Soluble epoxide hydrolase inhibitor \( \text{trans-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid} \) is neuroprotective in rat model of ischemic stroke

Jafar Sadik B. Shaik,1 Muzamil Ahmad,3 Wenjin Li,2,3 Marie E. Rose,2,3 Lesley M. Foley,4 T. Kevin Hitchens,4 Steven H. Graham,2,3 Sung Hee Hwang,5 Bruce D. Hammock,5 and Samuel M. Poloyac1

1Department of Pharmaceutical Sciences, University of Pittsburgh School of Pharmacy, Pittsburgh, Pennsylvania; 2Geriatric Research Educational and Clinical Center, VA Pittsburgh Healthcare System, Pittsburgh, Pennsylvania; 3Department of Neurology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania; 4Pittsburgh NMR Center for Biomedical Research, Carnegie Mellon University, Pittsburgh, Pennsylvania; and 5Department of Entomology and Comprehensive Cancer Center, University of California, Davis, California

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Shaik JS, Ahmad M, Li W, Rose ME, Foley LM, Hitchens TK, Graham SH, Hwang SH, Hammock BD, Poloyac SM. Soluble epoxide hydrolase inhibitor \( \text{trans-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid} \) is neuroprotective in rat model of ischemic stroke. Am J Physiol Heart Circ Physiol 305: H1605–H1613, 2013. First published September 16, 2013; doi:10.1152/ajpheart.00471.2013.—Soluble epoxide hydrolase (sEH) diminishes vasodilatory and neuroprotective effects of epoxyeicosatrienoic acids by hydrolyzing them to inactive dihydroxy metabolites. The primary goals of this study were to investigate the effects of acute sEH inhibition by \( \text{trans-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid (t-AUCB)} \) on infarct volume, functional outcome, and changes in cerebral blood flow (CBF) in a rat model of ischemic stroke. Focal cerebral ischemia was induced in rats for 90 min followed by reperfusion. At the end of 24 h after reperfusion rats were euthanized for infarct volume assessment by triphenyltetrazolium chloride staining. Brain cortical sEH activity was assessed by ultra performance liquid chromatography-tandem mass spectrometry. Functional outcome at 24 and 48 h after reperfusion was evaluated by arm flexion and sticky-tape tests. Changes in CBF were assessed by arterial spin-labeled-MRI at baseline, during ischemia, and at 180 min after reperfusion. Neuroprotective effects of t-AUCB were evaluated in primary rat neuronal cultures by Cytotox-Flour kit and propidium iodide staining.

Address for reprint requests and other correspondence: S. M. Poloyac, Dept. of Pharmaceutical Sciences, School of Pharmacy, Univ. of Pittsburgh, 807 Salk Hall, Pittsburgh, PA 15261 (e-mail: poloyac@pitt.edu).

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their ability to dilate arteries (7). Thus EETs have become an attractive target for the treatment of cerebrovascular complications such as cerebral ischemia.

One method of increasing EETs levels in the brain is by inhibiting their primary route of degradation to less active dihydroxy metabolites by sEH (31). sEH inhibitors have been shown to be neuroprotective; however, no studies have evaluated whether single dose acute administration of sEH inhibitors can improve neurofunctional outcomes after ischemic stroke. These data are essential to add to the growing data to establish whether sEH inhibition meets the STAIR criteria for further clinical development (14). Therefore, the primary goals of this study were to evaluate the effect of acute sEH inhibition by t-AUCB on infarct volume, functional outcome, and changes in CBF using a transient middle cerebral artery occlusion (MCAO) model in rats.

