Neurobiology of Disease

Polymorphisms in the Human Soluble Epoxide Hydrolase Gene *EPHX2* Linked to Neuronal Survival after Ischemic Injury

Ines P. Koerner,[1] Rachel Jacks,[1] Andrea E. DeBarber,[2] Dennis Koop,[2] Peizhong Mao,[3] David F. Grant,[4] and Nabil J. Alkayed[1,2]

[1]Departments of Anesthesiology and Peri-Operative Medicine, [2]Physiology and Pharmacology, and [3]Public Health and Preventive Medicine, Oregon Health & Science University, Portland, Oregon 97239-3098, and [4]Department of Pharmaceutical Sciences, University of Connecticut, Storrs, Connecticut 06269

Single nucleotide polymorphisms (SNPs) in the human *EPHX2* gene have recently been implicated in susceptibility to cardiovascular disease, including stroke. *EPHX2* encodes for soluble epoxide hydrolase (sEH), an important enzyme in the metabolic breakdown of arachidonic acid-derived eicosanoids referred to as epoxyeicosatrienoic acids (EETs). We previously demonstrated that EETs are protective against ischemic cell death in culture. Therefore, we tested the hypothesis that polymorphisms in the human *EPHX2* gene alter sEH enzyme activity and affect neuronal survival after ischemic injury *in vitro*. Human *EPHX2* mutants were recreated by site-directed mutagenesis and fused downstream of TAT protein transduction domain. Western blot analysis and immunocytochemistry staining revealed high-transduction efficiency of human TAT-sEH variants in rat primary cultured cortical neurons, associated with increased metabolism of 14,15-EET to corresponding 14,15-dihydroxyeicosatrienoic acid. A human variant of sEH with Arg103Cys amino acid substitution, previously demonstrated to increase sEH enzymatic activity, was associated with increased cell death induced in cortical neurons by oxygen-glucose deprivation (OGD) and reoxygenation. In contrast, the Arg287Gln mutation was associated with reduced sEH activity and protection from OGD-induced neuronal cell death. We conclude that sequence variations in the human *EPHX2* gene alter susceptibility to ischemic injury and neuronal survival in a manner linked to changes in the hydrolase activity of the enzyme. The findings suggest that human *EPHX2* mutations may in part explain the genetic variability in sensitivity to ischemic brain injury and stroke outcome.

Key words: **EETs; neuroprotection; stroke; ischemia; eicosanoids; protein transduction domain; P450 epoxygenase**

Introduction

Stroke is a multifactorial disease with a strong genetic predisposition. The precise genetic alterations underlying stroke susceptibility and outcome remain unknown (Markus and Alberts, 2006). A number of DNA sequence variations have been linked to stroke, but the role of these mutations in the pathogenesis of stroke remains unknown. The current study examines the impact of sequence variations in one such gene, *EPHX2*, on neuronal susceptibility to ischemic injury and its biochemical basis. Polymorphisms in the human *EPHX2* gene have been linked to the risk of cardiovascular disease, including stroke (Farin et al., 2001; Fornage et al., 2004, 2005; Sato et al., 2004; Dreisbach et al., 2005; Ohtoshi et al., 2005; Lee et al., 2006; Wei et al., 2006). The *EPHX2* gene encodes for soluble epoxide hydrolase, which metabolizes P450 arachidonic acid epoxygenase metabolites referred to as epoxyeicosatrienoic acids (EETs) to dihydroxyeicosatrienoic acids (DHETs). EETs are endogenous brain mediators produced by astrocytes (Alkayed et al., 1996). They exhibit a variety of biological activities that are beneficial in stroke, including vasodilation (Alkayed et al., 1996), anti-inflammation (Node et al., 1999), anti-apoptosis (Chen et al., 2001), anti-pyresis (Kozak et al., 2000), prevention of platelet aggregation (Heizer et al., 1991), and promotion of cell proliferation and angiogenesis (Davis et al., 2002). Furthermore, we have recently demonstrated that EETs exhibit direct cytoprotective property, as evidenced by their ability to reduce ischemic cell death induced in primary cultured astrocytes by oxygen-glucose deprivation (OGD) (Liu and Alkayed, 2005). Given the important protective role of EETs against cerebral ischemia, polymorphisms in *EPHX2* that affect soluble epoxide hydrolase (sEH) activity and alter EET levels in brain may have significant impact on neuronal sensitivity to ischemic events and stroke outcome.

A number of single nucleotide polymorphisms (SNPs) in human *EPHX2* have been linked to cardiovascular disease risk, including increased risk of coronary heart disease (Fornage et al., 2004; Lee et al., 2006), hyperlipoproteinemia (Sato et al., 2004), and type-2 diabetes (Ohtoshi et al., 2005). More relevant to the current study, different *EPHX2* haplotypes correlate differently

Received Sept. 25, 2006; revised March 20, 2007; accepted March 22, 2007.

This work was supported by National Institutes of Health NS44313 and NS049210 to N.J.A. and ES011630 and GM56708 to D.F.G.

Correspondence should be addressed to Dr. Nabil J. Alkayed, Associate Professor, Department of Anesthesiology and Peri-Operative Medicine, Oregon Health & Science University, 3181 S.W. Sam Jackson Park Road, UHS-2, Portland, OR 97239-3098. E-mail: alkayedn@ohsu.edu.

DOI:10.1523/JNEUROSCI.0056-07.2007

 0270-6474/07/274642-08$15.00/0

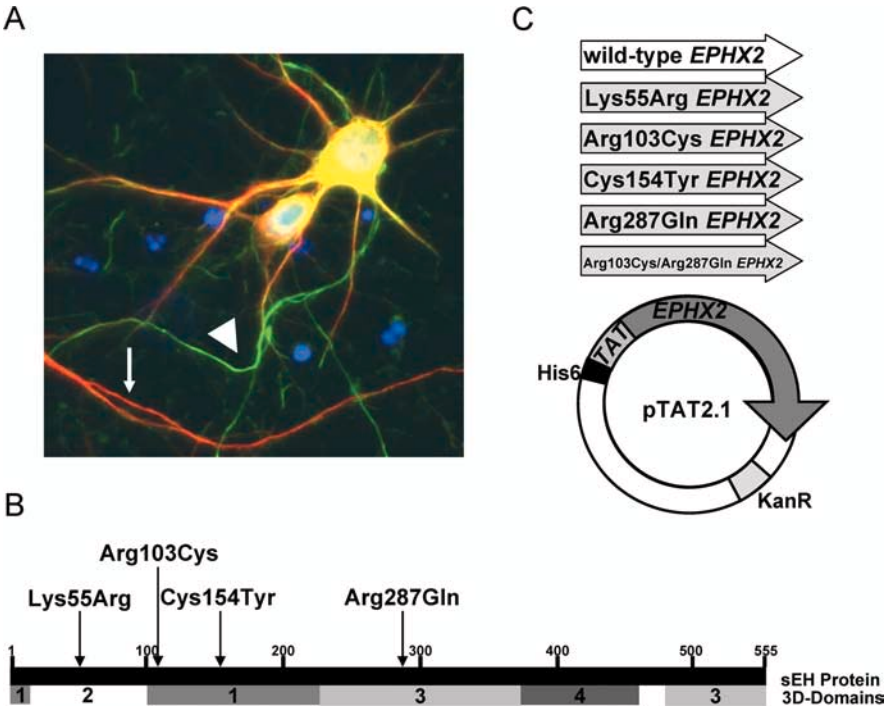

Figure 1. Genetic polymorphisms and localization of soluble epoxide hydrolase (sEH). ***A***, Colabeling with dendritic marker MAP-2 (red) localizes sEH immunoreactivity (green) in cytoplasm (yellow) and axons (arrowhead) but not dendrites (arrow) of primary cultured cortical neurons. ***B***, Amino acid changes associated with *EPXH2* mutations and their localization within the amino acid sequence (1–555) and structural domains (1–4) of human sEH. ***C***, Cloning strategy for TAT-hsEH fusion protein. Variants of human *EPXH2* were inserted downstream of TAT (47–57) protein transduction domain in pTAT2.1 vector. KanR, Kanamycin resistance.

