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Protective Role of P450 Epoxyeicosanoids in Subarachnoid Hemorrhage

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Abstract

Background—Patients recovering from aneurysmal subarachnoid hemorrhage (SAH) are at risk for developing delayed cerebral ischemia (DCI). Experimental and human studies implicate the vasoconstrictor P450 eicosanoid 20-hydroxyeicosatetraenoic acid (20-HETE) in the pathogenesis of DCI. To date, no studies have evaluated the role of vasodilator epoxyeicosatrienoic acids (EETs) in DCI.

Methods—Using mass spectrometry, we measured P450 eicosanoids in cerebrospinal fluid (CSF) from 34 SAH patients from 1 to 14 days after admission. CSF eicosanoid levels were compared in patients who experienced DCI versus those who did not. We then studied the effect of EETs in a model of SAH using mice lacking the enzyme soluble epoxide hydrolase, which catabolizes EETs into their inactive diol. To assess changes in vessel morphology and cortical perfusion in the mouse brain we used optical microangiography, a non-invasive coherence based imaging technique.

Results—Along with increases in 20-HETE, we found that CSF levels of 14, 15-EET were elevated in SAH patients compared to control CSF, and levels were significantly higher in patients who experienced DCI compared to those who did not. Mice lacking sEH had elevated 14, 15-EET and were protected from the delayed decrease in microvascular cortical perfusion after SAH, compared to wild type mice.

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Conflict of Interest Statements

Dominic A. Siler, Ross Martini, Jonathan Ward, Jonathan Nelson, Rohan Borkar, Kristen Zuloaga, Jesse Liu, Jeffrey Raskin, Stacy Fairbanks, Valerie Anderson, Aclan Dogan, Ruikang K. Wang, Nabil J. Alkayed, Justin S. Cetas declare that they have no conflict of interest

Conclusions—Our findings suggest that P450 eicosanoids play an important role in the pathogenesis of DCI. While 20-HETE may contribute to the development of DCI, 14, 15-EET may afford protection against DCI. Strategies to enhance 14, 15-EET, including sEH inhibition, should be considered as part of a comprehensive approach to preventing DCI.

Keywords

Subarachnoid Hemorrhage; Soluble Epoxide Hydrolase; Epoxyeicosatrienoic acid; 20-HETE; Delayed cerebral Ischemia

Introduction

An estimated 33,000 patients suffer from aneurysmal subarachnoid hemorrhage (SAH) in the US annually, which has a mortality rate of 20–40% and a very high rate of disability among survivors [1,2], primarily attributed to delayed cerebral ischemia (DCI). DCI occurs in 30% of survivors [3], usually between 3 and 14 days after the initial hemorrhage [4]. Current monitoring and treatment strategies require prolonged intensive care unit stays, at high institutional and patient cost. There are few known risk factors, no reliable predictive test, and few preventative treatments for the development of DCI.

The best characterized pathological feature associated with DCI is severe constriction of cerebral arteries, or vasospasm, which leads to hypoperfusion and ischemia in dependent brain regions [4]. These constrictions occur at several places along the vascular tree, from large conduit arteries, which are easily detectable by angiography, down to the smallest penetrating arterioles detectable only by perfusion computed tomography [5]. Different vasoactive molecular mediators exert varying levels of influence on vessel tone along the branches of the vascular tree [6]. While large vessel vasospasm has been largely attributed to alterations in endothelin-1 and nitric oxide signaling [7], less is understood about microvascular vasospasm despite its significant contribution to DCI [8].

Cytochrome P450 eicosanoids are produced by microvascular endothelium and astrocytes [9]. These lipid effector molecules were first implicated in DCI with the discovery of elevated CSF levels of 20-hydroxyeicosatetraenoic acid (20-HETE) in human SAH patients [10] and in animal models of SAH [11]. It is not known if other P450 eicosanoids with vasodilator properties play a potentially opposing role in SAH. Namely, 14, 15-epoxyeicosatrienoic acids (14, 15-EET) has been shown to preferentially dilate cerebral microvessels [12] and act as a neuroprotectant in models of cerebral ischemia [13]. Levels of 14, 15-EET in brain are regulated by their synthesis via cytochrome P450 epoxigenases in endothelium and astrocytes [14], and their metabolism primarily by the enzyme soluble epoxide hydrolase (sEH) [9].