**MATERIALS AND METHODS**

All animal experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC) and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Animals and experimental design.** Male Sprague-Dawley rats (250–300 g; Hilltop Laboratory Animals, Scottsdale, PA) were maintained on a 12-h:12-h light/dark cycle and were given food and water ad libitum. The rats were randomly assigned to either vehicle [lyophilized HP/βCD, 0.5 mg] groups. MCAO was performed on all rats. All HP/βCD lyophilized complexes (vehicle or t-AUCB) were reconstituted in phosphate-buffered saline, pH 7.2, and filtered before administration. Treatment group rats received a single t-AUCB 0.9 mg/Kg bolus dose via the femoral vein at the time of MCAO. Five experiments were performed: 1) effect of t-AUCB on cerebral infarct volume after MCAO (n = 9/group); 2) t-AUCB inhibitory potential on brain cortical sEH activity (n = 6/group); 3) acute t-AUCB effect on short-term behavioral outcome after MCAO (n = 9/group); 4) changes in CBF during and after MCAO with t-AUCB treatment were determined with arterial spin-labeled-MRI (ASL-MRI; n = 7/group); and 5) effect of t-AUCB on primary neuronal cultures after hypoxic injury (n = 6 wells/group). The surgeon and individuals involved in all above experiments were blinded to all treatment groups.

**MCAO in rats.** Rats received MCAO for 90 min followed by reperfusion as described previously (32). Briefly, rats were anesthetized via nose cone with 1% to 2% isoflurane, 50/50 N2/O2 through-oxygen surgery. The left common carotid artery was exposed, and the external common carotid artery was isolated and ligated using 5-0 silk (Ethicon). MCAO was achieved by inserting a 5.0 nylon suture (with tip coated with silicon ~280 μm diameter) into the internal carotid artery a distance of 16–19 mm from the bifurcation of the common carotid artery and internal carotid artery. The wound was closed, and the animals were allowed to recover with the suture in place. After 90 min, the rats were re-anesthetized and the suture removed, initiating reperfusion. Sham surgeries were performed in the same manner as MCAO surgeries but without insertion of suture. Throughout the surgical procedure core temperature was maintained at 37 ± 0.5°C using a thermo regulated heating pad.

**Infarct volume determination.** Rats (n = 9/group) were euthanized at 24 h after reperfusion, and infarct volume was assessed by staining with 2,3,5-triphenyl-tetrazolium chloride (TTC; Sigma, St. Louis, MO, 2% in phosphate-buffered saline). Brains were placed in a rat brain matrix (ASI Instruments, Warren, MI) and were sliced into 1-mm sections. The sections were immersed in the TTC for 30 min at room temperature. The sections were transferred to formalin and photographed. Infarct volume was measured using image analysis (MCID; St Catharines, Ontario, Canada). To minimize the effect of edema on the quantitative size, the method of Swanson et al. (36) was used. The percent infarct volume was calculated by dividing infarct volume by contralateral hemisphere volume.

**Tissue extraction and chromatographic analysis of AA metabolites.** Concentrations of various metabolites including HETEs (12-, 15-, and 20-HETE), EETs (8,9-, 11,12-, and 14,15-EET), DHETs (5,6-, 8,9-, 11,12-, and 14,15-DHET), PGs (6-keto-PGF1α, 11β-PGF2α, PGF2α, PGD2, PGI2, 15-deoxy-Δ12,14-PGJ2), 15-deoxy-Δ12,14-PGJ2, 15-F2t-PGF2α, PGF1α, PGE2, and TXB2 (11-dehydro-TXB2) were determined from brain cortical tissues of vehicle and t-AUCB (0.9 mg/kg iv; n = 6/group)-treated rats that underwent MCAO surgery using solid phase extraction as described previously with slight modifications (26, 28). Briefly, tissue samples were homogenized in deionized water containing 0.113 mM butylated hydroxytoluene and centrifuged for 30 min at 10,000 rpm. The supernatant was removed and spiked with 12.5 μl (containing 12.5 ng) of 20-HETE-d6 (for all HETEs, EETs, and DHETs), PGD2-d6, 15-deoxy-PGJ2-d6, 6-keto-PGF1α-d6, PGF1α-d6, 11-deoxy-TXB2-d6, PGE2-d6, and PGF2α-d6 as internal standards. The spiked supernatant samples were loaded onto Oasis hydrophilic-lipophilic balanced (30 mg) solid phase extraction cartridges (Waters, Milford, MA) that were conditioned and equilibrated with 1 ml of methanol and 1 ml of water, respectively. Columns were washed with three 1-mL volumes of 5% methanol and were eluted with 100% methanol. Extracts were spiked with 15 μl of 1% acetic acid in methanol, dried under nitrogen gas at 37°C, and reconstituted in 125 μl of 80:20 methanol/deionized water for chromatographic analysis as described previously (26).

