Altered Soluble Epoxide Hydrolase Gene Expression and Function and Vascular Disease Risk in the Stroke-Prone Spontaneously Hypertensive Rat

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Abstract—Soluble epoxide hydrolase (sEH) metabolizes epoxyeicosatrienoic acids and represents a novel therapeutic target in cardiovascular disease treatment. We investigated the relationship among sequence variation in the sEH gene (Ephx2), sEH function, and risk of end-organ injury in strains of spontaneously hypertensive rat (SHRs) differing in their susceptibility to develop brain vascular disease. Brain Ephx2 expression was significantly lower in stroke-prone (SHR/A3) than in stroke-resistant (SHR/N) SHRs (5-fold; P<0.0001). Resequencing of the Ephx2 promoter in the 2 strains identified 3 polymorphisms that significantly influenced promoter transcriptional activity in vitro. Measurements of brain sEH enzyme activity and plasma levels of arachidonate and linoleate metabolites of sEH further suggested significant differences between the 2 strains. Ratios of epoxyoctadecenoic acids to dihydroxyoctadecenoic acids were significantly higher, indicating a lower sEH activity in SHR/A3 than in SHR/N (P<0.0001). Plasma dihydroxyeicosatrienoic acid levels were lower in SHR/A3 than in SHR/N (P<0.0001), but plasma epoxyeicosatrienoic acid levels were similar in the 2 strains. Association analysis of Ephx2 polymorphism in the F2 progeny of an SHR/A3×SHR/N cross showed that animals carrying the SHR/A3 allele of Ephx2 had a greater risk of stroke and associated urinary proteinuria than animals that do not. Investigation of patterns of allelic similarities and differences among multiple stroke-prone and stroke-resistant SHR substrains showed that Ephx2 belongs to a haplotype block shared among all of the stroke-prone but no stroke-resistant substrains. These data support a role for Ephx2 polymorphism on sEH gene expression and function and risk of end-organ injury in the stroke-prone SHR. (Hypertension. 2008;51[part 2]:567-573.)

Key Words: stroke-prone SHR ■ genetics ■ cytochrome P450 ■ cardiovascular disease ■ animal model of human disease

Cytochrome P450 metabolism of arachidonic acid to epoxyeicosatrienoic acids (EETs) is emerging as a central mechanism in the regulation of cerebrovascular function. In the brain, both vascular endothelial cells and astrocytes provide a carefully regulated supply of EETs to the cerebral microvasculature and, thus, regulate cerebral blood flow.1,2 Endothelium-derived EETs cause vasorelaxation of cerebral vessels.3 EETs released from astrocytes mediate cerebral functional hyperemia and the coupling of blood flow to neuronal metabolic activity4 and induce mitogenesis and morphogenesis of cerebral capillary endothelial cells, resulting in angiogenesis.5,6 Hydrolysis of EETs to their corresponding diols by the soluble epoxide hydrolase (sEH) regulates EET levels and represents a major mechanism by which the biological effects of EETs are attenuated.7 The central role of sEH in controlling EET bioavailability underscores the potential of this enzyme as a novel therapeutic target in the treatment of cardiovascular disease and stroke.8

We previously reported single nucleotide polymorphisms in the coding sequence of the gene encoding sEH (Ephx2) among closely related inbred rat strains varying in their susceptibility to develop hypertension.9 We showed that Ephx2 sequence variation among these strains was significantly associated with variation in protein abundance and activity in the kidney but not blood pressure levels.9 In the present study, we examined the association and functional consequences of sequence variation in the Ephx2 gene in relation to hypertension-related end-organ injury in the spontaneously hypertensive rat (SHR).

