

Cardiovascular, Pulmonary and Renal Pathology

Soluble Epoxide Inhibition Is Protective Against Cerebral Ischemia via Vascular and Neural Protection

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Inhibition of soluble epoxide hydrolase (SEH), the enzyme responsible for degradation of vasoactive epoxides, protects against cerebral ischemia in rats. However, the molecular and biological mechanisms that confer protection in normotension and hypertension remain unclear. Here we show that 6 weeks of SEH inhibition via 2 mg/day of 12-(3-adamantan-1-ylureido) dodecanoic acid (AUDA) in spontaneously hypertensive stroke-prone (SHRSP) rats protects against cerebral ischemia induced by middle cerebral artery occlusion, reducing percent hemispheric infarct and neurodeficit score without decreasing blood pressure. This level of cerebral protection was similar to that of the angiotensin-converting enzyme inhibitor, enalapril, which significantly lowered blood pressure. SEH inhibition is also protective in normotensive Wistar-Kyoto (WKY) rats, reducing both hemispheric infarct and neurodeficit score. In SHRSP rats, SEH inhibition reduced wall-to-lumen ratio and collagen deposition and increased cerebral microvessel density, although AUDA did not alter middle cerebral artery structure or microvessel density in WKY rats. An apoptosis mRNA expression microarray of brain tissues from AUDA-treated rats revealed that AUDA modulates gene expression of mediators involved in the regulation of apoptosis in neural tissues of both WKY and SHRSP rats. Hence, we conclude that chronic SEH inhibition protects against cerebral ischemia via vascular protection in SHRSP rats and neural protection in both the SHRSP and WKY rats, indicating that SEH inhibition

has broad pharmacological potential for treating ischemic stroke. (Am J Pathol 2009, 174:2086–2095; DOI: 10.2353/ajpath.2009.080544)

Epoxyeicosatrienoic acids (EETs), lipid metabolites produced from arachidonic acid by CYP450 enzymes, are novel mediators that antagonize the sequela of hypertension,¹ match cerebral blood flow to increased neural activity and metabolic demand, promotes angiogenesis,² and protect against ischemia.^{3,4} Because ischemic stroke occurs with loss of cerebral blood flow and is strongly associated with hypertension, modulation of epoxide degradation has potential in managing ischemic stroke. Unfortunately, pharmacological utility of exogenous EETs is impractical because the epoxides are rapidly degraded by the soluble epoxide hydrolase (SEH) into their less active diol, dihydroxyeicosatrienoic acids.⁵ In fact, human SEH polymorphisms are linked to the incidence of ischemic stroke,⁶ and this association could be related to modifications in SEH activity, and thus epoxide catabolism.⁷ An alternative strategy that has been used to increase EETs systemically is SEH inhibition.¹

We previously showed that the SEH inhibitor 12-(3-adamantan-1-ylureido) dodecanoic acid (AUDA) protects against cerebral ischemia in spontaneously hypertensive stroke-prone (SHRSP) rats, an animal model of essential hypertension.⁸ Interestingly, chronic AUDA treatment in SHRSP rats effectively decreased infarct size induced by middle cerebral artery occlusion (MCAO)

Supported by the American Heart Association (established investigator to J.D.I.) and the National Institutes of Health (grants HL59699, NS053002, and F31 HL087723-01).

Accepted for publication February 6, 2009.

Disclosures: B.D.H. is the founder and J.D.I. is a member of the Scientific Advisory Board of Arête Therapeutics, a developer of SEH inhibitors for the treatment of hypertension.

Supplemental material for this article can be found on <http://ajp.amjpathol.org>.

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without decreasing blood pressure.⁸ Although blood pressure is an important variable in controlling infarct size in the SHRSP, the cerebrovasculature is also a key determinant of increased sensitivity to cerebral ischemia and thus infarct size.^{9,10} Our study provided preliminary evidence that AUDA protection in the SHRSP involved changes in vascular structure.⁸ More recently, a report suggested that administration of AUDA-butyl ester protects against cerebral ischemic reperfusion injury in normotensive mice by mechanisms that may involve neural protection rather than vascular protection.¹¹ As a result, the contribution of vascular and neural protection to the cerebral protective effects of SEH inhibition during states of hypertension and normotension are unclear and require further investigation. The present study tested the hypothesis that SEH inhibition provides cerebral protection via different mechanisms in normotensive WKY and hypertensive SHRSP rats.

Materials and Methods

Animals

All male animals were housed and fed a normal rat chow (Tekads 8604; Harlan, Indianapolis, IN) in the animal care facility at the Medical College of Georgia approved by the American Association for the Accreditation of Laboratory Animal Care and all protocols were approved by the institutional animal care and use committee at this institution. All drug treatments were administered via drinking water. Six- to seven-week-old SHRSP rats (Charles River, Wilmington, MA) were divided into four treatment groups as follows: 6 weeks of vehicle (500 mg/L of cyclodextrin and 0.075% of ethanol), 6 weeks of AUDA (2 mg/day via drinking water, 50 mg/L, dissolved using vehicle to aid in solubilization), 6 weeks of enalapril (2.5 mg/day via drinking water dissolved in vehicle), and 5 weeks of AUDA (2 mg/day via drinking water dissolved via vehicle followed by a 7- to 12-day washout period). In addition, a group of 12- to 13-week-old SHRSPs was treated with *trans*-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid (tAUCB), an SEH inhibitor,^{12,13} 2 mg/day for 7 to 12 days via drinking water (50 mg/L) prepared using vehicle to aid in solubilization. Six- to seven-week-old WKY (Harlan) were treated with either 6 weeks of vehicle or 6 weeks of AUDA, 2 mg/day. Blood pressure was monitored by tail plethysmography (IITC Life Science, Woodland Hills, CA) during the experimental period in acclimatized conscious rats. At the end of the treatment, all animals were anesthetized with pentobarbital (50 mg/kg) for middle cerebral artery occlusion (MCAO) experiments and tissue collection.

Plasma and Brain AUDA and Metabolite Measurements

AUDA levels were measured in homogenized brain, and AUDA metabolites were assessed in plasma samples by reverse phase high performance liquid chromatography

followed by negative mode electron spray ionization and tandem mass spectroscopy as previously described.¹¹

MCAO

MCAO was conducted as previously described by Longa and colleagues.¹⁴ In brief, anesthetized rats with body temperature maintained at 37°C had a 3–0 dermalon monofilament with a rounded tip inserted cranially until a drop in the Laser Doppler (Perimed, North Royalton, OH) attached to the skull 2 mm down and 5 mm lateral to bregma confirmed MCA occlusion.⁸ Physiological parameters (PO_2 , PCO_2 , hematocrit, and blood glucose) were measured via blood sampling with a femoral arterial line using a blood gas analyzer (GemPremier 3000; Instrumentation Laboratory, Lexington, MA). There were no differences in physiological parameters measured during the MCAO at baseline between vehicle and AUDA treatment (see Supplemental Table S1 at <http://ajp.amjpathol.org>).