To investigate if 14, 15-EET is altered after SAH in humans, we sampled the CSF in a cohort of SAH patients at high risk for DCI, whose neurologic status on admission necessitated the placement of an external ventricular drain. Along with the already documented increase in 20-HETE we also found that 14, 15-EET is elevated in SAH patients compared to non-hemorrhage controls. Patients with the highest levels of both eicosanoids were more likely to go on to experience DCI. To determine if increased 14, 15-

EET plays a protective role against the development of vasospasm after SAH, we subjected mice with genetic deletion of sEH, which has higher 14, 15-EET, to experimental SAH and found that these mice were protected from the decrease in microvascular perfusion after SAH compared to WT mice.

Methods

Approval statement

The clinical study was approved by the Institutional Review Board, and informed consent was obtained. All experimental animal procedures performed in this study conform to the guidelines of the US National Institutes of Health. The laboratory animal protocols were approved by the Animal Care and Use Committee of Oregon Health & Science University (Portland, OR, USA).

Patient population

Adult patients with aneurysmal subarachnoid hemorrhage confirmed with digital subtraction cerebral angiography between December 1, 2008 and August 1, 2013 were recruited from the Neurosciences Intensive Care Unit at Oregon Health & Science University (OHSU) – a stroke referral center in Portland, OR. All patients had ventriculostomies placed for hydrocephalus.

Clinical Data and Outcomes

Baseline demographic and physiologic data were collected from electronic medical records. Hunt Hess grade, modified fisher score and aneurysm location were collected from the admission history and physical and based on first CT scan or angiogram. Development of delayed cerebral ischemia (DCI) was also determined using patient records including clinical notes, laboratory data, and imaging studies. The primary outcomes of interest were the development of DCI and mortality. We defined DCI as an acute decline in neurologic status documented by a decrease in glasgow coma scale of at least 2 points, depressed level of consciousness or new focal neurologic sign lasting at least 1 hour, and not explained by other disease processes such as hydrocephalus, electrolyte abnormalities or infection, that was concomitant with evidence of vasospasm by cerebral angiography or transcranial doppler. The secondary outcome measured was patient disposition. We dichotomized disposition into those who went home (either to live independently or with assistance) and those who were discharged to rehabilitation centers, skilled nursing facilities, or who died.

CSF collection and processing

Cerebrospinal fluid (CSF) was obtained at intervals while the ventriculostomy was in place. CSF was obtained on day 1, and every other day after that up to day 14. The number of samples collected from each patient ranged from 1–7 depending on the availability of CSF with a median of 3 samples collected per patient. Samples were sorted into two-day bins with n = 22, 23, 23, 13, 19, 13, 8 for days 1–2, 3–4, 5–6, 7–8, 9–10, 11–12, and 13–14 respectively. Sample numbers were higher at earlier time-points because this is when ventricular drains were most likely to still be in place. A volume of 3 mL of CSF was collected directly from the ventricle using standard sterile procedure, placed immediately on

ice and spun in a chilled centrifuge at 10,000 RPM for 10 minutes. The supernatant was collected and 15 µl of 1% butylated hydroxytoluene (BHT) was added as an anti-oxidant agent to prevent EETs oxidation. Samples were stored at –80° C until analyzed by LC-MS/MS.

Control CSF

CSF samples from healthy age and sex matched controls (n = 10) were obtained through Joseph Quinn and the Oregon Alzheimer Center which were collected as part of an unrelated aging study [15]. Participants in this study had no clinically significant pathologies. Specifically, patients were excluded from the study if they had a history of stroke, TIA, myocardial infarction, diabetes, or a body mass index > 30. Additionally, the participants had no evidence or history of cognitive dysfunction and a mini mental status score of > 26. CSF was collected via lumbar puncture, flash frozen and stored at –80° C until analyzed by LC-MS/MS.

Preparation of Samples and Calibrators

CSF sample preparation was a slight modification of Poloyac et al. [10]. CSF samples were thawed on ice, 10 µl of 10 mg/ml BHT was added to each sample along with internal standard mix consisting of 1 ng of each of the following, d₈-15 HETE, d₆-20 HETE, d₈ 14, 15 EET, and d₁₁-14, 15 DHET. The samples were vortex mixed briefly and then spun at 2000×g for 5 minutes to pellet any solid debris. The samples were gravity loaded onto Oasis HLB 30 mg solid phase extraction (SPE) cartridges which had been pre-equilibrated with 1 ml of methanol, followed by 1 ml of water. Following loading the SPE were washed with 3 mls of 5% methanol. SPE were then dried for 15 minutes at maximum house vacuum of 5–15 Hg before being eluted with 3 mL of methanol. A brief application of vacuum finished the elution, and then the samples were dried under vacuum at for 1 hour 20 minutes at 35°C. The tube walls were washed with 1 ml of hexane, immediately re-dried and then brought up in 50 µl of start solvent which consisted of 45:55 (vol:vol) acetonitrile:water with 0.2 mg/ml triphenylphosphine (TPP), 0.01% BHT and 0.01% formic acid and placed in sample vials with inserts and analyzed by LC-MS/MS. The injection volume was 20 µl. An un-extracted standard curve was used for the majority of the studies after an initial experiment comparing spiked CSF to un-extracted samples showed similar values. Area ratios were plotted and unknown determined using the slopes. U-extracted standard curves were always prepared and compared to a spiked CSF sample because of the difficulty of obtaining blank CSF in sufficient volumes to prepare full standard curves.