Methods

Animals
Studies were performed on male, stroke-resistant SHR/Ns (CRiv) and male stroke-prone SHR/A3s (Heid) from a breeding colony maintained by the investigators and described previously.9 All of the animals were housed at 23°C on a 12-hour light-dark cycle and were
fed a standard rat chow and water ad libitum. All of the rats were euthanized at 12 weeks of age. These animals did not exhibit any evidence of stroke after behavioral assessment and histological examination at the time of sampling. Blood was collected by aortic puncture for measurements of plasma levels of oxytropins. The whole brain was excised for RNA collection. An F$_2$ cohort was bred from a cross between a male SHR/A3 and a female SHR/N. All of the animals were fed a standard rat chow and water ad libitum until age 8 weeks. Subsequently, they received a “stroke-permissive” diet (18.7% protein, 0.63% potassium, 0.37% sodium [Purina] and 1% NaCl drinking solution. From age 10 weeks until death (≤60 weeks of age), each animal was monitored daily for symptoms of stroke, including sudden weight loss, exploration and grooming deficits, torso rotation, urinary incontinence, and mild-to-severe lethargy.$^{10}$ In addition, each week, rats were individually housed in a metabolic cage for a 24-hour period for urine collection. Concurrently, food and water intake and weight were recorded. Twenty-four–hour urine protein concentrations were assayed in triplicate using the Bradford assay (Bio-Rad Laboratories). The study protocols were approved by the animal care committee of the University of Texas (Houston).

RNA Extraction and RT-PCR
Total RNA was isolated from the excised whole brain using the method of Chomczynski and Sacchi$^{11}$ adapted in the RNASTAT-60 protocol (TelTest). The 5′ nuclease (TaqMan) RT-PCR assay was used to quantitate the relative abundance between the 2 rat strains of Ephx2 mRNA. Probes and primers were custom designed so as to specifically detect the Ephx2 mRNA transcript. The sequences of the fluorescent-labeled probe and of the forward and reverse PCR primers were 5′-FAM-CCACGACTGTT, GGACGTGGC-CTATCCACA, and AAAGAATAACTCGGCGA, respectively. Reverse transcription was performed on 12.5 ng of the total RNA template (n = 6 for each strain), following the manufacturer’s protocol (Applied Biosystems). Each assay was run in triplicate. Ephx2 mRNA expression was normalized to GAPDH, and a fold change was calculated relative to SHR/N Ephx2 mRNA abundance.

Sequencing of the Ephx2 Promoter
The Ephx2 promoter was identified by bioinformatics analysis of the 5′ upstream region of the annotated Ephx2 gene in the rat genome database (Genomatix Software GmbH). Two primer pairs were designed to PCR-amplify the promoter region of Ephx2 (719 bp) from genomic DNA from each of the strains. The primer pairs were as follows: TCCTCCTAACTGGAGACCAC, CCAAGAAACT- GAAACCCCTGCA and TGGGATCCGACCATCAAGCA, GCA-CTCCGTCAGAGTGCA. PCR products were resequenced using the BigDye Terminator v1.1 Cycle Sequencing protocol (Applied Biosystems). Polymorphisms were detected through sequence alignment of PCR products obtained from each strain using the PolyPhred software.$^{12}$

Cloning of the Ephx2 Promoter
Promoter sequence of the Ephx2 gene was PCR amplified from rat genomic DNA using the forward primer CGGCTCGAGGG-AAGGACTCTGGGGCAAGGGG and the reverse primer CCAACGTTGATGGCCGGGCTGGCGGC and CCCAAGGTT-GATGGCCGGCCTGGCAGCG for SHR/N and SHR/A3, respectively. PCR products were cloned into a pG3L-Basic vector (Promega) according to manufacturer specifications.

Site-Directed Mutagenesis
In vitro site-directed mutagenesis was performed using QuikChange II Site-Directed Mutagenesis kit (Stratagene) on the previously constructed double-stranded plasmid containing the SHR/N promoter insert. Individual mutations were synthesized, as well as double-mutations, according to manufacturer instructions. All of the promoter construct sequences were verified by sequencing (Lone Star Labs, Inc).

Transfection and Luciferase Reporter Gene Assay
African Green Monkey kidney cells (Cos-1) were plated at a concentration of 1×10$^5$ cells per well in DMEM (American Type Culture Collection, Manassas, VA), supplemented with 10% FBS and 1% penicillin-streptomycin. Insert DNA (1 μg/μL) was transfected into cells using “Effectene” (Qiagen) according to manufacturer instructions. All of the transfections were performed with 5 replicates per construct, alongside a transfected empty pG3L vector and an untransfected control for background measurement. Within each transfection replicate, 3 replicate luciferase assays were conducted, totaling 15 measurements for each construct. All of the wells were cotransfected with a control β-galactosidase vector to account for possible differences in transfection efficiency. Luciferase activity was normalized against activity of the cotransfected β-galactosidase construct.