with stroke incidence, with some haplotypes associating with increased and others with decreased stroke risk (Fornage et al., 2005). Polymorphisms in human *EPHX2* have also been shown to alter EET conversion by purified sEH *in vitro* (Przybyla-Zawislak et al., 2003). In the current study, we examined the impact of altered sEH activity on neuronal survival. We introduced common human sEH variants into primary cultured neurons using TAT-mediated protein transduction and assessed their effects on OGD-induced ischemic cell death. Our findings indicate that different polymorphisms in *EPHX2* may increase or decrease susceptibility to ischemic cell death depending on whether the mutation increases or decreases sEH hydrolase activity and EETs metabolism. The findings are consistent with genetic studies linking these mutations to stroke risk in humans, and suggest that pharmacological inhibition of sEH may be used selectively to decrease stroke risk and stroke related brain damage in carriers of mutant alleles linked to increased sEH activity and stroke risk.

Materials and Methods

Timed-pregnant Sprague Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). The pTAT2.1 vector was kindly provided to us by Dr. S. Dowdy (University of California, San Diego, CA). Rabbit polyclonal antibody against soluble epoxide hydrolase was kindly provided by Dr. Bruce Hammock (University of California, Davis, CA). The antibody recognizes mouse, rat, and human sEH. All chemicals and reagents were purchased from Sigma (St. Louis, MO), unless otherwise indicated.

Primary cortical neuronal culture. Primary cultures of cortical neurons were prepared from fetal rats on embryonic day 18 (E18). Cortices were dissected in HEPES-buffered HBSS, containing sodium bicarbonate (0.35 g/L), glucose (6 g/L), BSA (0.3 g/L), and magnesium sulfate (0.97 g/L) and dissociated by digestion with trypsin (Invitrogen, Carlsbad, CA) and trituration. Cells were seeded at a density of 1.5×10^5 cells/cm^2 onto poly-D-lysine (100 μg/ml) coated 24-well plates (Corning, Corning, NY) and were maintained in a humidified incubator in air with 5% CO_2. Neurons were cultured in Neurobasal medium without phenol red (Invitrogen) supplemented with 2% B27 (Invitrogen), 1% Glutamax (Invitrogen), and 1% penicillin/streptomycin (Invitrogen). At day 3 *in vitro* (DIV 3) and DIV 7, one-half of the culture medium was replaced with fresh medium. Cytosine-1-β-D-arabino furanoside (1 μM; Fluka, Buchs, Switzerland) was added to culture medium on DIV 3 to suppress growth of glial cells. Cultures consisted of >98% microtubule-associated protein 2 (MAP2)-positive and <2% glial fibrillary acidic protein (GFAP)-positive cells (DIV 10).

Construction of vectors to express TAT-human sEH fusion proteins. Locations of EPHX2 mutations within the human soluble epoxide hydrolase gene and our cloning strategy are shown in Figure 1, *B* and *C*. Construction of plasmids containing wild-type (WT) and mutant human *EPHX2* is as described previously (Przybyla-Zawislak et al., 2003). Briefly, after PCR amplification and introduction of a 5′ *Bam*HI restriction site and a 3′ *Xho*I site, WT human *EPHX2* was subcloned into pCR-Script vector (Stratagene, La Jolla, CA) and subjected to site-directed mutagenesis using the QuikChange mutagenesis system (Stratagene). To generate the TAT-human sEH (hsEH) fusion proteins, WT and mutant *EPHX2* cDNAs were subcloned into *Bam*HI/*Xho*I sites of the pTAT2.1 vector downstream of TAT (47–57). Plasmids were sequenced to verify proper insertion.

Protein expression and purification. Plasmids containing WT or mutant *EPHX2* inserts in frame with TAT were used to transform BL21 Star *Escherichia coli* (Invitrogen). Bacteria were grown in LB medium (Invitrogen) containing kanamycin to an OD_{600} of 0.6, and protein expression was induced with 1 mM isopropyl-b-D-thiogalactopyranoside (EMD Biosciences, San Diego CA) for 4.5 h. Bacteria were pelleted and sonicated in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole, pH 8.0) containing 1 mg/ml lysozyme. After RNase A and DNase 1 digestion, lysates were centrifuged at 10,500 × *g* at 4°C for 30 min. For protein purification, TAT-hsEH fusion protein was bound to Ni-NTA agarose slurry (Qiagen, Valencia, CA), washed three times with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 20 mM imidazole, pH 8.0), and eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole, pH 8.0). Eluted proteins were dialyzed against three changes of PBS (Invitrogen) containing 2 mM $MgCl_2$ and 50 mM HEPES (Invitrogen) and filter sterilized. Protein concentration was measured with a Bio-Rad (Hercules, CA) protein assay using a BSA standard curve. Protein purity was verified by Coomassie staining (Bio-Rad) of SDS-PAGE gels and by Western blot using anti-sEH antibody.

Transduction of TAT-hsEH fusion proteins into primary neurons. To evaluate the ability of TAT-hsEH fusion proteins to penetrate neuronal cell membranes, TAT-hsEH was added to the medium of primary cortical neurons to final concentrations between 10 nM and 1 μM. After various incubation times, culture medium was removed, and cells were washed three times with PBS and fixed for immunocytochemistry or lysed for immunoblotting.

Immunocytochemistry. Neurons were cultured as above on poly-D-lysine-coated 12-mm-diameter glass coverslips. Coverslips were rinsed three times with PBS and fixed in 4% paraformaldehyde in PBS for 30 min. After three rinses, cells were blocked for 1 h with blocking solution containing 2% normal goat serum, 1% BSA, and 0.1% Triton-X in PBS

and rinsed twice with PBS. Cells were incubated overnight at 4°C with rabbit polyclonal antibodies against soluble epoxide hydrolase or against His-Tag (Rockland Immunochemicals, Gilbertsville, PA). Immunoreactivity was visualized using a biotin-conjugated goat anti-rabbit antibody and cyanine 2 (Cy2)-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA). Cells were counterstained using mouse antibodies against neuronal marker MAP2 (Millipore, Bedford, MA) or astrocytic marker GFAP (Millipore) and Cy3-conjugated goat anti-mouse (Jackson ImmunoResearch). Coverslips were mounted in ProLong Antifade Mounting Medium containing DAPI (4′,6′-diamidino-2-phenylindole dihydrochloride) (Invitrogen).