Briefly HETEs, EETs, and DHETs were separated on a ultra performance liquid chromatography BEH C18 column 1.7 μm (2.1 × 100 mm), and PGs were separated on a ultra performance liquid chromatography BEH C18, 1.7 μm (2.1 × 150 mm) reverse-phased column (Waters, Milford, MA) protected by a guard column (2.1 mm × 5 mm; Waters) of the same packing material. Column temperature was maintained at 55°C. Mobile phases consisted of 0.005% acetic acid, 5% acetonitrile in deionized water (A), and 0.005% acetic acid in acetonitrile (B). HETEs, EETs, and DHETs were separated by delivering mobile phase at 0.5 ml/min at an initial mixture of 65:35 A and B, respectively. Mobile phase B was increased from 35% to 70% in a linear gradient over 4 min, and again increased over 0.5 min where it remained for 0.3 min. This was followed by a linear return to initial conditions over 0.1 min with a 1.5 min pre-equilibration period before the next sample run. A slightly different gradient program was used for PGs separation where the mobile phase was delivered at 0.4 ml/min at an initial mixture of 65:35 A and B. Mobile phase B was maintained at 35% for 7.5 min and then increased to 98% in a linear gradient over 1.5 min, where it remained for 0.2 min. This was followed by a linear return to initial conditions over 0.1 min with a 2.7 min pre-equilibration period before the next sample run. Total run time per sample was 6.4 min for HETEs, EETs, and DHETs and 12 min for all PGs. All injection volumes were 7.5 μl.

Mass spectrometric analysis of analyte formation was performed using a TSQ Quantum Ultra (Thermo Fisher Scientific, San Jose, CA) triple quadrupole mass spectrometer coupled with heated electrospray ionization operated in negative selective reaction monitoring mode with unit resolutions at both Q1 and Q3 set at 0.70 full width at half maximum. Quantitation by selective reaction monitoring analysis on HETEs, EETs, DHETs, and PGs was performed by monitoring their ms/ transitions. Scan time was set at 0.01 s, and collision gas pressure was set at 1.3 mTorr. Analytical data was acquired and analyzed using Xcalibur software version 2.0.6 (Thermo Finnigan, San Jose, CA).

**Functional outcome assessment.** Functional outcome experiments were aimed at evaluating motor activity (primary motor cortex) and somatosensory activity of rats that underwent MCAO surgery. Behavioral deficits (functional outcome evaluation) in rats (n = 9/group) were examined at 24 and 48 h after reperfusion. A simple neurological scoring system was used to assess neurological damage following MCAO surgery as follows: 0 = no neurological deficit; 1 = failure to
extend left forepaw fully and torso turning to ipsilateral side when held by tail (a mild focal neurologic deficit); 2 = circling to the effected side (a moderate focal neurologic deficit); 3 = unable to bear weight on the effected side (a severe focal deficit); 4 = no spontaneous locomotor activity. Behavioral deficits were determined using the arm flexion and sticky tape test as described previously (3). The arm flexure test was conducted once daily by lifting rats by their tails so that their ventral surface was exposed for observation. The cumulative duration of asymmetrical arm flexure during a 10-s period after tail lifting was recorded using a stop watch. In the tape test, self-adhesive labels (1-cm-diameter circles) were placed on each forepaw to assess the time required for the rat to touch and remove each label. In addition, the order (contralateral vs. ipsilateral) of removal was also used to determine ipsilateral asymmetry. Preference for a given wrist was accounted for by affixing larger labels to the wrist less preferred and correspondingly smaller labels to the other wrist. The larger the ratio between surface of ipsilateral versus contralateral patches (from 1:1 to 1/8:15/8), the more extensive the damage (scored on a scale from 1 to 7 in the increasing order of severity of damage). One trial per day was conducted at 24 and 48 h after reperfusion.