Ephx2 Polymorphism Detection
Ephx2 polymorphisms detected in this and our previous study$^{9}$ are in complete linkage disequilibrium over the short gene distance because of full inbreeding of the 2 rat strains. Thus, any 1 of them is equally suited to discriminate the SHR/A3 and SHR/N allelic forms of the Ephx2 gene. We genotyped the previously reported G405A polymorphism in 106 F2 progeny from SHR/A3×SHR/N crosses. Genotyping was performed using the TaqMan assay (Applied Biosystems), as described.$^{13}$ A 69-bp product was amplified by PCR from 15 ng of DNA using 0.9 μmol/L each of forward primer AATCCCTGTAATCTCTACCTAG and reverse primer CACT-GTCGTCAGCGGT. The sequence-specific probes (FAM-CGGTGCAATTCAACCA and VIC-ACGGCAATTGTCACCA) were used in the allele discrimination assay, and allele detection and genotyping call were performed using the ABI7900 instrument and Sequence Detection System software.

Measurement of sEH Activity
Activity of sEH in subcellular fractions of whole brain homogenates was determined using 3H-trans stilbene oxide, as described previously.$^{14}$

Measurement of Plasma Oxytropin Levels
Plasma levels of the arachidonic acid metabolites, EETs and dihydroxyeicosatrienoic acids (DHETs), and plasma levels of the linoleic acid metabolites, epoxyeicosatrienoic acids (EpoMEs), and dihydroxyeicosatetraenoic acids (DHOMEs) were measured on 12-week–old stroke-resistant SHRs (SHRSRs) and stroke-prone SHRs (SHRSPs; n = 6, respectively) using a validated high-performance liquid chromatography/mass chromatography method, as described previously.$^{15,16}$

Statistical Analyses
A t test statistic was used to assess differences in means between the 2 SHR strains. Association analyses of Ephx2 genotype data in the F2 cohort with age at onset of stroke or proteinuria were performed using Cox proportional hazards.$^{17}$ Association analyses of Ephx2 genotype data in the F2 cohort with urinary protein levels were performed using generalized estimating equations to allow for correlations between the repeated measurements.$^{18}$ Covariates included in the model were age and sex (except in sex-specific analyses). A P value of 0.05 was considered statistically significant.

Results
We detected significant differences in brain Ephx2 gene expression between SHRSPs (SHR/A3) and stroke-resistant SHRs (SHRSRs; SHR/N). Brain Ephx2 mRNA transcript levels were 5-fold lower in SHR/A3s than in SHR/Ns (P < 0.0001). These results were in agreement with our previous microarray analysis using Affymetrix RAE230A arrays showing a significantly lower (2-fold) Ephx2 brain expression in SHR/A3s than in SHR/Ns (data not shown).
To determine whether the molecular basis of the altered Ephx2 gene expression seen in the stroke-prone SHR/A3 was because of sequence variation in the cis-regulatory region, 719 bp encompassing the gene promoter were resequenced. A comparison of the DNA sequences obtained from each of the 7 strains identified 3 polymorphisms at positions −255 (T>C), −111 (G>A), and +25 (C>T) from the first transcribed nucleotide (Figure 1).

We next examined the functional consequences of these promoter polymorphisms on Ephx2 gene expression using in vitro reporter assays. Cos-1 African green monkey kidney cells are known to express the xenobiotic epoxide hydrolases19 and were chosen for the reporter gene assays. Relative to the SHR/N promoter, the mean transcriptional activity of the SHR/A3 promoter was 2.5-fold lower (P<0.00001), consistent with the differences in brain Ephx2 gene expression seen in the stroke-prone SHR/A3 compared with SHR/N (Figure 3). In both subcellular fractions, sEH activity was significantly lower in the brain of SHR/A3 than in SHR/N (cytosolic: P=0.0003; peroxisomal: P=0.01).