Six hours after MCAO, neurodeficit was assessed using a combination of a modified form of the Bederson and colleagues¹⁵ score and an abbreviated adaptation of the modified Neurological Severity Score described by Chen and colleagues.¹⁶ The neurodeficit score for WKY animals consisted of a tail suspension test (score range, 1 to 4) to assess postural reflexes and a pad walk to assess motility (range, 1 to 3) that were combined for an overall neurodeficit score. SHRSP neurodeficit score also included an additional spontaneous activity score (1 to 4) to assess the more dramatic deficits displayed in the SHRSP rats, assessing general condition (calm to aggressive) and abnormal movements (curvilinear walk to seizure-like activity).

Next, brains were collected and sliced into 2-mm sections coronally from the frontal pole. Noninfarcted hemisphere, infarcted hemisphere, and infarct were measured using the National Institutes of Health (Bethesda, MD) Image software on digitized images of the slices stained with 2% triphenyltetrazolium chloride (TTC). Percent hemisphere infarct was calculated using the Swanson equation to account for swelling.¹⁷

Histology of MCA

Animals were perfused with a vasodilator cocktail (papaverine 0.3 mmol/L/L, adenosine 0.2 mmol/L, diltiazem 0.2 mmol/L/L) prepared in phosphate-buffered saline. A section of the MCA, 4 mm away from the Circle of Willis, was embedded. Serial sections of 5 μ m in thickness were taken equidistantly along 650 μ m, totaling 16 measurements per vessel. Outer and inner perimeters were measured via by a blinded reviewer using Axiovision 4.0 software (Axio Vision Rel.4.6.3; Carl Zeiss, Thornwood, NY). These measurements were used to calculate wall thickness and wall to lumen (W:L) ratio using the equation of a circle. The MCA was also stained for collagen with the Masson's trichrome stain and picrosirius red stain. The MCA was scored on a scale of 1 to 10 by two blinded observers.

Microvessel Density

Because the reduction in infarct size evident in SEH knockout mice was associated with increased perfusion in the cortex and striatum during MCA occlusion and early reperfusion¹⁸ and we previously found that AUDA treatment reduced infarcted tissue in cortex and striatum,⁸ we measured microvessel density in the cortex and the striatum of treated and control rats. To measure the microvessel density, the brains were collected and sliced into five 2-mm slices in the same manner used for TTC staining and infarct quantification. The slices are flash-frozen in a methylbutane dry-ice bath. Serial sections, 5 μm thick, were cut of each slice. After fixation with 10% formalin and blocking in 10% normal goat serum, frozen 5- μm serial sections of brain were incubated overnight with von Willebrand factor antibody (1:250, F3520; Sigma, St. Louis, MO) at 4°C, totaling five sections per animal. After washing with phosphate-buffered saline and incubating with secondary antibody, Cy3 goat anti-rabbit IgG conjugate (1:200; Zymed, South San Francisco, CA), slides were mounted with a coverslip using Prolong Antifade Reagent mounting media (Molecular Probes, Carlsbad, CA). Fluorescence was visualized with a Zeiss LSM 510 Meta confocal laser-scanning microscope (Thornwood, NY 543 nm wavelength). Twenty-five pictures of the cortex and striatum per animal at $\times 200$ magnification were analyzed using Axiovision 5.2 (Carl Zeiss, Thornwood, NY) by two blinded reviewers. Data were reported as area (mm^2) of fluorescence per area of field (mm^2).

Real-Time Polymerase Chain Reaction (PCR) Apoptosis Gene Expression Array

As previously described,¹⁹ total RNA was extracted from 50 mg of whole brain from male rats using the RNeasy lipid mini kit and DNase digestion (Qiagen, Valencia, CA) according to the manufacturer's protocol and RNA concentrations were determined using absorbance at 260 nm. Using RT² PCR array first strand kit (SuperArray Bioscience, Frederick, MD), 1 μg of RNA was converted to cDNA. The cDNA was then incubated with RT² real-time SYBR Green PCR mastermix (SuperArray Bioscience) into a 96-well PCR array plate, one sample per plate (three samples per experimental group). Thermal cycling and real-time detection via a Bio-Rad iCycler (Bio-Rad Laboratories, Hercules, CA) was conducted following the instructions of apoptosis array (Superarray Bioscience). The Superarray RT² PCR arrays are equipped with controls for genomic DNA contamination, reverse transcription, and PCR. Threshold cycle (C_t) values were normalized to β -actin. The significance analysis of microarrays (SAM) software (Stanford University, Stanford, CA) was used to determine the significance of changes in gene expression in the apoptosis microarray²⁰ with a median false discovery rate of 17% and q -value (comparable with P value) of 10%.

Statistics

All data are expressed as mean \pm SEM. Differences were assessed using analysis of variance and Student's t -tests with P values < 0.05 being statistically significant.

Results

Brain AUDA and Plasma AUDA Metabolite Levels

AUDA is metabolized by β oxidation into an inactive metabolite 12-(3-adamantyl-ureido)-butyl acid (AUBA). AUBA levels are used as an indication of AUDA exposure. Because AUDA reversibly and competitively inhibits the SEH enzyme, measuring SEH activity in the tissue to determine the degree of SEH inhibition is not feasible.^{13,21,22} Plasma levels of AUBA reached 4 ng/ml in the SHRSP rats and 13 ng/ml in the WKY rats after 6 weeks of SEH inhibition. In the brain, AUDA levels reached 2 $\mu\text{mol/g}$ in the SHRSP rats and 3 $\mu\text{mol/g}$ in the WKY rats. These levels suggest that treatment was sufficient to inhibit SEH enzymatic activity.