Immunoblotting. Primary cortical neurons were cultured as above in poly-D-lysine coated six-well plates (Corning). On DIV 10, cells were scraped and lysed with 100 μl of boiling lysis buffer (100 mM Tris, pH 6.8, 2% SDS, 20% glycerol, 10% β-mercapto-ethanol, 0.025% bromphenol blue). Samples were separated by SDS-PAGE (90 min, 200 V) and transferred to PVDF membranes (Invitrogen) at 35 V for 2 h. Transfer efficiency was verified by Ponceau S staining. After blocking with 5% dry milk in PBS containing 0.1% Tween, blots were incubated overnight at 4°C with primary rabbit antibody against soluble epoxide hydrolase (1:5000 in 5% dry milk). Signal was detected using a biotinylated secondary antibody (GE Healthcare, Piscataway, NJ) with an ECL plus (GE Healthcare) chemiluminescence detection kit. Blots were re-probed for neuronal marker β-tubulin (Millipore) to ensure equal loading. Exposed x-ray films were scanned and analyzed using QuantityOne (Bio-Rad) software.

sEH activity assay. Activity of TAT-hsEH fusion proteins was evaluated by the conversion of 14,15-EETs to corresponding 14,15-DHETs in TAT-hsEH-transduced neurons. Cortical neurons grown in six-well plates were transduced with TAT-hsEH fusion protein (100 nM final concentration) on DIV 7. On DIV 10, cells were exposed for 1 h to 1 μM 14,15-EET, medium was removed, and cells were scraped in 1 ml of PBS per well. Medium and cells were stored at −80°C. To measure 14,15-DHET, culture medium, and cells were treated with 40 μl of 1N formic acid and 5 μl of 1% butylated hydroxytoluene in methanol. Untreated medium and cells spiked with 500 pg of 14,15-DHET were used as positive controls. After spiking with the internal standard d_8-15-hydroxyeicosatetranoic acid (d_8-15-HETE), samples were extracted twice with a (1:1) hexane:ethyl acetate mixture. The organic layers were dried, and the residue was re-dissolved in (55:45) water:acetonitrile and subjected to liquid chromatography (LC)-tandem mass spectrometry (MS) performed using a triple stage TSQ Quantum Discovery quadrupole mass spectrometer (Thermo Electron, San Jose, CA) equipped with an electrospray ionization source. The ionization interface was operated in the negative mode using the following settings: spray voltage, 1.8 kV; sheath gas pressure, 30 U; auxiliary gas pressure, 10 U; capillary temperature, 270°C; capillary offset, −35 V; and tube lens offset, 103 V. The Q2 collision gas pressure was set to 0.8 mTorr. The LC-MS system included an in-line Surveyor autosampler at 4°C and HPLC pump (Thermo Electron). The samples were resolved using a 2.1 × 250 mm, 5 μm BetaBasic C18 HPLC column with guard (ThermoHypersil) maintained at 30°C. Gradient elution at a flow rate of 0.25 ml/min was used with water (A) and acetonitrile (B) both at 0.002% acetic acid, using the following conditions: 45–60% B over 1 min, linear to 65% B over 15 min, isocratic at 65% B for 10 min, linear to 95% B over 0.1 min, isocratic at 95% B for 5.8 min, linear to 45% B over 0.1 min, isocratic at 45% B for 14 min. The first 12 min of the HPLC run were diverted to waste and all available DHET regioisomers were resolved with acceptable peak shape. Infusion experiments were conducted to optimize ionization and fragmentation for each analyte and a selected reaction monitoring instrument method was created for analyte detection. Transitions monitored were m/z 327.2 → 226 for d_8-15-HETE (collision energy, 15 V) and m/z 337.2 → 237 for 14,15-DHET (collision energy, 17 V) Scan event settings were scan width 1 m/z, scan time 0.2 s, and Q1 and Q3 peak widths at 0.6 and 0.8, respectively. Production of 14,15-DHET was compared by obtaining the peak area ratios for 14,15-DHET to the internal standard for all samples. Data acquisition and quantitative processing was accomplished using Xcalibur software, version 2.0 (Thermo Electron).

Effects of OGD on sEH activity were assessed in a similar manner, except that 14,15-DHET was measured by the 14,15-DHET immunoassay kit ELISA (Detroit R&D, Detroit, MI). Neuronal cultures were prepared as above. On DIV 10, cells were exposed for 1 h to 1 μM 14,15-EET (baseline), or were exposed to 2 h of OGD, followed by 1 h exposure to 1 μM 14,15-EET (OGD). Medium was then removed, and cells were scraped in 500 μl of PBS per well and sonicated for 5 s. Medium and cells were purified on a 14,15-DHET affinity column (Detroit R&D), vacuum-dried, and redissolved in methanol. 14,15-DHET concentration was measured according to the manufacturer's instructions.

Oxygen-glucose deprivation and cell death assay. To simulate ischemia, on DIV 10, primary cortical neurons were exposed to OGD. OGD was performed in a Coy anoxia chamber filled with an anoxic gas mixture containing 5%H_2/5%CO_2/90%N_2 at 37°C. Anoxic conditions were confirmed using disposable anaerobic indicator strips (BBL GasPak; BD Biosciences, San Jose, CA). Culture medium was removed inside the anoxia chamber and cells were washed once to remove traces of oxygen and glucose. Culture medium was replaced with degassed balanced salt solution (140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 10 mM HEPES, and 30 μM glycine). After 2 h of OGD, balanced salt solution was exchanged for prewarmed culture medium, and cells were returned to the normoxic incubator. Control cells were kept under normoxic conditions, and medium was exchanged for fresh culture medium. After 24 h of reoxygenation, neuronal cell death was assessed by lactate dehydrogenase (LDH) release into the culture medium (LDH cytotoxicity detection kit; Roche Diagnostics, Indianapolis, IN). Data from 3 to 5 wells per condition per experiment were averaged to $n = 1$. Experiments were repeated on 20–25 independent cultures from different litters. Neuronal viability was also determined spectrophotometrically using the 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) assay. The assay is based on the reduction of MTT to purple-colored formazan by mitochondrial dehydrogenases. As such, the assay provides an assessment of mitochondrial function and is most sensitive to mitochondrial mechanisms of cell death. MTT (16.2 μl/ml) and stock solution (5 mg/ml) were added to the medium, and the reaction was terminated 1 h later. Cells were lysed with DMSO, and MTT reduction was determined fluorometrically at 540 nm.

To evaluate the effects of human sEH mutations on neuronal cell death, neurons were transduced with TAT-hsEH fusion proteins (100 nM final concentration) on DIV 7 before being exposed to oxygen-glucose deprivation on DIV 10. As control, cells were also transduced with TAT-linked enhanced green fluorescent protein (TAT-EGFP) to ensure specificity. To ensure that effects of the TAT-hsEH fusion proteins were caused by soluble epoxide hydrolase activity, excess substrate 14,15-EET (Cayman Chemical, Ann Arbor, MI) was added to the transduced cells, or sEH activity was inhibited by 4-phenylchalcone oxide (4-PCO; Biomol, Plymouth Meeting, PA) (Mullin and Hammock, 1982; Draper and Hammock, 1999). 14,15-EET (1 μM) or 2 μM 4-PCO were added to the culture medium 1 h before OGD as well as during OGD and during reoxygenation. To rule out the possibility that changes in neuronal survival were related to DHET, we also tested the effect of 14,15-DHET (1 μM) on OGD-induced cell death.

Data analysis and statistics. Results are expressed as mean ± SEM. Differences in cell death values among different sEH mutations were evaluated using one-way ANOVA, and interactions of sEH mutations with drug treatments were evaluated with two-way ANOVA. Significance was set at $p < 0.05$.