To determine whether the lower Ephx2 gene expression and lower sEH enzyme activity observed in the brain between SHR/A3 and SHR/N have functional implications on circulating levels of arachidonic and linoleic acid metabolites, we measured plasma levels of EET and EpOME isomers and their corresponding diol DHET and DHOME isomers in the 2 strains (Figure 4). EpOME:DHOME ratios are sensitive and specific biomarkers of sEH activity, with higher ratios indicative of lower epoxide hydrolase activity. There was no difference in circulating 14,15-EET, 11,12-EET, and 8,9-EET isomers between SHR/A3 and SHR/N (P=0.16, 0.50, and 0.39, respectively). However, plasma levels of 5,6-DHET, 11,12-DHET, and 14,15-DHET were significantly lower in SHR/A3 compared with SHR/N (P=0.0001; 2-fold: 3.6-fold: P<0.0001; and 3.6-fold: P<0.0001, respectively; Figure 4A). 12,13-EpOME:DHOME and 9,10- EpOME:DHOME ratios were significantly higher (4.2-fold: P<0.0001; 2-fold: P<0.0001; and 3.6-fold: P<0.0001, respectively; Figure 4A).
P<0.0001, respectively), consistent with lower sEH activity in SHR/A3 than in SHR/N (Figure 4B).

We next investigated whether sequence variation in the Ephx2 gene cosegregates with the onset of stroke, as measured by the onset of symptoms, and related phenotypes in the F2 hybrids from SHRSP×SHRSR crosses. The onset of urinary proteinuria is highly predictive of and can be measured more accurately than the onset of stroke20; therefore, quantitative measures of urinary protein and onset of severe proteinuria (defined as urinary protein levels >100 mg/d) were used as stroke-related phenotypes in the association analyses. There was a significant association between Ephx2 genotypes and the onset of stroke in females (hazard rate ratio [HRR]: 1.6; 95% CI: 1.2 to 2.6), but statistical significance was not reached in males (HRR: 1.1; 95% CI: 0.7 to 1.6).

There was a significant association between Ephx2 genotypes and urinary protein levels adjusting for age and sex (P<0.02). Animals with 2 SHR/A3 alleles had a significantly higher urinary protein than those carrying none, whereas animals with 1 SHR/A3 allele had intermediate levels. Analyses stratified by sex showed similar results, although statistical significance was reached only for the female group (HRR: 1.1; 95% CI: 0.7 to 1.6). There was a significant association between Ephx2 genotypes and urinary protein levels adjusting for age and sex (P=0.02). Animals with 2 SHR/A3 alleles had a significantly higher urinary protein than those carrying none, whereas animals with 1 SHR/A3 allele had intermediate levels. Analyses stratified by sex showed similar results, although statistical significance was reached only for the female group (P=0.03); however, a similar trend was observed in males (Figure 5). In agreement with these data, there was a significant association of Ephx2 genotypes with onset of severe proteinuria in the whole cohort (HRR: 1.5; 95% CI: 1.2 to 2.1) and similarly in each sex group (females: HRR: 1.4; 95% CI: 0.99 to 2.2; males: HRR: 1.5; 95% CI: 1.0 to 2.5).

Discussion

We report that the brain sEH mRNA transcripts were expressed at a lower level in the stroke-prone SHR/A3 as compared with the stroke-resistant SHR/N. This difference in Ephx2 mRNA expression between the 2 strains resulted from sequence variation in the cis-regulatory Ephx2 gene promoter. The transcriptional activity of the SHR/A3 native promoter was recapitulated through additive effects of 3 Ephx2 promoter polymorphisms. Ephx2 gene regulation and the mechanisms by which these polymorphisms may affect Ephx2 mRNA expression are unknown. In silico analysis21 of the Ephx2 promoter sequence revealed potential transcription factor binding sites that are altered by the identified polymorphisms. The −255T>C polymorphism lies in a sequence recognized by the promyelocytic leukemia zinc-finger PLZF transcription repressor; the −111G>A polymorphism lies in a sequences recognized by TGIF, a 3-amino acid loop extension (TALE) homeodomain-containing transcription factor that functions both as a corepressor of the transforming growth factor-β pathway and as a competitor of the retinoic acid pathway. Additional studies are needed to investigate the relevance of these transcription factors to Ephx2 promoter function and to characterize the molecular factors governing the regulation of the Ephx2 gene.