AUDA Decreases Infarct Size Without Preventing Hypertension

AUDA treatment in the SHRSP rats did not prevent the development of hypertension in the 12-week-old SHRSP rats (Figure 1) consistent with our previous findings.⁸ To determine whether the protective effects of AUDA were independent of blood pressure lowering, additional SHRSP rats were treated with an angiotensin-converting enzyme inhibitor and served as a blood pressure control group for the SHRSP AUDA-treated rats. As expected, administration of the angiotensin-converting enzyme inhibitor enalapril (2.5 mg/day) for 6 weeks was effective at preventing the development of hypertension in the SHRSP rats. Despite the absence of an effect on blood pressure, the reduction in infarct size and neurodeficit achieved with AUDA was similar to that achieved by 6 weeks of enalapril treatment (Figure 1, A–D).

Previous studies have suggested that high concentrations of AUDA activate the orphan nuclear receptor PPAR- α .^{23,24} Thus, we treated a separate set of SHRSP rats with tAUCB, a potent SEH inhibitor^{12,13} that lacks potential for PPAR agonistic activity (Figure 1). As expected, tAUCB protected against cerebral ischemia in the SHRSPs, reducing infarct size to $43 \pm 3\%$, $P < 0.05$, hemispheric infarct size and neurodeficit score to 6.3 ± 0.7 , $n = 9$, $P < 0.05$. These results indicate that the protective properties of AUDA are not attributable to PPAR agonistic activity but are the result of SEH inhibition.

Like the SHRSPs, AUDA treatment in the WKY did not alter blood pressure in the 12-week-old WKY rats (143 ± 1 mmHg versus 141 ± 3 mmHg). However, we still found a protective effect against cerebral ischemia in the WKY animals treated with AUDA. SEH inhibition significantly

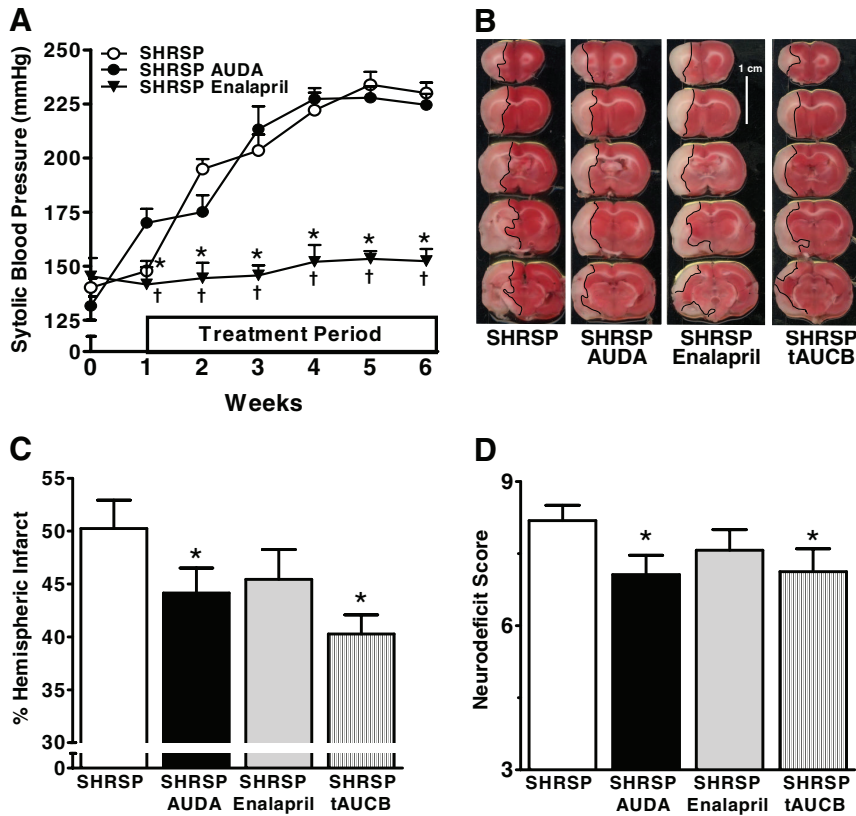


Figure 1. AUDA, enalapril, and tAUCB treatment decrease infarct size and neurodeficit in SHRSP. **A:** Systolic blood pressure in AUDA- and enalapril-treated SHRSP. †*P* < 0.05 relative to SHRSP enalapril, **P* < 0.05 relative to SHRSP AUDA. **B:** AUDA, enalapril, and tAUCB decrease infarct size after MCAO in SHRSP as demonstrated by the representative TTC-stained coronal slices arranged caudally starting at the frontal pole from each treatment group (black line delineates infarct from viable tissue). **C:** Quantification of percent hemispheric infarct size in SHRSP groups. **P* < 0.05 relative to SHRSP. **D:** Quantification of neurodeficit score in SHRSP groups. **P* < 0.05 relative to SHRSP. SHRSP, *n* = 16; SHRSP AUDA, *n* = 16; SHRSP enalapril, *n* = 14; and SHRSP tAUCB, *n* = 9.

reduced percent infarct size 40% and neurodeficit 13% (Figure 2, A–C).

AUDA Provides Vascular Protection in the SHRSP Rats

Previous studies have suggested that AUDA may mediate cerebral protection by increasing MCA compliance.⁸ However, direct evidence implicating AUDA in vascular structure and remodeling is lacking. Thus, we analyzed blood vessel structure in the MCA in the SHRSP and WKY rats. The wall thickness and W:L ratio, two measures of vascular remodeling, and collagen deposition around the MCA were increased in the adult SHRSP in comparison with the adult WKY animals. Treatment with AUDA attenuated remodeling of MCA in SHRSP rats, manifest as a

reduction in wall thickness (26%) and the W:L ratio (27%) (Figure 3, A and B). In addition, there was an appreciable reduction in collagen deposition around the MCA of SHRSP AUDA-treated animals as determined by the Masson's trichrome stain and picosirius red staining (Figure 3, C–J). Semiquantitative scoring by blinded reviewers determined that untreated SHR-SP had a collagen score of 7.8 ± 0.3 and that this significantly decreased to 4.6 ± 1.3 in AUDA-treated SHRSP. In contrast, we found that MCA wall thickness and W:L ratio of the WKY rats treated with AUDA was not altered (Figure 3, A and B). There was also no noticeable reduction in collagen deposition around the MCAs of AUDA-treated WKYs (Figure 3, C–J).