Results

Soluble epoxide hydrolase expression and distribution in primary cortical neurons

All experiments were conducted in rat primary cultured cortical neurons. To determine whether sEH is endogenously expressed in cortical neurons, we used immunocytochemistry with anti-sEH antibody. Strong sEH immunoreactivity was detected in primary cortical neurons, but not in astrocytes, as evidenced by colocalization with neuronal MAP2 (Fig. 1*A*, yellow color) but not with astrocytic marker GFAP (data not shown). Because MAP2 specifically stains neuronal cell bodies and dendrites, but not axons, Figure 1*A* demonstrates that sEH is localized in

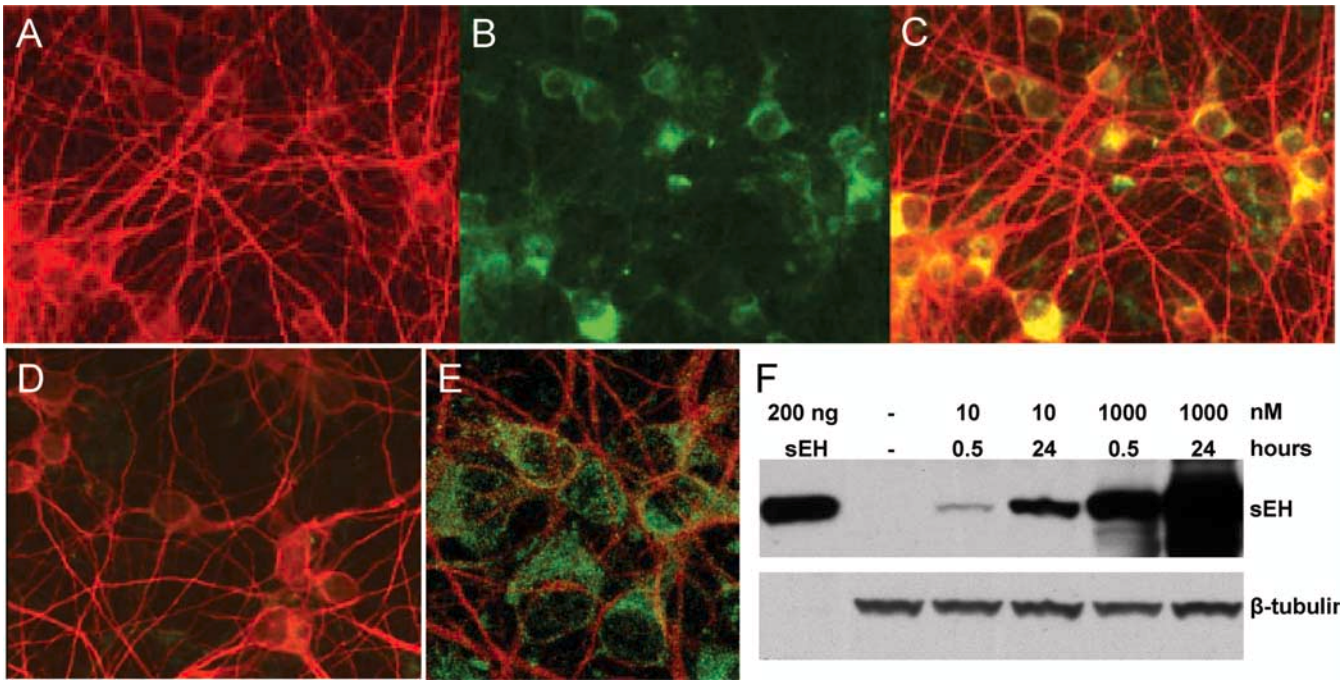

Figure 2. Efficient transduction and cytoplasmic localization of TAT-hsEH fusion protein in primary cultured cortical neurons. ***A–C***, neurons at 6 h after transduction with 1 μM wild-type TAT-hsEH. ***A*** is stained with anti-MAP2 antibody; ***B*** is stained with anti-HIS-tag antibody; and ***C*** is an overlay of ***A*** and ***B***, illustrating cytoplasmic localization of HIS-tagged TAT-hsEH fusion protein in transduced neurons. ***D***, Untreated cells; ***E*** is a confocal image through ***C***. ***F***, Western blot with anti-sEH antibody showing dose- and time-dependent increase in sEH level after transduction with wild-type TAT– hsEH fusion protein.

MAP2-negative axons (green processes) but not MAP2-positive dendrites (red processes).

TAT-hsEH transduction in primary cortical neurons

To determine the effect of *EPHX2* polymorphisms on ischemic neuronal death *in vitro*, we introduced WT and mutant forms of human sEH protein into primary cultured neurons. We took advantage of the ability of HIV (human immunodeficiency virus)-derived TAT protein transduction domain to penetrate and shuttle high molecular weight proteins across neuronal cell membranes (Nagahara et al., 1998; Becker-Hapak et al., 2001). Figure 1*C* illustrates the strategy we used to construct and express fusion proteins containing TAT-linked hsEH variants. Five sEH mutants containing amino acid changes Lys55Arg, Arg103Cys, Cys154Try, Arg287Gln, and Arg287Gln plus Arg103Cys (double mutant) encoded by common human *EPHX* SNPs (Przybyla-Zawislak et al., 2003), in addition to WT, were fused downstream of TAT protein transduction domain (11 aa). Figure 1*B* illustrates the locations of amino acid changes. Primary cultured cortical neurons were transduced with purified fusion proteins, and transduction efficiency was verified by immunoblotting with anti-sEH antibody and immunocytochemistry with anti-His tag antibody, to distinguish between recombinant and endogenous sEH. Strong immunoreactivity for the His tag was detected in the cytoplasm of all neurons examined at 6 h after incubation with purified WT TAT-hsEH fusion protein (1 μM final concentration in culture media) (Fig. 2*A–E*). Immunoblotting revealed a time- and dose-dependent increase in sEH immunoreactivity in primary cortical neurons after incubation with WT TAT-hsEH. Figure 2*F* shows that high levels of sEH are detected in neurons as early as 30 min after incubation with 10 nM WT TAT-hsEH, and that levels are further increased with increasing protein concentration and incubation time. Transduced proteins were stable and did not adversely affect baseline neuronal survival. Expression of WT TAT-hsEH was detected at high levels for up to 4 d after application, which did not induce any detectable cell death for up to 1 μM concentrations (data not shown).

Transduced TAT-hsEH is functionally active in primary cortical neurons

To determine whether transduced sEH was functionally active in neurons, we measured the ability of sEH-transduced primary cortical neurons to convert exogenously applied 14,15-EET to corresponding 14,15-DHET. When incubated with 14,15-EET, untransduced neurons produced a small peak of 14,15-DHET, reflecting baseline sEH activity in primary cortical neurons (Fig. 3, bottom left chromatogram). In contrast, the conversion of 14,15-EET to 14,15-DHET was markedly increased in primary neurons transduced with WT TAT-hsEH (Fig. 3, bottom middle chromatogram), indicating that the transduced soluble epoxide hydrolase was functionally active. Compared with naive, untransduced cultures, levels of 14,15-DHET were 131 $\pm$ 39 and 283 $\pm$ 12% in cells ($n = 6$) and media ($n = 8$) from cultures transduced with WT TAT-hsEH. To determine whether amino acid changes were associated with changes in sEH activity, we compared DHET levels in cultures transduced with TAT-hsEH variants to levels produced by WT TAT-hsEH. As depicted in Figure 3*B*, the most striking difference from WT sEH activity was observed for Lys55Arg and Arg287Gln mutations, which were associated with increased (216 $\pm$ 72% vs WT, $n = 3$) and decreased (34 $\pm$ 17% vs WT, $n = 5$) (Fig. 3*A*, bottom panel, right vs middle chromatograms) hydrolysis of 14,15-EET, respectively. Enzymatic activities associated with all other mutants were not different from WT ($p = 0.329$).

