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

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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 trans-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid (t-AUCB) inhibits focal cerebral ischemia in a dose-dependent manner (vehicle: 70.9 ± 4.5% vs. 14.5 ± 4.5%). Elevated cumulative epoxyeicosatrienoic acids-to-dihydroxyeicosatrienoic acids ratio in brain cortex by twofold (4.40 vs. 2.7%), and improved functional outcome at 24 and 48 h after reperfusion. 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 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. t-AUCB significantly reduced cortical infarct volume by 35% (14.5 ± 2.7% vs. 41.5 ± 4.5%), elevated cumulative epoxyeicosatrienoic acids-to-dihydroxyeicosatrienoic acids ratio in brain cortex by twofold (4.40 ± 1.89 vs. 1.97 ± 0.85), and improved functional outcome in arm-flexion test (day 1: 3.28 ± 0.5 s vs. 7.50 ± 0.9 s; day 2: 1.71 ± 0.4 s vs. 5.28 ± 0.5 s) when compared with that of the vehicle-treated group. t-AUCB significantly reduced neuronal death in a dose-dependent manner (vehicle: 70.9 ± 7.1% vs. t-AUCB0.1 (CYP450) pathway metabolizes AA into linear metabolites by incorporating one oxygen atom to form HETEs (hydroxyeicosatetraenoic acids) and epoxyeicosatrienoic acids (EETs). CYP4A and CYP4F enzymes form terminal and various mid-chain HETEs. CYP2C and CYP2J enzymes form four different regio-isomers by epoxidation of the unsaturated double bonds at the 5,6-, 8,9-, 11,12-, and 14,15- carbons, thereby forming four epoxide isomers. EETs are predominantly metabolized in vivo to less active dihydroxyeicosatrienoic acids (DHETs; 5,6-, 8,9-, 11,12-, and 14,15-DHET) by soluble epoxide hydrolase (sEH) enzyme (10, 34).

Unlike most of ω-6 FFA metabolites, which are pro-inflammatory mediators of ischemic damage, EETs have been shown to protect cells during ischemic insult. EETs act as important cellular lipid mediators in the cardiovascular, renal, and nervous systems (16, 19). In cerebral vasculature, EETs play an important role in CBF regulation (2) and neurovascular coupling (21). EETs have been implicated as mediators of vascular tone and inflammatory processes (35) and are also referred to as endothelium-derived hyperpolarizing factors because of phospholipids mainly by phospholipase A2 (PLA2) (29). Accumulation of the saturated and polyunsaturated FFA (PUFA) may indicate regional lipid membrane damage, which further leads to progressive infarction after cerebral ischemia (1, 5, 6, 15). The accumulation of FFA and the subsequent synthesis of oxygenated metabolites of these FFAs contribute to functional impairment after cerebral ischemia. It has been shown that the accumulation of FFAs after cerebral ischemia correlates locally with the severity of the insult (4). The cascade of events leading to ischemic injury-associated secondary brain damage due to accumulation of FFAs is mediated by direct and indirect mechanisms. Direct mechanisms include disruption of cellular energy metabolism, induction of blood-brain barrier breakdown, and edema formation (9, 30, 38). Indirect mechanisms include generation of active oxygenated metabolites of liberated FFA that affect cerebral blood flow (CBF) and vascular tone (11, 27).

Arachidonic acid (C20:4, ω-6; AA) is one of the PUFA released from the phospholipids of cell membranes into the cytosol in response to stimuli such as ischemia (8). Free AA is metabolized to biologically active products by cyclooxygenase (COX), lipoxygenase, and cytochrome P-450 (CYP450) pathways (25). Of the three pathways, the CYP450 pathway metabolizes AA into linear metabolites by incorporating one oxygen atom to form HETEs (hydroxyeicosatetraenoic acids) and epoxyeicosatrienoic acids (EETs). CYP4A and CYP4F enzymes form terminal and various mid-chain HETEs. CYP2C and CYP2J enzymes form four different regio-isomers by epoxidation of the unsaturated double bonds at the 5,6-, 8,9-, 11,12-, and 14,15-DHET) by soluble epoxide hydrolase (sEH) enzyme (10, 34).