**Cerebral blood flow assessment using ASL-MRI imaging.** CBF measurements were assessed by arterial spin-labeled (ASL)-MRI. Rats ($n = 7$) underwent femoral artery catheterization and were placed in a prone position on the cradle. MRI was performed using a 4.7-Tesla, 40-cm bore Bruker BioSpec AVI system (Billerica), equipped with a 12-cm shielded gradient insert. A 72-mm volume coil with 2.5 cm actively decoupled brain surface coil was used for imaging. Continuous ASL was used to quantify CBF (12, 39). A single shot SE-EPI sequence was used with a TR = 2 s, 64 × 64 matrix, FOV = 2.3 cm, 2-s labeling pulse. The labeling pulse for the inversion plane was positioned ± 2 cm from the perfusion detection plane. For each experiment, a map of the spin-lattice relaxation time of tissue water ($T_{1,obs}$) was generated from a series of spin-echo images with variable TR (FOV = 2.3 cm, 4 averages, 64 × 64 matrix) (17). CBF was assessed at three time points: at baseline, 70 min (during the occlusion period), and at 270 min (3 h after reperfusion). Blood gases were sampled at each time point and analyzed (Radiometer, Westlake, OH). For the duration of the experiment mean arterial blood pressure and EKG were continuously monitored. Rectal temperature was maintained at 37°C using a warm air system (SA Instruments, Stony Brook, NY).

The effect of t-AUCB on primary neuronal cultures after hypoxic injury. Rat primary cortical neurons were prepared as described previously (23). Briefly, cortical primary neuronal cultures were prepared from E17 fetal rats. Brains were removed and cortices dissected. Brain cortical tissue was freed of meninges and trypsinized. Brains were removed and cortices prepared from E17 fetal rats. Brains were removed and cortices prepared from E17 fetal rats. Cells were then plated at a density of $6 \times 10^4$ cells/well. The medium was replaced the following day and every 3 days thereafter with Neurobasal A medium (Invitrogen). The cultures were used for hypoxia experiments after 11 days. On the 12th day, the cells were either pretreated for 1 h with vehicle (neurobasal medium) or t-AUCB diluted in neurobasal medium at a final concentration of 0.1 and 0.5 μM. After pretreatment, the culture plates were placed into a hypoxic glove box (Coy Laboratories, Grass Lake, MI) flushed with argon for a period of 3 h, resulting in ~50% cell death after 24 h of reperfusion under normal incubation conditions. Staurosporine (20 μM, a 100% cell death internal standard) and MK801 (1 μM, a 100% cytoprotective internal standard) were used as positive and negative controls, respectively. Cytotoxicity was evaluated using a CytoTox-Fluor kit (Promega, Madison, WI) assay and propidium iodide (PI) staining (Molecular Probes) in two different experiments. At the end of the 24-h incubation period, cells were imaged under a fluorescent microscope for Hoechst (blue) and PI (red) staining and counted. Cell death in all treatment groups was normalized to that of the staurosporine-treated group.

**Statistical analysis.** Significant differences between treatment groups in experiments measuring infarct volume and brain sEH activity were assessed by Student’s t-test, and for in vitro neuronal culture experiments one-way ANOVA with Dunnett’s post-hoc test was used. Significant differences for ASL-MRI blood flow measurements and functional outcome assessments were determined via two-way ANOVA analysis. A *$P < 0.05$ was considered significant.

**RESULTS**

**Effect of t-AUCB on infarct volume after MCAO.** The effect of acute t-AUCB pretreatment on infarct volume after MCAO was evaluated and compared against vehicle. Figure 1A depicts representative rat brain sections stained with TTC. A significant reduction in percent infarct volume was observed in t-AUCB as compared with vehicle-treated (14.5 ± 2.7% vs. 41.5 ± 4.5%; ***$P < 0.001$) rats (Fig. 1B).