To determine whether hydrolase activity was altered by OGD, we measured the level of 14,15-DHET produced by neuronal cultures using ELISA, as described in Materials and Methods. We found that in the presence of excess 14,15-EET (1 μM), the level of

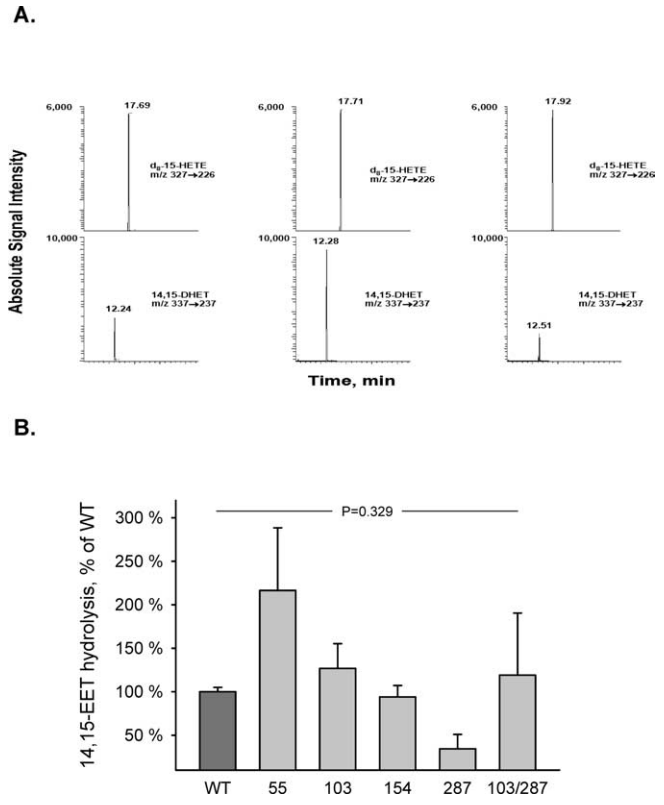

Figure 3. TAT-mediated protein transduction produces high levels of functionally active sEH in primary cultured neurons. ***A***, Detection of 14,15-DHET by selected reaction monitoring results in extracted ion chromatograms showing enhanced conversion of 14,15-EET (1 μM) to 14,15-DHET (peak at 12.28 min) in neurons transduced with wild-type TAT-hsEH (bottom middle panel) compared with untreated cells (bottom left panel). 14,15-EET conversion in Arg287Gln TAT-hsEH transduced neurons (bottom right panel) was not different from untreated cells. Top, Internal standard extracted ion chromatograms for each assay. ***B***, Summary graph depicting changes in 14,15-EET metabolism when neurons are transduced with various sEH variants compared with wild-type (WT) sEH.

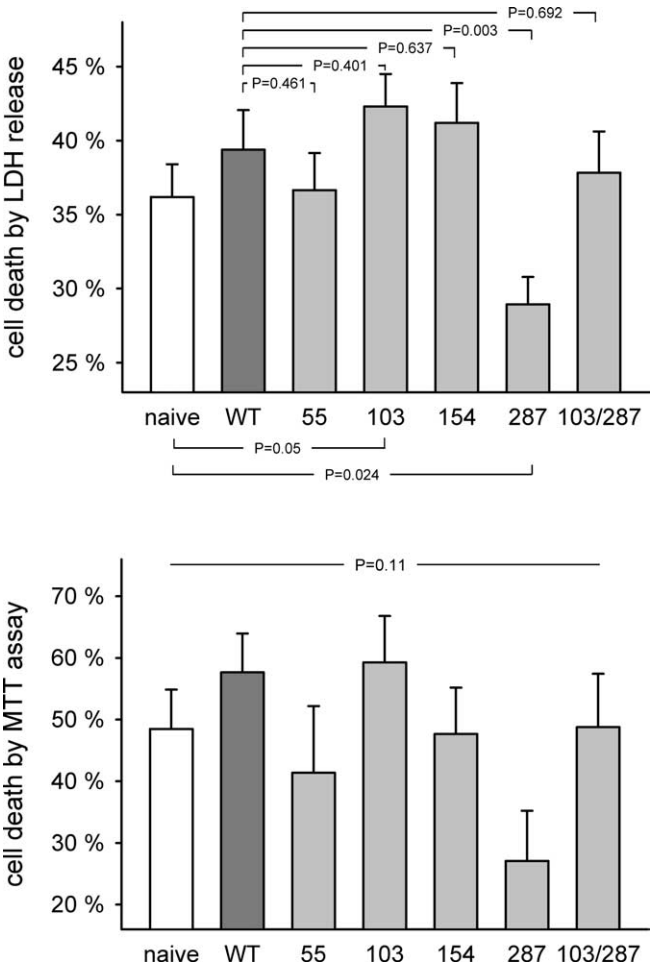

Figure 4. Variants of soluble epoxide hydrolase differentially affect neuronal survival after oxygen-glucose deprivation. ***A***, ***B***, Cell death after oxygen-glucose deprivation was measured using the LDH (***A***) and MTT assays (***B***). ***A***, Cell death was increased in neurons transduced with Arg103Cys TAT-hsEH and decreased in neurons transduced with Arg287Gln TAT-hsEH compared with untreated cells ($p = 0.05$ and 0.024, respectively). Only the Arg287Gln variant was statistically different compared with wild-type (WT) sEH variant ($p = 0.003$). ***B***, Similar trends were observed when mitochondrial mechanisms of cell death were assessed by the MTT assay, although differences among groups were not statistically significant ($p = 0.11$).

14,15-DHET produced by untransduced neuronal cultures was increased after OGD by 60%, from 5059 ± 236 to 8184 ± 342 pg/ml ($n = 3$).

Effect of EPHX2 mutations on OGD-induced neuronal cell death

OGD was used to induce ischemic cell death in primary cortical neurons transduced with WT and mutant TAT-hsEH. Two hours of OGD induced 36.2 ± 2.2% death in untreated primary cortical neurons, as assessed by lactate dehydrogenase release at 24 h of reoxygenation (Fig. 4*A*, naive) ($n = 25$). Transduction with TAT-hsEH fusion proteins impacted neuronal after OGD in a differential manner (Fig. 4), whereas transduction with TAT-EGFP had no effect on cell death (12 wells per condition; $n = 2$ different cultures). Overexpression of WT TAT-hsEH increased the percentage of neuronal cell death to 39.4 ± 2.7% compared with untransduced cells, although this difference was not statistically significant ($p = 0.08$). Similarly, neurons transduced with the Arg103Cys "103" TAT-hsEH variants showed a trend for increased cell death (42.3 ± 2.2%%; $n = 22$). These values were significantly higher than cell death in untransduced cells, although the differences from WT were not statistically different. In contrast, neurons transduced with the Arg287Gln TAT-hsEH mutant were significantly protected from OGD-induced cell death, compared with either untransduced neurons (28.9 ± 1.9%; $n = 22$; $p < 0.05$) or neurons transduced with WT sEH. The Lys55Arg variant (36.7 ± 2.5%) and the double-mutant TAT-hsEH (37.8 ± 2.8%) had no effect on neuronal survival after OGD. We also estimated cell death using the MTT assay, which provides an assessment of mitochondrial functional integrity and is most sensitive to mitochondrial mechanisms of cell death. In agreement with the LDH assay, cell death was increased in neurons transduced with WT and Arg103Cys TAT-hsEH, decreased in cells transduced with Arg287Gln and unchanged in cells transduced with Lys55Arg, Cys154Tyr, and the double mutant. None of these differences were statistically significant (Fig. 4*B*) ($p = 0.11$).