To further investigate the impact of AUDA on the vasculature, we assessed cerebral microvessel density

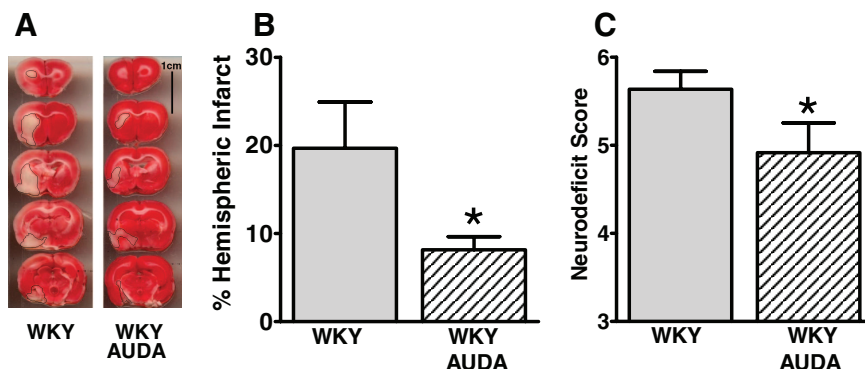


Figure 2. AUDA treatment decreases infarct size and neurodeficit in WKY rats. **A:** AUDA decreases infarct size after MCAO in WKY rats as demonstrated by the representative TTC-stained coronal slices arranged caudally starting at the frontal pole from each treatment group (black line delineates infarct from viable tissue). **B:** Quantification of percent hemispheric infarct size in WKY groups. **P* < 0.05 relative to WKY. **C:** Quantification of neurodeficit score in WKY groups. **P* < 0.05 relative to WKY. WKY, *n* = 11; and WKY AUDA, *n* = 12.

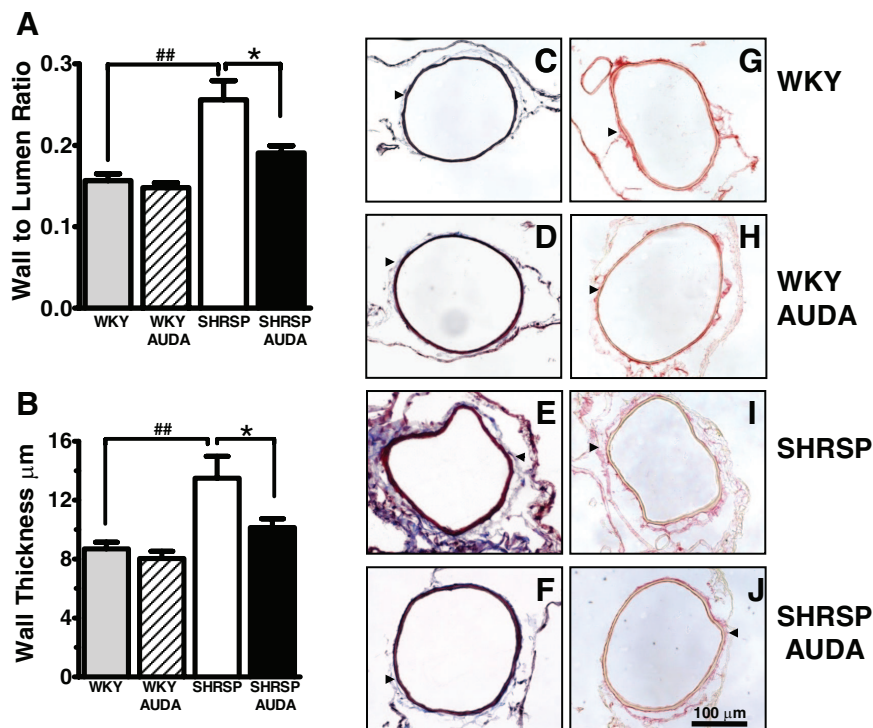


Figure 3. Middle cerebral artery (MCA) remodeling and collagen deposition is attenuated by AUDA treatment in SHRSP. **A:** AUDA attenuates the increase in wall to lumen ratio in the SHRSP. $^{**}P < 0.05$ relative to WKY, $^{*}P < 0.05$ relative to SHRSP. **B:** AUDA attenuates the increase in wall thickness in the SHRSP. $^{**}P < 0.05$ relative to WKY, $^{*}P < 0.05$ relative to SHRSP. **C–F:** Staining the MCAs for collagen (pointed to by the **black arrowhead**) via Masson's trichrome stain. **G–J:** Staining the MCAs for collagen via picrosirius red stain (collagen red). Stainings demonstrate that AUDA attenuates the increase in collagen deposition around the MCA as depicted by representative images taken at $\times 200$ magnification. WKY (**C** and **G**), AUDA-treated WKY (**D** and **H**), SHRSP (**E** and **I**), AUDA-treated SHRSP (**F** and **J**). WKY, $n = 4$; WKY AUDA, $n = 5$; SHRSP, $n = 6$; and SHRSP AUDA, $n = 6$.

[area (mm²) of fluorescence per area of field (mm²)]. Assessment of immunofluorescently labeled von Willebrand factor in the brain parenchyma of SHRSP and WKY rats at the end of the treatment period revealed that the area of microvessel density in the adult SHRSP was 33% lower than the adult WKY rats (Figure 4, A, B, and D). AUDA was again protective by increasing the microvessel density by 20% in the SHRSP rats (Figure 4, A, D, and E). This supports the notion that SEH inhibition could be providing cerebral protection by reducing the area at risk to ischemia in the SHRSP rats. WKY rats did not exhibit any change in cerebral microvessel density in the WKY animals treated with AUDA (Figure 4, A–C).

To determine whether the protection achieved by chronic AUDA treatment could be sustained after dis-

continuation of treatment, a separate group of SHRSP rats were treated with AUDA, 2 mg/day, for 5 weeks followed by 7 to 12 days of AUDA withdrawal. The area under the curve (AUC) of orally administered AUDA is 0.4×10^4 nmol/L·minute,¹² and the half-life for AUDA after oral gavage is 7.3 hours.²⁹ As a result, the withdrawal period should be sufficient to exclude the effects of the plasma presence of the drug AUDA. As anticipated, a trend for protection was still evident after the withdrawal period ($46 \pm 9\%$ hemispheric infarct, $n = 5$, $P < 0.26$ and 7.6 ± 0.8 neurodeficit, $n = 5$, $P < 0.20$) further supporting the notion that chronic AUDA treatment protection from ischemic damage was in part attributable to structural changes in the SHRSP cerebrovasculature.