**Effect of t-AUCB administration on brain sEH activity after MCAO.** The effect of acute sEH inhibition by t-AUCB in brain cortex after MCAO was assessed by measuring concentrations of various HETEs, EETs, and DHETs as well as various PGs to verify the specificity of t-AUCB inhibition. A significant increase in the ratio of cumulative EETs (11,12- and 14,15-EET) to DHETs (11,12- and 14,15-DHET) was observed in t-AUCB as compared with vehicle-treated (4.40 ± 1.89 vs. 1.97 ± 0.85; *$P < 0.05$) rats (Fig. 2A). No significant differences were observed in the concentrations of representative metabolites from the HETE and PG family such as 20-HETE.
Fig. 2. The effect of acute t-AUCB treatment on brain cortical soluble epoxide hydrolase (sEH) activity after temporary MCAO in rats (n = 6). Rats treated with t-AUCB showed a significant increase in the ratio of cumulative epoxyeicosatrienoic acids (EETs)/dihydroxyeicosatrienoic acids (DHETs) (11,12- and 14,15-EET/DHET) (A) but no significant changes in 20-hydroxyeicosatetraenoic acids (HETE; B), 6-Keto-PGF1α, a metabolite of prostacyclin (Fig. 2C: vehicle: 22.26 ± 4.35 vs. t-AUCB: 24.98 ± 6.21 pmol/gm tissue; P = 0.41); and PGF2α (Fig. 2D: vehicle: 57.73 ± 9.92 vs. t-AUCB: 64.68 ± 13.73 pmol/gm tissue; P = 0.35) in the cortex of t-AUCB and vehicle-treated rats. Control values of these metabolites (without stroke) were also depicted in respective figures. Representative LC/MS chromatograms depicting the levels of EET and 20-HETE before and after treatment with t-AUCB are shown in Fig. 3.

Effect of t-AUCB treatment on functional outcome after MCAO. The effect of t-AUCB pretreatment on short-term behavioral deficits after MCAO was evaluated in arm flexion and sticky tape behavioral tests. Rats receiving t-AUCB treat-
t-AUCB treatment significantly lowered neurological deficit scores on days 1 and 2 compared with the vehicle-treated group (day 1: 1.71 ± 0.9 vs. 2.75 ± 0.4; *P < 0.05; day 2: 1.14 ± 0.3 vs. 2.14 ± 0.3; ***P < 0.001; Fig. 4B). Sticky tape tests also revealed a significant impact of t-AUCB on days 1 and 2 compared with the vehicle-treated group (Fig. 4, C and D). Time to remove (in seconds) sticky tape from the contralateral arm was 140.37 ± 15 s vs. 92.8 ± 3.5 s on days 1 and 2, respectively, in the vehicle group, which was significantly reduced in the t-AUCB group on both days 1 and 2 (98.15 ± 6 s and 64.6 ± 8 s, ***P < 0.001). Tape surface area ratio of contralateral to ipsilateral arm in the vehicle group was 6.96 ± 0.77 vs. 5.97 ± 1.2 on days 1 and 2, respectively. This was significantly reduced with t-AUCB treatment on both days (2.53 ± 0.6 vs 1.19 ± 0.2; ***P < 0.001).

Effect of t-AUCB treatment on cerebral blood flow changes after MCAO. The effect of t-AUCB treatment on changes in CBF during and after ischemic injury was assessed with ASL MRI. Representative brain perfusion maps of rats treated with t-AUCB or vehicle at three different time points are shown in Fig. 5A. There is mild to moderate improvement in perfusion around the infarcted tissue during the post-ischemic hypoperfusional state. The representative brain perfusion maps of rats treated with vehicle or t-AUCB at baseline (pre-MCAO), during MCAO (70 min), and after post-ischemic reperfusion (270 min) are shown in Fig. 5A. The dark blue area on the right cerebral cortex signifies formation of infarct. CBF values (reported as ml/100 g tissue/min) in the cortex ipsilateral to infarct are shown in Fig. 5B. Cerebral blood flow (CBF) during and after MCAO were assessed with arterial spin labeling (ASL) MRI. A: Representative CBF maps of rats treated with vehicle or t-AUCB before MCAO (pre), during MCAO (70 min), and after post-ischemic reperfusion (270 min). Dark blue area on right cerebral cortex signifies formation of infarct. B: CBF values (reported as ml/100 g tissue/min) in the cortex ipsilateral to infarct. C: Physiological parameters [mean arterial blood pressure (MABP) and blood pCO2]. Data represented as means ± SD.
fusion period (270-min MRI scan) in t-AUCB-treated rats. CBF values calculated from perfusion and T1obs maps revealed no differences in the CBF values between the two groups at baseline and during ischemic injury (Fig. 5B). However, a nonsignificant trend toward increased CBF was seen during the post-ischemic hypoperfusion period (180 min after reperfusion) in t-AUCB-treated rats compared with vehicle control (Fig. 4B; t-AUCB: 73.3 ± 35 vs. vehicle: 42.2 ± 17 ml/100 g/min; P = 0.079). No statistically significant differences between physiological variables such as mean arterial blood pressure and pCO2 were observed between the two treatment groups (Fig. 5C).