Exogenous 14,15-EET prevents the increase in cell death by TAT-hsEH

To determine whether impaired neuronal survival in TAT-hsEH transduced neurons can be rescued by excess 14,15-EET, we co-applied 14,15-EET (1 μM) with TAT-hsEH. 14,15-EET prevented the increase in neuronal cell death in cultures caused by sEH overexpression. The difference was statistically significant for

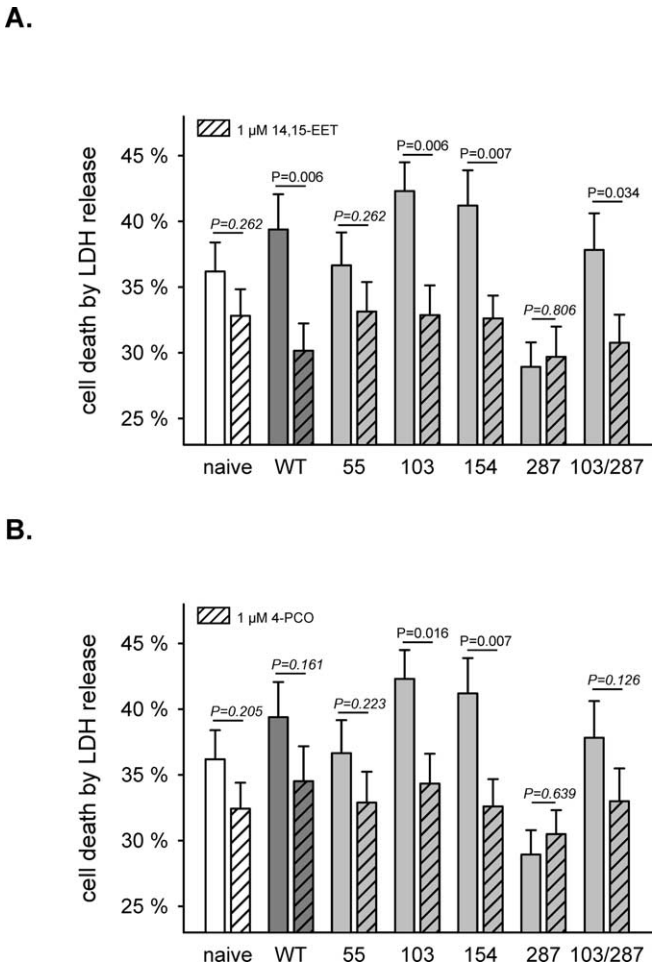

Figure 5. Neuronal rescue from TAT-hsEH induced cell death by sEH substrate addition and pharmacological inhibition. ***A***, Excess 14,15-EET, the substrate for soluble epoxide hydrolase, prevents the increase in cell death induced by TAT-hsEH. ***B***, Inhibition of soluble epoxide hydrolase by 4-PCO prevents the increase in OGD-induced cell death caused by TAT-hsEH. WT, Wild type.

four variants: WT, Arg103Cys, Cys154Tyr, and Arg103Cys/Arg287Gln variants of TAT-hsEH (Fig. 5*A*). No effect of 14,15-DHET was observed on neuronal survival after OGD. These findings suggest that the increase in cell death in transduced neurons is likely related to increased EETs hydration by sEH overexpression.

Pharmacological inhibition of sEH prevents the increase in cell death by TAT-hsEH

To determine whether the effects of EPHX2 SNPs on neuronal survival are specifically linked to sEH hydrolase activity, cells transduced with sEH variants were treated with the epoxide hydrolase inhibitor 4-PCO (2 μM) (Mullin and Hammock, 1982; Draper and Hammock, 1999). 4-PCO treatment prevented the increase in cell death caused by the overexpression of sEH; the difference being statistically significant for Arg103Cys and Cys154Tyr TAT-hsEH (Fig. 5*B*). Cell death in neurons transduced with Arg287Gln TAT-hsEH was not altered by 4-PCO.

Discussion

We present three major findings: (1) human polymorphisms in EPHX2 that alter sEH hydrolase activity impact neuronal survival after ischemic injury, (2) 14,15-EET rescues the increase in ischemic neuronal death caused by sEH overexpression, and (3) pharmacological inhibition of sEH reverses the detrimental effects of EPHX2 polymorphisms on neuronal survival. These findings suggest that polymorphisms in human EPHX2 gene may be linked to increased susceptibility to ischemic brain injury. The findings also suggest that EETs are endogenous neuroprotectants in brain and that sEH may serve as a therapeutic target in stroke.

Single nucleotide polymorphisms in human *EPHX2* have been linked to cardiovascular disease risk, including coronary heart disease (Fornage et al., 2004; Lee et al., 2006), hyperlipoproteinemia (Sato et al., 2004), and type-2 diabetes (Ohtoshi et al., 2005). The association of *EPHX2* polymorphisms with cerebrovascular disease, however, is less clear. *EPHX2* haplotypes have been linked to both increased and decreased incidence of ischemic stroke (Fornage et al., 2005). In the current study, we tested the hypothesis that SNPs linked to increased stroke risk are "gain-of-function" mutations leading to increased sEH enzyme activity, whereas those linked to decreased stroke risk are "loss-of-function" mutations leading to decreased sEH hydrolase activity. The idea that hydrolase activity may play a detrimental role in ischemic cell death stems from our previous observations that neuroprotection by ischemic preconditioning is associated with increased expression of EET-biosynthetic enzyme (Alkayed et al., 2002a), that EETs are protective against ischemic cell death (Liu and Alkayed, 2005), and that pharmacological inhibition and gene deletion of sEH are protective against ischemic brain damage *in vivo* (Alkayed et al., 2002b). These studies suggested that EETs are neuroprotective, and that sEH plays a detrimental role against ischemic cell death, primarily by hydrolyzing EETs. Therefore, any sequence variation in EPHX2 gene, which encodes for sEH, that alters its hydrolase activity can potentially affect neuronal susceptibility to ischemic cell death. To test this hypothesis, we recreated common human SNPs using site directed mutagenesis, and introduced sEH variants into primary cortical neurons to determine their impact on sEH hydrolase activity and neuronal survival after ischemic injury induced by OGD. Studies involving ectopic gene expression in primary cultured neurons are hampered by extremely low transfection efficiency. This can be overcome by viral vector-assisted gene delivery. However, toxicity and immunological reactions caused by viral vectors led to the development of nonviral methods for assisted delivery of genes and proteins, including TAT-mediated delivery of proteins into neurons *in vitro* (Soane and Fiskum, 2005) and into brain *in vivo* (Cao et al., 2002). Protein transduction domains, including TAT, are short peptide sequences that can cross plasma membranes and transport large macromolecules, including recombinant proteins (Nakanishi et al., 2003; Kilic et al., 2006). Using immunoblotting and ICC staining, we showed that TAT is capable of delivering high levels of sEH protein across neuronal membranes. The ectopic expression of WT sEH protein was enzymatically active, as demonstrated by increased conversion of its preferred substrate, 14,15-EET, to the corresponding hydrolysis product 14,15-DHET. Furthermore, we found that one amino acid substitution, Arg287Gln, was associated with decreased conversion of 14,15-EET, whereas another, Lys55Arg, was associated with increased hydrolysis of 14,15-EET compared with WT sEH. These changes are in agreement with previous reports examining the impact of these mutations on EET conversion by purified sEH *in vitro* (Przybyla-Zawislak et al., 2003). More importantly, the increase in sEH hydrolase activity associated with overexpression of WT sEH was accompanied by an increase in OGD-induced neuronal cell death, which was reversed by excess 14,15-EET and by pharma-