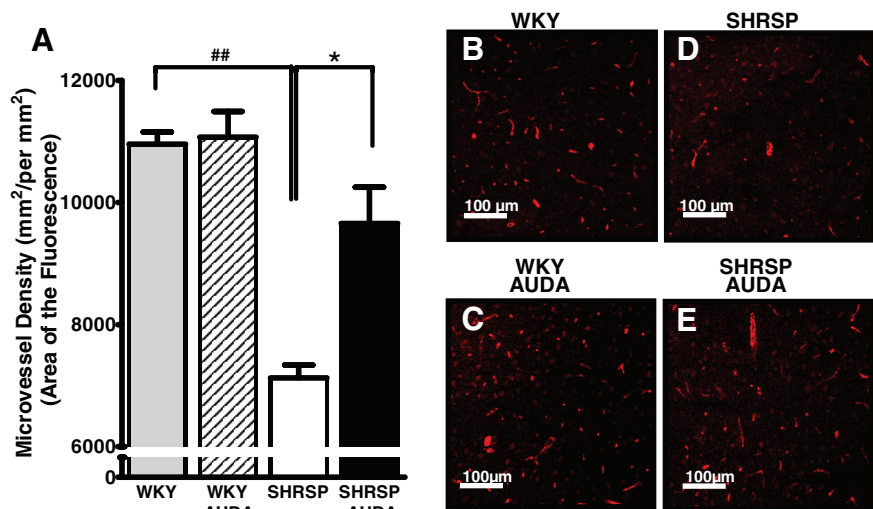


Figure 4. Cerebral microvascular density is increased by AUDA treatment in SHRSP. **A:** The area of fluorescently labeled von Willebrand factor (mm²) per area of the field (mm²) in the WKY ($n = 4$), WKY AUDA ($n = 4$), SHRSP ($n = 4$), and SHRSP AUDA ($n = 5$). $^{**}P < 0.05$ relative to WKY, $^{*}P < 0.05$ relative to AUDA. **B–E:** Representative von Willebrand factor-labeled immunofluorescent images (red). Original magnifications, $\times 200$.

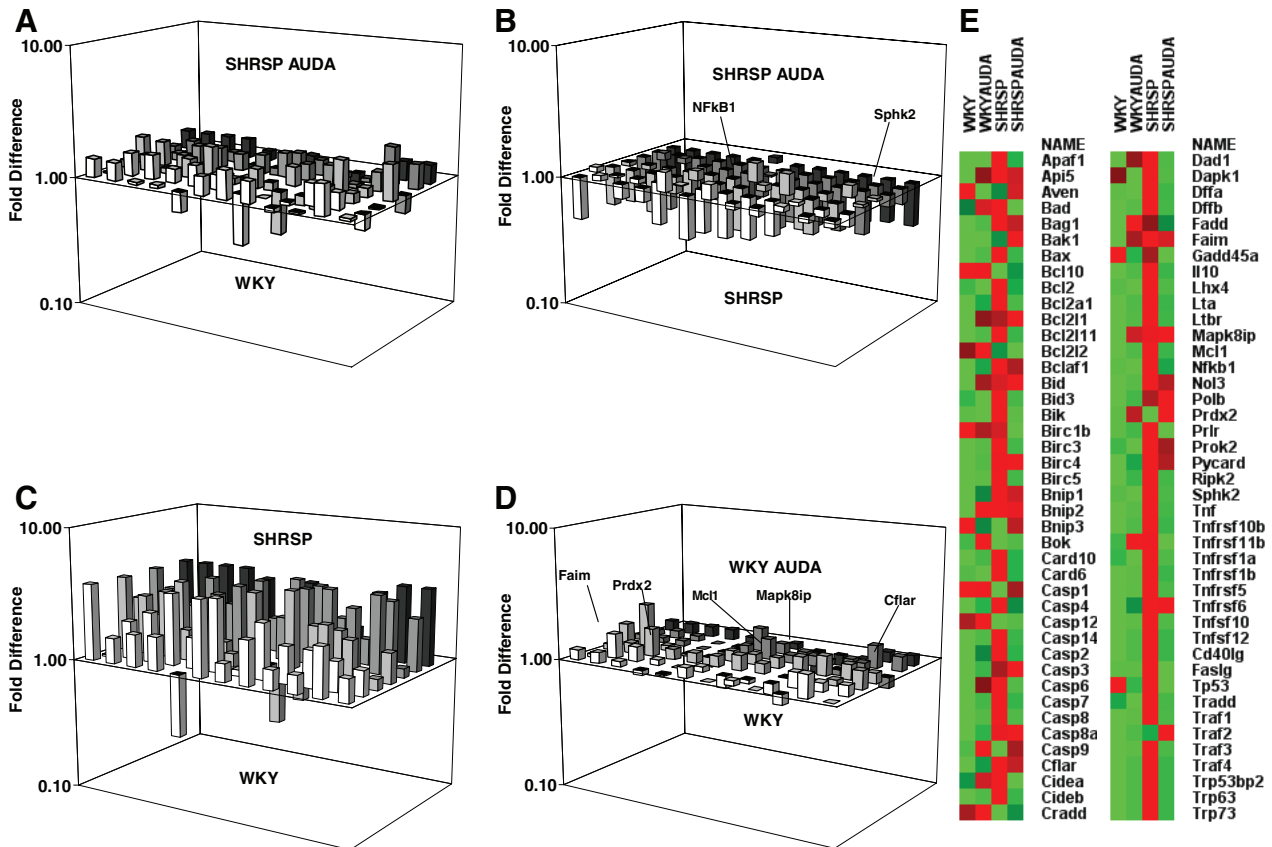


Figure 5. Expression of apoptotic genes in neural tissue was compared with mRNA PCR microarray. *n* = 3 per experimental group. **A:** Gene expression of 25 apoptotic factors increased more than or equal to twofold in SHRSP control versus WKY. **B:** AUDA down-regulated 32 apoptotic factors more than or equal to twofold in SHRSP, including *nuclear factor (NF)-kB1* and *sphingosine kinase 2 (Sphk2)*. **C:** Difference in gene expression in AUDA-treated SHRSP versus WKY. **D:** Gene expression of seven apoptotic factors increased ≥ 1.5 -fold in AUDA-treated WKY versus WKY. **E:** Heat map of gene expression profiles of each treatment group and relative changes in expression of the genes between the four experimental groups (green lowest expression to bright red highest expression between the four experimental groups).