LC-MS/MS Analysis of Eicosanoid Metabolites

DHETs, HETEs and EETs levels were analyzed using a 5500 Q-TRAP hybrid/triple quadrupole linear ion trap mass spectrometer (Applied Biosystems) with electrospray ionization (ESI) in negative mode as described previously [16]. The mass spectrometer was interfaced to a Shimadzu (Columbia, MD) SIL-20AC XR auto-sampler followed by 2 LC-20AD XR LC pumps and analysis on an Applied Biosystems/SCIEX Q5500 instrument (Foster City, CA). The instrument was operated with the following settings: source voltage –4000 kV, GS1 40, GS2 40, CUR 35, TEM 450 and CAD gas HIGH. The scheduled MRM transitions were monitored with a 1.5 minutes window. Compounds were infused

individually and instrument parameters optimized for each MRM transition. The gradient mobile phase was delivered at a flow rate of 0.5 ml per min, and consisted of two solvents, A: 0.05% acetic acid in water and B: acetonitrile. Initial concentration of solvent B was 45%, this was held for 0.1 minutes before being increased to 60% over 5 minutes, then increased to 61.5% over 5 minutes, followed by an increase to 95% over 1.1 minutes, held at 95% for 2 minutes, decreased to start conditions of 45% B over 0.4 minutes and then equilibrated for 5 minutes. The Betabasic-18 100×2, 3μ column was kept at 40 °C using a Shimadzu CTO-20AC column oven. Data were acquired and analyzed using Analyst 1.5.1 software. The standard curves were from 0–1000 pg/sample and the limit of quantification was 10 pg per sample except for 19-HETE and 20-HETE where the limit of quantification was 25 pg per sample where the relative standard deviation was less than 20%. Values that were above detection thresholds but below quantification thresholds were assigned a value of 4pg/ml.

Animals

All mice were housed on a 12:12-h light:dark cycle and given free access to standard rodent chow and water. Homozygous sEHKO mice were generated in-house by breeding homozygous sEHKO mice. Homozygous mice are viable, fertile, normal in size and do not display any gross physical or behavioral abnormalities. Genotype was confirmed by PCR, as previously described [17]. Homozygous sEHKO mice have been backcrossed to C57BL/6J for at least 7 generations. Therefore, sEHKO mice were compared to wild-type (WT) C57BL/6J mice obtained from Jackson Laboratories. All experiments were conducted with male mice 8 – 12 weeks of age. Animals underwent either survival or non-survival surgery, as described below. Both surgeries were performed identically, differing only in that non-survival animals had a femoral artery catheter inserted for arterial blood pressure monitoring, and that animals were culled for hemorrhage size measurements after thirty minutes. Animals in the survival study had no femoral artery catheter and were survived for imaging studies.

Mouse SAH model

For both acute and longitudinal studies, SAH was induced in mice using the endovascular perforation technique [18] [19]. Briefly, mice were anesthetized with isoflurane (1.5 to 2% in O₂-enriched air by face mask), and maintained at 37±0.5°C via rectal temperature monitoring and warm water pads. A small laser-Doppler probe (Moor Instruments) was affixed to the skull to monitor cortical perfusion and confirm vascular rupture. To induce hemorrhages, a sharpened nylon suture (5-0) was introduced into the internal carotid artery via the external carotid artery and advanced ~10mm into the Circle of Willis. The suture was then advance slightly further to induce a hemorrhage and removed immediately. The common carotid artery was maintained patent at all times to maximize flow to the ruptured artery immediately following arterial perforation. In sham-operated animals, the suture was advanced into the internal carotid artery and then removed without arterial perforation.