cological inhibition of sEH. These findings suggest that EETs are endogenous neuroprotectants produced in neurons and that sEH overexpression exacerbates neuronal cell death by increasing the metabolism of EETs. In support of this conclusion, we also found that the decrease in sEH hydrolase activity was associated with protection from OGD-induced neuronal cell death. However, the association between hydrolase activity and cell death was not as clear when neurons were transduced with other mutants. For example, transduction with Arg103Cys variant, which did not significantly affect hydrolase activity, lead to the highest increase in neuronal cell death, whereas the Lys55Arg variant, which caused the highest increase in hydrolase activity, had no effect on neuronal cell death. The discrepancy between hydrolase activity and cell death in these mutants suggests that these amino acid changes may affect other functions of sEH unrelated to EET metabolism. For example, the increase in cell death in the Lys55Arg may be linked to changes in the N-terminal phosphatase activity of sEH (Cronin et al., 2003; Newman et al., 2003). Alternatively, these polymorphisms may differentially alter the ability of sEH to metabolize different regioisomers of EETs. For example, if Arg103Cys would preferentially affect the metabolism of a regioisomer other than 14,15-EET, such a change in activity would not be detected in our assay, which uses 14,15-EET. In support of this idea, the increase in cell death by Arg103Cys could be reversed by pharmacological inhibition of sEH with 4-PCO.

These findings suggest that in addition to their reported association with stroke risk, polymorphisms in EPHX2 gene alter neuronal sensitivity to ischemia, and may, therefore, alter stroke outcome. These findings are in agreement with our previous work demonstrating that EETs are protective against ischemic cell death in astrocytes (Liu and Alkayed, 2005) and that pharmacological inhibition and gene deletion of sEH are protective against ischemic brain injury *in vivo* (Alkayed et al., 2002b).

There are other possible explanations for our results. Increased cell death by sEH overexpression may alternatively be related to the accumulation of toxic products, such as leukotoxin diol metabolites (Kosaka et al., 1994; Moghaddam et al., 1997; Sandberg et al., 2000). This possibility, however, seems unlikely, because toxicity by these metabolites should not be reversed by EETs or 4-PCO. We also considered the possibility that increased cell death was related to the toxic effects of 14,15-DHET. We found no effect of 14,15-DHET on neuronal survival after OGD. It is important, however, to recognize that EETs are metabolized through pathways other than sEH, such as β-oxidation, and therefore, the protection obtained by exogenous application of EETs may result from their metabolic conversion through such pathways. Similarly, 4-PCO may increase neuronal survival through mechanisms unrelated to sEH inhibition, for example, increased production of reactive oxygen species (Sellers et al., 2005).

Although exacerbation of cell death by sEH variants with increased hydrolase activity can readily be accounted for by increased EET metabolism, the protection afforded by "loss-of-function" mutation Arg287Gln cannot easily be explained by reduced EETs hydrolysis. For protection to take place because of a reduction in hydrolase activity, the activity must be reduced to below baseline levels. The nonconservative substitution of Arg^{287} with Gln^{287} has been proposed to interfere with the formation of stable dimers (Sandberg et al., 2000; Srivastava et al., 2004). Therefore, if Arg287Gln sEH heterodimerizes with endogenous sEH monomers, it may destabilize and therefore suppress endogenous sEH through a "dominant-negative" effect.

The following limitations should be considered when interpreting our study. First, we introduced mutant protein variants on a background of endogenous sEH. This situation is more representative of the setting in individuals, who are heterozygous for the mutation, which is the most commonly encountered variant in humans (Przybyla-Zawislak et al., 2003; Fornage et al., 2005). Effects may be more pronounced in homozygous situations, and we may have, therefore, missed effects of some SNPs. Nevertheless, our data suggest that *EPHX2* polymorphisms may be relevant even for individuals possessing only one mutant allele. Second, we do not know whether the introduction of human mutations in rodent cells may have biased our results. Mammalian sEH proteins, however, are highly conserved (Sandberg et al., 2000), suggesting that effects are likely to be similar across species. Nevertheless, future studies are needed to confirm the effects of *EPHX2* SNPs on cell death in human neurons. Finally, a recent case-cohort study examining the relationship between EPHX2 polymorphisms and coronary heart disease reported that the frequencies of some of the EPHX2 variants examined in our study, for example, the Cys154Try variant and the double mutant, are rare in the human population, whereas other more common variants, such as R402Ins/Del and E470G, were not investigated in our study (Fornage et al., 2005; Lee et al., 2006). Despite these limitations, our study provides proof of principle that genetic variations in EPXH2 can affect neuronal survival and as such, adds functional insights to human studies of statistical association between haplotypes of the EPHX2 gene and ischemic stroke risk.

We conclude that polymorphisms in the human *EPHX2* gene alter neuronal susceptibility to ischemic cell death *in vitro*, likely related to changes in the hydrolase activity of the enzyme. The findings suggest that EPHX2 SNPs may be linked to altered sensitivity to ischemic brain injury and stroke outcome. The findings also suggest that sEH plays an important role in the mechanism of ischemic cell death and may serve as a potential therapeutic target for stroke related brain damage, especially in individuals with gain-of-function SNPs associated with increased hydrolase activity.

References

Alkayed NJ, Birks EK, Hudetz AG, Roman RJ, Henderson L, Harder DR (1996) Inhibition of brain P-450 arachidonic acid epoxygenase decreases baseline cerebral blood flow. Am J Physiol 271:H1541–H1546.

Alkayed NJ, Goyagi T, Joh HD, Klaus J, Harder DR, Traystman RJ, Hurn PD (2002a) Neuroprotection and P450 2C11 upregulation after experimental transient ischemic attack. Stroke 33:1677–1684.

Alkayed NJ, Goyagi T, Peng X, Harder DR, Hurn PD, Traystman RJ, Koehler RC (2002b) In: Pharmacology of cerebral ischemia (Krieglstein J, ed), pp 303–310. Stuttgart, Germany: Medpharm Scientific Publishers.

Becker-Hapak M, McAllister SS, Dowdy SF (2001) TAT-mediated protein transduction into mammalian cells. Methods 24:247–256.

Cao G, Pei W, Ge H, Liang Q, Luo Y, Sharp FR, Lu A, Ran R, Graham SH, Chen J (2002) *In vivo* delivery of a Bcl-xL fusion protein containing the TAT protein transduction domain protects against ischemic brain injury and neuronal apoptosis. J Neurosci 22:5423–5431.

Chen JK, Capdevila J, Harris RC (2001) Cytochrome p450 epoxygenase metabolism of arachidonic acid inhibits apoptosis. Mol Cell Biol 21:6322–6331.