Synthesis of EETs, the major substrates of sEH, represents an important mechanism in the regulation of cerebrovascular function. Despite evidence of a decreased Ephx2 mRNA expression and hydrolase enzyme activity, plasma EET levels in the stroke-prone SHR/A3 were similar to those in the stroke-resistant SHR/N. Comparative analysis of brain gene expression of CYP2J2 and CYP2C11, major EET-synthesizing enzymes in brain, showed a slightly lower level for the former (−1.2 fold; P=0.004) and no significant difference for the latter (P=0.05) in SHR/A3 versus SHR/N (data not shown). This suggests that the similar levels of plasma EETs in SHR/A3 and SHR/N are unlikely to be because of feedback regulation of EET synthesis by cytochrome P450 enzymes. Moreover, this implies that sEH function does not exclusively influence plasma EET levels and may have broader physiological implications than that of regulation of EET metabolism.

EETs exhibit a variety of biological properties that may be beneficial in stroke, including vasodilation and cerebral blood flow regulation,2,4 anti-inflammation,22 promotion of cell

Figure 4. Plasma levels of EET and DHET isomers (A) and plasma EpOME:DHOME ratios (B) from SHR/N and SHR/A3 rats aged 12 weeks.

Figure 5. Association of urinary total protein (milligrams per day) in the F2 cohort of SHR/N×SHR/A3 cross. Mean urinary protein levels per Ephx2 genotype category are shown for the total cohort and for subsamples stratified on sex. *Adjusted for age and sex (except in the stratified analyses).
proliferation and angiogenesis, prevention of platelet aggregation, antiapoptosis, and cytoprotection, as demonstrated in vitro by astrocyte tolerance to ischemia-like injury induced by oxygen-glucose deprivation. The lack of differences in plasma EET levels between SHR/A3 and SHR/N suggests that the increased risk of stroke and stroke-related phenotypes conferred by the SHR/A3 Ephx2 allele may not be principally mediated through known EET effects on vascular function. Several hypotheses may be proposed to explain the relationship of decreased sEH function and increased risk of target organ injury in the SHR/A3. Although conversion of EETs to DHETs by the sEH is the primary pathway of EET metabolism, distinct but interactive pathways have been shown to metabolize EETs under various conditions. Pharmacological inhibition of the enzyme was shown to produce an increase in cellular incorporation and retention of EETs into endothelial phospholipids and to enhance the flux of EETs into alternative β-oxidation and chain elongation metabolic pathways. Pharmacological inhibition of the enzyme was also shown to lead to increased EET-mediated production of reactive oxygen species, possibly through a mechanism involving CYP450 enzymes. These data raise the possibility that altered sEH expression and/or function in SHR/A3 may promote EET metabolism through alternate pathways and that activation of these alternate pathways may adversely affect brain vascular function in these animals.

Activation of phosphatidylinositol 3-kinase/AKT and mitogen-activated protein kinase pathways by EETs has been described recently as an important novel mechanism for protection from ischemic injury. We have previously reported significant perturbations in kinase-mediated signaling in the SHR/A3, raising the possibility that interaction between altered sEH function and altered kinase-mediated signaling may play a role in susceptibility to brain vascular damage in the SHR/A3.

Compared with SHR/N, SHR/A3 exhibited significantly lower plasma DHETs. Although the vascular effects of EETs are increasingly well characterized, the significance of their metabolic products on vascular function is poorly understood. It was originally thought that DHETs are waste excretion products with no biological activity. However, a relevant role in vascular function was recently proposed for these oxylipids. 14,15-DHET was shown to activate peroxisome proliferator-activated receptor-α through a ligand-dependent mechanism. Peroxisome proliferator-activated receptor-α is a nuclear hormone receptor with known effects on lipid metabolism, atherosclerosis, and vascular inflammation. Other studies have also shown that DHETs are vasoactive and may contribute to the regulation of vascular tone in some vascular beds. These data raise the possibility that the decreased amount of DHETs produced in the SHR/A3 may impair important vascular functions and affect stroke susceptibility in this strain.