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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 hydroxypropyl-β-cyclodextrin (HPβCD), 15%] or treatment (lyophilized t-AUCB in HPβCD, 0.5 mg) groups. MCAO was performed on all rats. All HPβCD 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 N₂O/O₃ through-out 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 quantification of infarct 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, PGE₂, PGD₂, PGI₂, 15-deoxy-Δ12,14-PGD₂, 15-deoxy-Δ12,14-PGJ₂, 15PGE₂, 15PGF₂α, PGE₂, PGD₂, 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-d₅, 15-deoxy-PGJ₂-d₅, 6-keto-PGF₁-d₅, 11β-PGF₂-d₅, PGI₂-d₅, 11-deoxy-TXB₂-d₅, PGE₂-d₅, and PGF₂-d₅ 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 C-18 column 1.7 μm (2.1 × 100 mm), and PGs were separated on a ultra performance liquid chromatography BEH C-18, 1.7 μm (2.1 × 150 mm) reverse-phase 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 μl/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 to 100% over 0.5 min where it remained for 3.5 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 μl/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 m/z 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

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extend left forepaw fully and torso turning to ipsilateral side when held by tail (a mild focal neurologic deficit); 2 = circling to the affected side (a moderate focal neurologic deficit); 3 = unable to bear weight on the affected 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/group) underwent femoral arterial 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 was equipped with a 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 inversion plane was positioned.

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. 1. The effect of acute trans-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]benzoic acid (t-AUCB) treatment on brain infarct volume after temporary middle cerebral artery occlusion (MCAO) in rats (n = 9). A: representative rat brain sections stained with 2,3,5-triphenyltetrazolium chloride following vehicle (HPBCD) or t-AUCB. B: percent infarct volume in rats treated with vehicle (HPBCD) or t-AUCB. Percent infarct volume was calculated by dividing infarct volume by contralateral hemisphere volume. Rats were euthanized 24 h after MCAO, and brain sections were obtained for infarct volume determination. Data represented as means ± SD. ***Significant values for P < 0.01.](https://example.com/fig1.png)

**Fig. 1.** The effect of acute trans-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]benzoic acid (t-AUCB) treatment on brain infarct volume after temporary middle cerebral artery occlusion (MCAO) in rats (n = 9). A: representative rat brain sections stained with 2,3,5-triphenyltetrazolium chloride following vehicle (HPBCD) or t-AUCB. B: percent infarct volume in rats treated with vehicle (HPBCD) or t-AUCB. Percent infarct volume was calculated by dividing infarct volume by contralateral hemisphere volume. Rats were euthanized 24 h after MCAO, and brain sections were obtained for infarct volume determination. Data represented as means ± SD. ***Significant values for P < 0.01.
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-

Fig. 3. Representative LC/MS chromatograms of rat brain cortical sample showing EETs and 20-HETE before (A) and (B) treatment with t-AUCB.
ment showed significantly improved outcome in the arm flexure test on days 1 and 2 as compared with the vehicle-treated group (day 1: 3.28 ± 0.5 s vs. 7.50 ± 0.9 s, ***P < 0.001; day 2: 1.71 ± 0.4 s vs. 5.28 ± 0.5 s, ***P < 0.001; Fig. 4A). Similarly, 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 hypoper-
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: 56.9 ± 7%; P = 0.057) and significant reduction at 0.5 μM (cell death: vehicle: 67 ± 11.6 vs. t-AUCB: 48.5 ± 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

![Graph A](image1.png)

**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-AUCB0.1mg/kg iv: 58 ± 5.11; vs. t-AUCB0.5mg/kg iv: 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)-dodecanic 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). This study for the first time showed that infarct volume reduction was associated with a twofold elevation of the cumulative EETs-to-DHETs ratio in brain cortex with t-AUCB treatment. Analysis of brain cortices by our validated ultra performance liquid chromatography-tandem mass spectrometry method demonstrated no significant changes in the levels of metabolites produced from CYP4A, CYP4F, and the COX pathway, thereby indicating the specificity and selectivity of sEH inhibition by t-AUCB. Together, these findings suggest that low-dose t-AUCB selectively alters EETs concentrations and significantly reduces infarct volume after a single-dose administration in the rat MCAO model.