Apoptosis

EETs and CYP overexpression reportedly antagonize intrinsic and extrinsic apoptosis.^{30–33} Also, EETs and SEH inhibition have been shown to promote cell survival during hypoxic reperfusion injury.³⁴ As a result, we undertook a global approach to assess expression of apoptotic genes (see Supplemental Table S2 at <http://ajp.amjpathol.org> for gene description of the 84 genes analyzed in the PCR plate) in brain samples collected from controls and AUDA-treated SHRSP and WKY rats using an apoptosis PCR microarray (Figure 5 and Table 1). We have identified 57 genes up-regulated ≥ 1.5 -fold and 25 ≥ 2 -fold in the SHRSP in comparison with the WKY encoding *tumor necrosis factor (TNF)* ligands and receptors, members of the Bcl-2 family, caspases, *nuclear factor (NF)-kB1*, and factors involved in DNA damage-induced apoptosis (Table 1 and Figure 5, A and E). In contrast, AUDA-treated SHRSP had only 1 gene up-regulated ≥ 2 -fold and 11 ≥ 1.5 -fold in comparison with WKY (Figure 5, C and E; and Table 1). Furthermore, AUDA reduced gene expression of 39 apoptotic factors ≥ 1.5 -fold and 32 ≥ 2 -fold versus SHRSP vehicle-treated rats (Figure 5, B and E). AUDA down-regulated genes in the SHRSP rats encoding *NF-kB1*, *sphingosine kinase 2 (Sphk2)*, TNF ligands (*CD40L*, *Lta*, *Lbtr*, *Tnf*, *FasL*), and TNF receptors

(1a, 1b, and 11b), mediators of DNA damage-induced apoptosis (*Dad1*, *Cidea*, *Cideb*, *Dffb*, *Trp53bp2*, *Trp63*, and *Trp73*), members of the Bcl-2 family (*Bcl2*, *Bid3*, *Bax*, *Bcl2l11*, *Mcl1*, and *Bik*), caspases, and additional mediators listed in Table 1. In the WKY, AUDA up-regulated seven genes ≥ 1.5 -fold as listed in Table 1 (Figure 5, D and E). Interestingly, AUDA increased anti-apoptotic mediators that have also been shown to be neurotrophic and/or neuroprotective, including *mitogen-activated protein kinase 8* interacting protein (*Mapk8ip*),^{35–38} *Fas apoptotic inhibitory molecule (Faim)*,^{39,40} *caspase 8*, and *FADD-like apoptosis regulator/Fas-associated death domain-like interleukin-1 β -converting enzyme inhibitory protein (Cflar/Flip)*,^{41,42} *myeloid cell leukemia sequence 1 (Mcl-1)*,⁴³ and an antioxidant *peroxiredoxin 2 (Prdx2)* in the WKY animals (Table 1 and Figure 5E).

Discussion

In this study, we investigated vascular and nonvascular mechanisms by which AUDA can protect against cerebral ischemia in normotension and hypertension. Although chronic SEH inhibition via AUDA protects SHRSP rats via vascular and neural protection, only neural protection was observed in WKY rats.

Table 1. Using PCR Microarray, N-Fold Changes in mRNA Expression Levels Were Determined of Genes Encoding Mediators Involved in the Apoptotic Pathway ($n = 3$ Per Experimental Group)

Fold up- or down-regulation apoptotic genes									
Gene	SHRSP/ WKY	WKY AUDA/WKY	SHRSP AUDA/WKY	SHRSP AUDA/ SHRSP	Gene	SHRSP/ WKY	WKY AUDA/WKY	SHRSP AUDA/WKY	SHRSP AUDA/SHRSP
<i>Apaf1</i>	1.5	1.0	1.1	-1.1	<i>Dad1</i>	1.4*	1.2*	1.0	-1.2*
<i>Api5</i>	2.3*	1.5*	1.7*	-1.1	<i>Dapk1</i>	1.3	-1.2	-1.1	-1.3
<i>Aven</i>	1.4*	-1.2	-1.0	1.1	<i>Dffa</i>	2.5	-1.0	1.2	-1.7
<i>Bad</i>	1.4*	1.0*	-1.1	-1.1	<i>Dffb</i>	3.5*	1.1	1.3	-2.1*
<i>Bag1</i>	2.2*	1.1	1.5	-1.1	<i>Fadd</i>	1.8	2.2*	1.5*	-1.1
<i>Bak1</i>	1.4	1.0	1.7*	1.3	<i>Faim</i>	2.4*	1.5*	1.6*	-1.1
<i>Bax</i>	3.8*	1.2	1.4	-2.1*	<i>Gadd45a</i>	1.2	-1.2	-1.4	-1.2
<i>Bcl10</i>	-2.9*	-1.0	-1.4	1.4	<i>Il10</i>	3.8*	1.2*	1.4	-2.1*
<i>Bcl2</i>	1.8*	-1.1	1.1	-1.3*	<i>Lhx4</i>	2.9*	-1.1	1.1	-2.1*
<i>Bcl2a1</i>	1.8*	1.1	1.0	-1.3	<i>Lta</i>	3.8*	1.2*	1.4	-2.1*
<i>Bcl2l1</i>	1.6*	1.2*	1.4	1.1	<i>Ltbr</i>	3.6*	1.1	1.3	-2.1*
<i>Bcl2l11</i>	3.8*	1.2	1.4	-2.1*	<i>Mapk8ip</i>	2.1*	1.4*	1.5*	1.0
<i>Bcl2l2</i>	1.5*	1.2*	-1.3	-1.2	<i>Mcl1</i>	3.8*	1.2*	1.4	-2.1*
<i>Bclaf1</i>	1.7*	1.2*	1.3	-1.1	<i>Nfkb1</i>	3.3*	-1.0	1.3	-1.5*
<i>Bid</i>	1.9*	1.3*	1.5	1.1	<i>Nol3</i>	2.7*	1.0	1.4	-1.1
<i>Bid3</i>	1.9	-1.2	-1.1	-1.8*	<i>Polb</i>	1.3	1.1	1.4	1.1
<i>Bik</i>	2.6	-1.1	1.0	-2.0*	<i>Prdx2</i>	1.2	1.1*	1.1	1.1
<i>Birc1b</i>	1.1	-1.2	-2.4	-2.2*	<i>Prlr</i>	3.8*	1.1	-1.0	-1.6
<i>Birc3</i>	3.5*	1.1	1.3	-2.1*	<i>Prok2</i>	2.5*	1.3	1.5*	-1.3
<i>Birc4</i>	1.7*	1.0	1.4	1.0	<i>Pycard</i>	3.2*	1.5*	2.0*	-1.2
<i>Birc5</i>	3.5*	1.1	1.3	-2.1*	<i>Ripk2</i>	1.6*	1.1*	1.1	-1.3*
<i>Bnip1</i>	2.4*	1.3*	1.5*	-1.1	<i>Sphk2</i>	2.1*	-1.1	1.1	-2.3*
<i>Bnip2</i>	1.9*	1.5*	1.5	-1.0	<i>Tnf</i>	3.6*	1.1	1.3	-2.1*
<i>Bnip3</i>	-1.0	-1.1	-1.0	1.1	<i>Tnfrsf10b</i>	3.8*	1.2*	1.4	-2.1*
<i>Bok</i>	1.2	1.4*	-1.0	1.0	<i>Tnfrsf11b</i>	1.8*	1.5*	-1.1	-1.7*
<i>Card10</i>	2.1	1.1	1.2	-1.4	<i>Tnfrsf1a</i>	2.3	-1.4	-1.1	-2.1*
<i>Card6</i>	2.6*	-1.2	1.2	-1.8*	<i>Tnfrsf1b</i>	2.1*	1.0	1.2	-1.7*
<i>Casp1</i>	-1.5	-1.0	-1.2	1.5	<i>Tnfrsf5</i>	3.2*	1.1	1.2	-2.2*
<i>Casp4</i>	-2.3*	1.2*	-2.1	1.1	<i>Tnfrsf6</i>	1.9*	1.1*	1.3	-1.0
<i>Casp12</i>	3.8*	1.3*	1.4	-2.1*	<i>Tnfsf10</i>	2.4*	1.1	1.1	-1.9*
<i>Casp14</i>	1.3	1.2*	1.1	-1.3	<i>Tnfsf12</i>	3.8*	1.2*	1.4	-2.1*
<i>Casp2</i>	2.2*	1.2*	1.8*	1.2	<i>Cd40lg</i>	3.8*	1.2*	1.4	-2.1*
<i>Casp3</i>	1.7	1.2*	1.4	-1.4	<i>Faslg</i>	3.2*	1.0	-1.1	-2.3*
<i>Casp6</i>	3.1*	1.6*	1.1	-2.0*	<i>Tp53</i>	1.6	-1.2	-1.3	-1.3
<i>Casp7</i>	2.8*	1.2*	1.3	-1.8*	<i>Tradd</i>	1.7*	-1.5	-1.2	-2.0*
<i>Casp8</i>	3.7*	1.1*	1.1	-2.0*	<i>Traf1</i>	3.3*	1.1	1.2	-2.1*
<i>Casp8ap2</i>	1.7*	1.2*	1.9*	1.0	<i>Traf2</i>	1.3	1.0	1.2	1.1
<i>Casp9</i>	-1.0	1.3*	1.2	1.2	<i>Traf3</i>	3.2*	1.1	1.2	-2.1*
<i>Cflar</i>	2.2*	1.4*	1.8*	-1.1	<i>Traf4</i>	3.8*	1.2*	1.4	-2.1*
<i>Cidea</i>	2.2*	1.3*	-1.4	-2.0*	<i>Trp53bp2</i>	3.8*	1.2*	1.4	-2.1*
<i>Cideb</i>	3.8*	1.2*	1.4	-2.1*	<i>Trp63</i>	3.8*	1.2*	1.4	-2.1*
<i>Cradd</i>	-1.3	1.2*	-1.2	1.4	<i>Trp73</i>	3.8*	1.2*	1.4	-2.1*