Finally, a novel phosphatase activity has been discovered recently in the N-terminal domain of the sEH and may regulate its physiological function. Isoprenoid phosphates have been proposed as substrates of the N-terminal domain of the enzyme, suggesting a possible physiological function in sterol synthesis or inflammation. The possibility that the relationship between increased risk for target organ injury and altered sEH function in the SHR/A3 is mediated through effects of N-phosphatase activity remains to be explored.

The complex relationship among EETs, sEH, and ischemic brain injury is further underscored by a recent pharmacological study, reporting that sEH inhibition was associated with lower infarct volume in the mouse brain subjected to middle cerebral artery occlusion. This effect was not mediated through the known effects of EETs on the regulation of cerebral blood flow. Moreover, previous treatment with an inhibitor of EET synthesis prevented the protection from ischemic damage associated with sEH inhibition. However, there was no effect of inhibition of EET synthesis on ischemic damage, raising the possibility that the beneficial effects of sEH inhibition in this model may be mediated by mechanisms unrelated to the modulation of EET levels, including activation of peroxisome proliferator-activated receptor-α and/or vasodilation of resistance vessels.

Ephx2 polymorphism was associated with variation in the risk of stroke and stroke-related phenotypes among SHR strains. We have demonstrated previously that Ephx2 gene sequence variation and function were not primary determinants of blood pressure levels in SHR strains, thus, possible confounding effects of blood pressure on these associations are unlikely. There is increasing evidence from this and other studies that the Ephx2 gene polymorphism influences the risk for cardiovascular disease and stroke. We have demonstrated previously that common Ephx2 haplotypes are associated with both increased and decreased incidence of stroke in human populations. Functional assays in vitro have provided insights about the impact of human Ephx2 polymorphisms on neuronal survival after ischemic injury induced by oxygen-glucose deprivation, with some variants of the gene associated with increased neuronal survival and others with increased neuronal cell death. Although the neuroprotective/neurotoxic effects of Ephx2 variants were directly linked to enzyme activity, our data suggest that the relationship between sEH and risk for stroke in vivo may be more complex than predicted by in vitro experiments.

Perspectives

Although we cannot exclude the possibility that the observed association may be because of sequence variation in another linked gene(s), evidence from this and other studies for a role of Ephx2 in vascular injury in the SHRSP is further reinforced by our analysis of patterns of allelic similarities and differences among various stroke-prone and stroke-resistant strains using the genomic resources of the National BioResource Project for the Rat in Japan. We compared alleles at microsatellites mapping to chromosome 15 among 8 substrains of SHRSP, including animals from the A3 substrains and 8 substrains of SHRSR, to determine whether the Ephx2 gene region may belong to a haplotype block shared among all of the stroke-prone SHR that differs from that observed in SHRSRs. We found that the Ephx2 gene belongs to a haplotype block shared among all of the SHRSP substrains but not SHSRs, further suggesting that ≥1 gene in this haplotype block may contribute to the risk for vascular...
injury (Figure 6). Importantly, other than Ephx2, no known gene mapping in this haplotype block was found differentially expressed between the SHR/A3 and SHR/N (data not shown). In addition, a QTL influencing cholesterol levels in SHRSP was shown recently to map to this region of chromosome 15.46 Similar to our data, the effects of this QTL showed a significant sexual dimorphism and were stronger in females than in males. Whether the increased risk for end-organ injury associated with altered sEH function in SHR/A3 is mediated through effects on cholesterol is intriguing but speculative. Recent data reporting a role for sEH in the catalysis of phosphorylated lipid precursors of cholesterol biosynthesis and isoprenylation40 and the association of variation in the human Ephx2 gene with familial hypercholesterolemia47 are consistent with such a possibility. Our data demonstrating a lack of a relationship between altered Ephx2 gene function and plasma EET levels further underline that the scope of epoxide hydrolase function extends beyond that on EET level regulation. Additional studies in transgenic and congenic animals are needed to address this hypothesis and to further investigate the molecular mechanisms underlying the relationship between altered sEH and increased risk for vascular injury in the stroke-prone SHR/A3.

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Disclosures

None.

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