Based on these results, we delineated the impact acute sEH inhibition had on behavioral performance. Our findings suggest that acute administration of t-AUCB significantly improved behavioral performance following post-ischemic reperfusion. The behavioral tests used to assess MCAO damage have been shown to correlate with the duration of ischemia and relate to the degree of behavioral deficit (3). Acute treatment with t-AUCB at the time of ischemic injury exerted beneficial effects on days 1 and 2 following reperfusion. The data from these behavioral performance tests suggest that the extent of cortical damage was more severe in vehicle-treated rats after MCAO when compared with t-AUCB-treated animals and that the rats recovered significantly from cortical damage and sensory neglect after acute t-AUCB administration. Taken together, these data demonstrate that selectively increasing brain cortical EETs concentrations by acute sEH inhibition may not only reduce infarct volume but also improves the neurofunctional outcome in rats after MCAO, thereby providing additional evidence in support of sEH inhibitors as potential therapeutic intervention for neuroprotection after ischemic injury.

The data from infarct volume and behavioral performance assessments led us to investigate the factors contributing to the neuroprotective effects of t-AUCB. We used a noninvasive ASL-MRI imaging technique to evaluate real-time CBF analysis. We found that acute t-AUCB treatment produced a nonsignificant trend toward improved CBF around the infarcted area during the post-ischemic hypoperfusion period with no major differences in CBF observed either during or after ischemia. Our findings after ischemic injury were similar to the results reported previously showing no significant differences in regional CBF rates as measured by [14C]-iodoantipyrine [IAP] autoradiography between AUDA-nBE and vehicle-treated groups (41). Conversely, in another study, Zhang et al. (42) reported that brain tissue perfusion was significantly higher in sEH null mice (sEH−/−) compared with wild-type mice during and after vascular occlusion by Laser-Doppler perfusion and [14C]-IAP autoradiography. One of the likely explanations for the contradictory findings between sEH−/− phenotype and chemical sEH inhibition is related to acute chemical sEH inhibition versus chronic loss of activity in sEH−/− mice producing differential effects on CBF. It is also possible that developmental differences or loss of phosphatase activity in the sEH−/− animals may explain the observed CBF effects as suggested by Keseru et al. (20) in a study evaluating hypoxia-induced pulmonary hypertension. In this study, Keseru et al. (20) observed increased right heart hypertrophy and pulmonary artery muscularization in sEH−/− mice subjected to chronic hypoxia than the wild-type mice treated chronically with sEH inhibitors. Collectively, these studies support that the phenotype differences exist between sEH chemical inhibition and sEH−/− animals. More studies focusing on CBF changes should be completed to elucidate the underlying mechanisms of these phenotypic differences and to better understand the degree to which changes in CBF within microvessels contribute to overall neuroprotection within the neurovascular unit.

Given that acute administration did not significantly affect CBF in the rat MCAO model, we sought to determine if t-AUCB directly protects neurons from ischemic damage in vitro. Our in vitro experiments with naïve rat primary cortical neurons showed that t-AUCB significantly reduced neuronal cell death in a concentration-dependent manner after hypoxic injury, thereby suggesting a direct neuroprotective effect of t-AUCB treatment under hypoxic conditions. t-AUCB did not show any effect under nonhypoxic conditions, suggesting that the effect is specific to the injury mechanisms initiated during hypoxia. In our assay negative control MK-801, a potent NMDA receptor antagonist prevented neuronal death significantly after hypoxic injury, suggesting that involvement of excitotoxic mechanisms for neuronal death; however, it is unknown whether the protective mechanisms of the EETs are identical in vivo versus in vitro. Future studies comprehensively evaluating the protective mechanisms will help understand the similarity of in vitro protective mechanisms to the in vivo mechanisms. Furthermore, our findings are consistent with the data published by Koerner et al. (22), who reported that polymorphisms in the sEH gene that alter hydrolase
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 EET 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 showed 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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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