Effect of t-AUCB on cytotoxicity of in vitro neuronal cultures under hypoxic conditions. The effect of t-AUCB on cytotoxicity of rat primary cortical neuronal culture was assessed by treating neurons with 0.1 and 0.5 μM t-AUCB followed by hypoxic injury. Pretreatment before hypoxic injury resulted in a slight nonsignificant reduction in cell death at 0.1 μM (cell death: vehicle: 67 ± 11.6 vs. t-AUCB: 56.91 ± 7%; P = 0.057) and significant reduction at 0.5 μM (cell death: vehicle: 67 ± 11.6 vs. t-AUCB: 45.56 ± 9.5%; **P < 0.01) compared with their respective vehicle-treated groups as assessed by the CytoTox-Flour kit assay (Fig. 6A). Under nonhypoxic conditions of incubation t-AUCB did not alter neuronal survival compared with vehicle-treated group (cell death: vehicle: 45.56 ± 9.4%; t-AUCB0.1μM: 44.09 ± 7.5%; t-AUCB0.5μM: 52.04 ± 6.1%; P = 0.124) when tested at the same concentration range (Fig. 6A). In another experiment, neurons treated with t-AUCB at 0.1 and 0.5 μM before hypoxic injury were imaged under a fluorescent microscope for Hoechst (blue) and PI (red) staining (Fig. 6B). t-AUCB pretreatment before hypoxic injury resulted in significant reduction in cell death in a

Fig. 6. The effect of t-AUCB on cytotoxicity of primary rat cortical neuronal cultures. Cytotoxicity was assessed by using a Cytotox-Flour kit and propidium iodide (PI) staining. Cytotoxicity was represented as percent cell death normalized to positive control staurosporine (SP). A: percent cell death with t-AUCB or vehicle treatment under hypoxic and nonhypoxic conditions. B: representative images of rat primary cortical neuronal cultures imaged under fluorescence microscope after Hoechst (blue) and PI (red) staining. C: cytotoxicity assessment after hypoxic injury by PI staining. Staurosporine and MK-801 were used as positive and negative controls. Data represented as means ± SD. **Significant values for P < 0.01; ***significant values for P < 0.001, respectively.
dose-dependent manner (percent PI-positive cells: vehicle: 70.9 ± 7.1 vs. t-AUCB0.1μM: 58 ± 5.11; vs. t-AUCB0.5μM: 39.9 ± 5.8; ***P < 0.001) compared with the vehicle-treated group (Fig. 6C).

**DISCUSSION**

In this study, we investigated the neuroprotective effects of acute sEH inhibition with a single low-dose t-AUCB (0.9 mg/kg iv) in focal ischemic stroke and the factors contributing to the neuroprotection. Specifically, we have established causative relationship between acute sEH inhibition, EETs concentrations, and neuroprotection. Furthermore, this study is the first to report the impact of acute sEH inhibition on behavioral performance using comprehensive behavioral tests evaluating motor and somatosensory activity. The major findings of this study are that a single low-dose administration of t-AUCB 1) significantly decreases infarct volume and elevates brain cortical EETs concentrations, 2) does not significantly alter CBF as determined by ASL-MRI, and 3) significantly increases neuronal survival under hypoxic conditions. Collectively, these data suggest that acute inhibition of sEH by t-AUCB offers neuroprotection primarily through a direct cytoprotective effect on neurons with a minor contribution from alterations in CBF.

Our finding of significant reduction in infarct volume after MCAO is consistent with the previous data reported using urea-based derivative 12-(3-adamantan-1-yl-ureido)-dodecanedicarboxylic acid butyl ester (AUDA-nBE) in a mouse (10 mg/kg ip) (13, 18, 41) and rat (2 mg/day) (33) model of ischemic stroke. Also our results are consistent with other non-urea-based sEH inhibitors such as 4-PCO (4-phenyl chalcone oxide) that have been shown to produce neuroprotection in stroke models (18a). We found that acute administration of t-AUCB 1) significantly decreases infarct volume and elevates brain cortical EETs concentrations, 2) does not significantly alter CBF as determined by ASL-MRI, and 3) significantly increases neuronal survival under hypoxic conditions. Collectively, these data suggest that acute inhibition of sEH by t-AUCB offers neuroprotection primarily through a direct cytoprotective effect on neurons with a minor contribution from alterations in CBF.