Fold up- or down-regulation (N-fold Δ in expression level) in between the compared groups identified in each column is given. *Factors significantly up- or down-regulated are in bold.

Chronic AUDA treatment of SHRSP rats protected through vascular protection by attenuating the hypertrophic remodeling and collagen deposition that occurs in the large cerebral vessel of the SHRSP rats.^{9,10} Several reports suggest that EETs and SEH inhibition may attenuate vascular remodeling by modulating intracellular signaling pathways in vascular smooth muscle cells⁴⁴ and fibroblasts.⁴⁵ Our laboratory has previously shown that SEH inhibition with 1-cyclohexyl-3-dodecyl-urea (CDU) decreased collagen deposition in kidneys of angiotensin-infused hypertensive rats and decreased renal vascular remodeling.¹ Furthermore, we have previously found increased MCA compliance in SHRSP rats chronically treated with AUDA.⁸ This corroborates our current findings of reduced W:L ratio, wall thickness, and collagen deposition in the SHRSP.

In addition to its effects on the MCA, AUDA treatment increased cerebral microvessel density in the SHRSP rats. Jesmin and colleagues^{27,28} reported that SHRSP rats have reduced regional cerebral blood flow, angiogenic factors, and cerebral microvessel density in the cerebral cortex at 6 weeks of age in comparison with age-matched WKY rats.²⁸ In our study, vehicle-treated SHRSP rats also demonstrated reduced cerebral microvessel density at 12 weeks of age, the time of occlusion, in comparison with the WKY rats to a degree similar to the previous report. EETs have been shown to induce angiogenesis in several *in vivo* models and on co-culture of astrocytes and endothelial cells.² Because hypoxia and increased metabolic demand are potential triggers for astrocyte EET-mediated angiogenesis,⁴⁶ we speculate that SEH inhibition resulted in increased cerebral

microvessel density by countering the deficiencies present in the SHRSP rats. Interestingly, there was a trend for maintenance of the cerebral protection in SHRSP rats after AUDA was withdrawn for 7 to 12 days. Taken together, these results suggest that cerebral protective effects of AUDA in SHRSP rats were in part attributable to structural changes in the vasculature.

Unlike the effects of AUDA on vascular remodeling and microvessel density, SEH inhibition provided neural protection in both normotensive and hypertensive rat strains by modulating gene expression of mediators involved in the regulation of apoptosis in the brain tissue of both WKY and SHRSP rats. We propose that the up-regulation of the anti-apoptotic mediators and neuroprotectants in the WKY and dampening of overexpression of pro-apoptotic mediators in the SHRSP rats may set the stage for increased tolerance of cellular stress. Although the studies conducted here were on tissue pre-ischemic insult and therefore do not allow for the determination of the specific genes that may be involved in providing protection from cerebral ischemia, they do show that SEH inhibition causes a global change in the expression of mediators involved in the apoptotic pathway. The range of fold changes in expression levels we detected are in accordance with prior reports³¹ of apoptotic gene expression and protein expression pre-insult, which report smaller inductions pre-insult that prime the cells for a dramatic up-regulation on induction of apoptosis, and thus promotion of cell survival.³¹ However, because of the limitations of our study, we cannot determine the cell type or specific genes that could be attributed to possible neuroprotection. The SAM statistical analysis is limited by the occurrence of false-positives, and we have not confirmed changes in gene expression by cellular localization, sub-cellular localization, or protein analysis.