Cronin A, Mowbray S, Durk H, Homburg S, Fleming I, Fisslthaler B, Oesch F, Arand M (2003) The N-terminal domain of mammalian soluble epoxide hydrolase is a phosphatase. Proc Natl Acad Sci USA 100:1552–1557.

Davis BB, Thompson DA, Howard LL, Morisseau C, Hammock BD, Weiss RH (2002) Inhibitors of soluble epoxide hydrolase attenuate vascular smooth muscle cell proliferation. Proc Natl Acad Sci USA 99:2222–2227.

Draper AJ, Hammock BD (1999) Inhibition of soluble and microsomal epoxide hydrolase by zinc and other metals. Toxicol Sci 52:26–32.

Dreisbach AW, Japa S, Sigel A, Parenti MB, Hess AE, Srinouanprachanh SL, Rettie AE, Kim H, Farin FM, Hamm LL, Lertora JJ (2005) The preva-

lence of CYP2C8, 2C9, 2J2, and soluble epoxide hydrolase polymorphisms in African Americans with hypertension. Am J Hypertens 18:1276–1281.

Farin FM, Janssen P, Quigley S, Abbott D, Hassett C, Smith-Weller T, Franklin GM, Swanson PD, Longstreth Jr WT, Omiecinski CJ, Checkoway H (2001) Genetic polymorphisms of microsomal and soluble epoxide hydrolase and the risk of Parkinson's disease. Pharmacogenetics 11:703–708.

Fornage M, Boerwinkle E, Doris PA, Jacobs D, Liu K, Wong ND (2004) Polymorphism of the soluble epoxide hydrolase is associated with coronary artery calcification in African-Am subjects: the coronary artery risk development in young adults (CARDIA) study. Circulation 109:335–339.

Fornage M, Lee CR, Doris PA, Bray MS, Heiss G, Zeldin DC, Boerwinkle E (2005) The soluble epoxide hydrolase gene harbors sequence variation associated with susceptibility to and protection from incident ischemic stroke. Hum Mol Genet 14:2829–2837.

Heizer ML, McKinney JS, Ellis EF (1991) 14,15-Epoxyeicosatrienoic acid inhibits platelet aggregation in mouse cerebral arterioles. Stroke 22:1389–1393.

Kilic E, Kilic U, Hermann DM (2006) TAT fusion proteins against ischemic stroke: current status and future perspectives. Front Biosci 11:1716–1721.

Kosaka K, Suzuki K, Hayakawa M, Sugiyama S, Ozawa T (1994) Leukotoxin, a linoleate epoxide: its implication in the late death of patients with extensive burns. Mol Cell Biochem 139:141–148.

Kozak W, Kluger MJ, Kozak A, Wachulec M, Dokladny K (2000) Role of cytochrome P-450 in endogenous antipyresis. Am J Physiol Regul Integr Comp Physiol 279:R455–R460.

Lee CR, North KE, Bray MS, Fornage M, Seubert JM, Newman JW, Hammock BD, Couper DJ, Heiss G, Zeldin DC (2006) Genetic variation in soluble epoxide hydrolase (EPHX2) and risk of coronary heart disease: the atherosclerosis risk in communities (ARIC) study. Hum Mol Genet 15:1640–1649.

Liu M, Alkayed NJ (2005) Hypoxic preconditioning and tolerance via hypoxia inducible factor (HIF) 1alpha-linked induction of P450 2C11 epoxygenase in astrocytes. J Cereb Blood Flow Metab 25:939–948.

Markus HS, Alberts MJ (2006) Update on genetics of stroke and cerebrovascular disease 2005. Stroke 37:288–290.

Moghaddam MF, Grant DF, Cheek JM, Greene JF, Williamson KC, Hammock BD (1997) Bioactivation of leukotoxins to their toxic diols by epoxide hydrolase. Nat Med 3:562–566.

Mullin CA, Hammock BD (1982) Chalcone oxides–potent selective inhibitors of cytosolic epoxide hydrolase. Arch Biochem Biophys 216:423–439.

Nagahara H, Vocero-Akbani AM, Snyder EL, Ho A, Latham DG, Lissy NA, Becker-Hapak M, Ezhevsky SA, Dowdy SF (1998) Transduction of full-length TAT fusion proteins into mammalian cells: TAT-p27Kip1 induces cell migration. Nat Med 4:1449–1452.

Nakanishi M, Eguchi A, Akuta T, Nagoshi E, Fujita S, Okabe J, Senda T, Hasegawa M (2003) Basic peptides as functional components of nonviral gene transfer vehicles. Curr Protein Pept Sci 4:141–150.

Newman JW, Morisseau C, Harris TR, Hammock BD (2003) The soluble epoxide hydrolase encoded by EPXH2 is a bifunctional enzyme with novel lipid phosphate phosphatase activity. Proc Natl Acad Sci USA 100:1558–1563.

Node K, Huo Y, Ruan X, Yang B, Spiecker M, Ley K, Zeldin DC, Liao JK (1999) Anti-inflammatory properties of cytochrome P450 epoxygenase-derived eicosanoids. Science 285:1276–1279.

Ohtoshi K, Kaneto H, Node K, Nakamura Y, Shiraiwa T, Matsuhisa M, Yamasaki Y (2005) Association of soluble epoxide hydrolase gene polymorphism with insulin resistance in type 2 diabetic patients. Biochem Biophys Res Commun 331:347–350.

Przybyla-Zawislak BD, Srivastava PK, Vazquez-Matias J, Mohrenweiser HW, Maxwell JE, Hammock BD, Bradbury JA, Enayetallah AE, Zeldin DC, Grant DF (2003) Polymorphisms in human soluble epoxide hydrolase. Mol Pharmacol 64:482–490.

Sandberg M, Hassett C, Adman ET, Meijer J, Omiecinski CJ (2000) Identification and functional characterization of human soluble epoxide hydrolase genetic polymorphisms. J Biol Chem 275:28873–28881.

Sato K, Emi M, Ezura Y, Fujita Y, Takada D, Ishigami T, Umemura S, Xin Y, Wu LL, Larrinaga-Shum S, Stephenson SH, Hunt SC, Hopkins PN (2004) Soluble epoxide hydrolase variant (Glu287Arg) modifies plasma total cholesterol and triglyceride phenotype in familial hypercholesterolemia: intrafamilial association study in an eight-generation hyperlipidemic kindred. J Hum Genet 49:29–34.

Sellers KW, Sun C, Diez-Freire C, Waki H, Morisseau C, Falck JR, Hammock BD, Paton JF, Raizada MK (2005) Novel mechanism of brain soluble epoxide hydrolase-mediated blood pressure regulation in the spontaneously hypertensive rat. FASEB J 19:626–628.

Soane L, Fiskum G (2005) TAT-mediated endocytotic delivery of the loop deletion Bcl-2 protein protects neurons against cell death. J Neurochem 95:230–243.

Srivastava PK, Sharma VK, Kalonia DS, Grant DF (2004) Polymorphisms in human soluble epoxide hydrolase: effects on enzyme activity, enzyme stability, and quaternary structure. Arch Biochem Biophys 427:164–169.

Wei Q, Doris PA, Pollizotto MV, Boerwinkle E, Jacobs Jr DR, Siscovick DS, Fornage M (2006) Sequence variation in the soluble epoxide hydrolase gene and subclinical coronary atherosclerosis: interaction with cigarette smoking. Atherosclerosis 190:26–34.