Fig. 6. Effect of preincubating the wild type and the double mutant (Arg287Gln/Arg103Cys) sEHs on specific activity. Enzymes were preincubated at 37°C in 100 mM Tris, pH 8.0. Total protein concentrations in the incubations were 23.1, 23.7, 22.9, and 80.3 μ g/ml for wild type (\blacktriangle), Arg103 (\blacksquare), Arg287 (\triangle), and double mutant (\times), respectively. All values are the mean of triplicates with bars representing the standard deviation. *, points statistically significantly different ($p < 0.05$) from wild type, Arg103Cys, and Arg287Gln.

In vitro expression systems are useful for identifying functionally important SNPs. However, variant enzymatic phenotypes defined in vitro may not correlate with wild-type phenotypes defined in vivo. Theoretically, many possible factors besides sEH genotype can account for the broad range (e.g., 500-fold for hsEH) of sEH enzyme activity in vivo, among them stability of mRNA, interactions with other gene products, and changes in inducibility caused by sequence variations in noncoding regions. Enzyme activity, which was the focus of this study, is one of many possible factors determining protein function. Other factors affecting enzyme function in a metabolic pathway can be controlled through covalent modification and rate of the enzyme synthesis and degradation as well as allosteric interactions.

SNPs occur approximately once per 500 to 1000 base pairs and are recognized as a major source of variation in the human genome (Wang et al., 1998). Mutations in coding and regulatory sequences of genes are of special interest in association analyses (Sherry et al., 2000). A total of seven protein variants in the hsEH have now been identified (this study and Sandberg et al., 2000); four of these (Lys55Arg, Arg287Gln, Arg287Gln/Arg103Cys, and Arg402–403ins) have significant effects on enzyme activity in vitro, and the double mutant Arg287Gln/Arg103Cys has significant effects on apparent K_m , V_{max} , and enzyme stability. It is also important to note that the enzyme activity results are based on assays using “homozygous” mutant proteins and that the variants reported in this study exist predominantly as heterozygotes in vivo.

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Address correspondence to: David F. Grant, Department of Pharmaceutical Sciences, University of Connecticut, 372 Fairfield Road, Unit 2092, Storrs, CT 06269-2092. E-mail: david.grant@uconn.edu OR Darryl C. Zeldin, Division of Intramural Research, NIEHS, National Institutes of Health, 111 T.W. Alexander Dr., Bldg. 101, Research Triangle Park, NC 27709. Email: zeldin@niehs.nih.gov