The changes that we report with SEH inhibition do however corroborate previous findings of the anti-apoptotic properties of EETs, CYP overexpression, and SEH inhibition. EETs and CYPs reportedly inhibit apoptosis induced by Fas ligand,³⁰ TNF- α induced,^{31,32} serum deprivation,³⁰ etoposide, arachidonic acid, ceramide production, inhibition of reactive oxygen species,³³ and hypoxic reperfusion possibly by antagonizing reactive oxygen species.³⁴ Our data are also in agreement with a previous report that MAPK and PI3/Akt signaling pathways protect endothelial cells from TNF- α induced apoptosis.³¹ Closely related, a recent study linked human SEH polymorphisms to cortical neuronal sensitivity to oxygen-glucose deprivation. Overexpression of SEH and human polymorphisms associated with variations in SEH activity increased cell death induced by oxygen-glucose deprivation.⁴⁷ To the best of our knowledge, our study is the first to provide a possible link to the neuroprotection achieved via SEH inhibition with AUDA in hypertension and normotension with a shift in the balance in the gene expression of pro- and anti-apoptotic mediators, producing possible ischemic tolerance.

Previous reports indicate that AUDA has PPAR- α activity at high concentrations most probably attributable to its long alkyl chain.^{23,24} Its structural similarity to fatty acids makes the compound lipophilic, which increases

its ability to bind to the hydrophobic ligand-binding domain of PPAR- α .^{23,24} Although we cannot exclude the possibility of PPAR- α , the AUDA levels achieved in the present study were in the nanomolar range, which would indicate that AUDA levels were not sufficient to induce PPAR- α activity. We also found protection with a non-alkanoic acid SEH inhibitor, tAUCB. Not only does tAUCB lack the long fatty acid chain required for PPAR- α binding, tAUCB is more polar than AUDA.^{12,13} As a result, tAUCB lacks potential for binding to the hydrophobic PPAR- α binding domain.^{48,49} Moreover, mice that had the gene responsible for SEH production deleted also were protected from cerebral ischemic reperfusion injury.⁵⁰ Based on our current findings and published findings in the *Ephx2*^{-/-} mice, we conclude that the protection from cerebral ischemia with AUDA treatment is most likely to be attributable to SEH inhibition.

In our study, the SHRSP and WKY animals were treated with AUDA in the drinking water, as in our prior study.⁸ In our prior study, the AUDA and AUDA plasma levels were sufficient to show adequate inhibition of the SEH enzyme as demonstrated by an increase in the urinary epoxide to diol ratio. In the current study, we do not have confirmatory data to show the level of SEH inhibition via the epoxide to diol ratio in the brain or plasma. However, the plasma and brain AUDA metabolite and AUDA drug levels are similar to that previously established as therapeutic in our prior study.⁸ In addition, measuring the degree of SEH inhibition directly is more difficult because AUDA reversibly and competitively inhibits the SEH enzyme.^{13,21,22} In the current study, the AUDA plasma levels were variable between the WKY and SHRSP animals, and could be attributable to differences in the drinking water intake between the strains. We cannot rule out possible differences in pharmacokinetics and AUDA metabolism between the rat strains. However, the brain tissue levels of AUDA were very similar between the two groups in our study. The reason for similar brain AUDA levels is not known but could be related to the blood brain barrier or to brain compartmentalization of AUDA. We did not measure brain AUDA levels in the prior study. As a result, we cannot determine relative differences in between dose and brain tissue levels of AUDA in this study in comparison with our prior study. Yet, the doses of the SEH inhibitor were similar to those also shown to be protective against LPS challenge in mice.¹² In addition, the plasma levels in both studies were above the rat IC₅₀ for AUDA (8 nmol/L).¹²

Moreover, in our study we did not investigate the other potential protective effects of the ACE inhibitor enalapril or its possible effects in the WKY rats. Here, we used enalapril as a blood pressure-lowering agent to provide a blood pressure control in the SHRSP rats and therefore did not treat the normotensive WKY rats with enalapril. However, it is important to recognize that ACE inhibitors such as enalapril have the potential to protect against cerebral ischemia by mechanisms that may not be directly related to blood pressure lowering.

In the present study, we evaluated the cerebral protective effects of SEH inhibition in normotensive and hypertensive animals because both presentations are clin-

ically relevant. Conducting these studies in an animal model with hypertension is essential for addressing the main patient population affected by ischemic stroke.⁵¹ Based on the TOAST study subclassifications, 36 to 56.6% of ischemic strokes are reportedly related to vascular disease associated with hypertension, such as large vessel atherosclerosis and small artery disease.^{52,53} Moreover, human SEH polymorphisms have been found to alter SEH activity⁷ and not only affect the incidence of ischemic stroke but also hypertension and atherosclerosis.^{1,6} Smoking, another risk factor for ischemic stroke, synergistically increases the prevalence of hypertension and atherosclerotic disease with the SEH polymorphisms.¹ Because SEH polymorphisms are linked to both ischemic stroke and its modifiable risk factors, modulating SEH enzymatic activity may be beneficial in decreasing the incidence and severity of ischemic stroke in high-risk patients. It is also important to address the nonhypertensive population and the patients without evidence of vascular disease that have cerebral ischemic events. The ability of SEH inhibition to protect in the absence of hypertension and vascular disease implicates that SEH inhibition may also be beneficial in treating the nontraditional ischemic stroke patients. This hypothesis is further confirmed by the epidemiological association of increased risk for ischemic stroke with *Ephx2* gene polymorphisms in patients with large artery disease and patients lacking the traditional etiology for ischemic stroke.⁵⁴

In summary, we show that chronic SEH inhibition is protective during cerebral ischemia in hypertensive and nonhypertensive rats. SEH inhibition normalized cerebrovascular structure in hypertensive animals and provided neural protection in normotensive and hypertensive rats. Overall, the ability of AUDA to provide protection in the presence or absence of hypertension is clinically relevant because ischemic stroke occurs in hypertensive and nonhypertensive patients. The TOAST subclassifications demonstrates the heterogeneity of the ischemic stroke patient population,^{52,53} and as a result, using a treatment that can act through multiple mechanisms of protection under varying clinical presentations further increases the patient population that can be effectively treated. As a result, SEH inhibition has broad pharmacological potential for ischemic stroke management, and its utility should be further investigated.

Acknowledgments

We thank Jeffrey Quigley and Dr. David Stepp for technical assistance.

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