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activity of sEH are linked to neuronal survival in an in vitro oxygen-glucose deprivation study with primary rat cortical neurons. In this experiment, Koerner et al. (22) showed that overexpression of wild-type sEH resulted in an increase in OGD-induced neuronal death, which was reversed by exogenous addition of excess 14,15-EET. Also, a mutant of sEH with decreased hydrolase activity showed significant reduction in cell death compared with untreated cells. In addition to the cytoprotective effect on neurons, EETs also appear to exert beneficial effects on other brain cells in ischemia as reported by Liu and Alkayed (24), who showed cytoprotective effects of exogenous administration of EETs on cortical astrocyte culture in an OGD model. Although, we studied the neuroprotective mechanisms in vitro and CBF changes in vivo, we observed significant improvements in infarct volume reduction and behavioral performance in the absence of a significant CBF improvement, suggesting that the protective effects are due to both direct effect on neurons and additional contributions from CBF changes. Future studies evaluating EET agonists and antagonists that evaluate microvascular flow will aid in the elucidation of the mechanisms of neuroprotection. Furthermore, evaluating the effect of sEH inhibition on oxidative stress and inflammation accompanying ischemic damage of cerebral tissue will elucidate underlying effector mechanisms involved in the pathogenesis. Although we did not observe significant changes in the levels of key prostaglandin metabolites across treatment groups, future studies evaluating the levels of key oxidative stress markers such as 8-isoprostane will help in elucidating the underlying mechanisms of pathogenesis of ischemic injury. Collectively, these data suggest that altering EETs levels by acute inhibition of sEH is likely to produce the largest benefits by affecting multiple components of neurovascular unit such as astrocytes, neurons, and microvascular flow.

One of the limitations in our current study was the administration of t-AUCB at the time of initiation of ischemia. Due to the difficulty in administering t-AUCB post-ischemic injury in our MCAO model in CBF assessment experiments by ASL-MRI, we administered t-AUCB immediately before initiation of ischemic injury in all of our experiments. Future direction of our work includes evaluating the neuroprotective effects of t-AUCB administered with the same dosing regimen (0.9 mg/kg iv) during the post-ischemic reperfusion period. A second limitation of our study was that in our in vitro cell culture experiments, we used high concentrations of t-AUCB (0.5 μM) to account for possible loss due to nonspecific binding to cells and metabolism after uptake. This concentration may not be reflective of intracellular concentrations achieved with our dosing regimen (37). Future goals of our work will aim at assessing the in vitro neuroprotective efficacy of t-AUCB over a wider concentration and time exposure range. A third limitation of our study was that t-AUCB neuroprotection was evaluated in male rats alone. A previous study has shown that sEH expression in females was lower than males and that the gene deletion of sEH did not reduce infarct volume in females, presumably due to lower sEH expression (40). Future studies are needed to evaluate acute and/or chronic sEH inhibition in female rats to understand whether gender difference plays a crucial role in sEH mediated neuroprotective effects.

Conclusion

In summary, in the current study we have demonstrated the neuroprotective effects of t-AUCB in a rat MCAO model at a low dose and have produced the first evidence that t-AUCB alters EETs-to-DHETs ratio without significant changes in other AA metabolites from CYP4A, CYP4F, and COX pathways. In addition, these data are first to demonstrate improved short-term behavioral performance by t-AUCB, thereby providing evidence that sEH inhibitors meet the STAIR criteria of improved functional outcome in the rat. Furthermore, we demonstrated that the neuroprotection by t-AUCB is likely due to direct neuronal effects with minor contributions from alterations in CBF. Chronic sEH inhibition by pharmacological inhibitors is an area of the future study to better elucidate long-term behavioral performance and evaluate sEH inhibitors as a potential novel intervention for focal ischemic insults.

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The present address of Muzamil Ahmad: Indian Institute of Integrative Medicine, Neuropharmacology Laboratory, Srinagar (J&K), India.

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