Acute SAH studies

WT and sEHKO mice were subjected to SAH while simultaneously being monitored for mean arterial blood pressure (MAP) (n = 4 all groups) and cortical perfusion (n = 4 all

groups) for 30 minutes after SAH in a non-survival surgery. Arterial blood pressure was monitored via femoral artery catheter. Laser Doppler measurements were collected from a probe affixed to the skull above the middle cerebral artery (MCA). Thirty minutes after SAH induction, the animals were perfused transcardially with heparinized cold saline and the brains analyzed for hemorrhage grade (n = 8 all groups) using a system adapted from Sugawara et al [20]. Briefly, an image of the ventral surface of the perfused brain was obtained using a Nikon Coolpix camera. Images were subdivided into 6 sections (Fig. 2C) and each section given a score of 0–3 by a blinded investigator according to the amount of blood present. A score of 0 was assigned to sections with no visible blood, while a score of 3 was assigned to sections with thick blood clots that blocked visualization of underlying vasculature.

Longitudinal studies using optical microangiography (OMAG)

To monitor changes in vessel diameters and CBV *in vivo*, we used the OCT-based imaging technique OMAG [21–23]. Briefly, at baseline and on days 1 and 3 after SAH (sEHKO n = 4, WT n = 6) or sham (n = 6) surgery, mice were anesthetized with isoflurane (1.5 to 2% in O₂-enriched air by face mask). The skin over the skull was reflected, the cortex illuminated through the skull at 1,310 nm, and the resulting backscattered and reference light detected to produce spectral interferograms. Volumetric imaging data were collected by scanning the probe beam through a 1,000 × 500 × 512-voxel cube, representing 2.5 × 2.5 × 2 mm³ (x-y-z) of tissue (Fig.1). After scanning, the skin over the skull is closed and the animal is allowed to recover. Global CBV images were rendered in the 3-D software AMIRA (Visual Imaging GmbH) and analyzed for mean pixel intensity changes over time using Image-J [24]. Pixel intensity histograms were generated in Image-J. For vessel diameter measurements, the same data was rendered in IMARIS (Bitplane) software. Using the filament tracing function and the mean diameter calculator, average vessel diameters were calculated at each branching segment of the MCA within the scan area (n = 198, 98, and 154 vessel segments for WT, sEHKO and sham respectively). Vessels were chosen for analysis based on baseline scans by an individual blinded to 24h and 72h outcomes.

Whole brain mouse eicosanoid measurements

(n = 10 all groups) were kept on dry ice until homogenization. Each sample was placed into 1.5 mls of PBS with 15 µl of an anti-oxidant mix consisting of 0.2mg/ml BHT, 2 mg/ml TPP, and 2 mg/ml indomethacin. They were then homogenized on ice using a polytron, setting 2–3 for 20–30 seconds till homogenous. Samples were kept on dry ice prior to homogenization and wet ice at all times thereafter. The samples were immediately re-frozen on dry ice methanol after the aliquot was removed for analysis. 1 ml of 15% KOH was added to each tube, and an aliquot corresponding to 10 mg of brain tissue was added, the tube was briefly vortexed, capped tightly and then hydrolyzed at 40°C for 1 hour. Samples were cooled briefly (<=5 minutes) and then spiked with internal standard mix consisting of 1 ng of each of the following, d₈-15 HETE, d₆-20 HETE, d₈ 14, 15 EET, and d₁₁-14, 15 DHET. Samples were acidified with 200 µl of glacial acetic acid, and the pH checked using pH paper for a desired range of 3–4. Samples were extracted with 3 ml of ethyl acetate, followed by 3 ml of hexane:ethyl acetate 1:1, followed by 2 ml of hexane. The extracts were combined and dried under vacuum for 35 minutes at 35 °C. 150 µl of 0.1N HCl was added

to residue in each tube, followed by the addition of 1 ml of hexane. Samples were vortexed for 2× 20 sec, spun at 2000×g for 5 minutes and then hexane was transferred to a fresh tube. Samples were then dried under vacuum for approximately 7 minutes till dry and immediately brought up in 100 µl of start solvent which consisted of 45:55 (vol:vol) acetonitrile:water with 0.2 mg/ml TPP, 0.01% BHT and 0.01% formic acid and filtered through 0.22 micron placed in sample vials with inserts and analyzed immediately by LC-MS/MS. The injection volume was 20 µl. An un-extracted standard curve was used for these studies.

Western blot

sEH protein expression was measured as previously described [25]. In brief, mice were perfused with ice-cold heparinized saline to remove blood, and brains were collected. Brains were homogenized in lysis buffer, centrifuged, and supernatant collected. Protein samples (40 µg) were separated by gel electrophoresis and then transferred to Polyvinylidene Difluoride (PVDF) membranes. Blots were blocked in 5% dry milk, and incubated at 4°C overnight with a primary rabbit polyclonal antibody against murine sEH (1:500; Cayman Chemical, Ann Arbor, MI). The signal was visualized using a horseradish peroxidase-linked (HRP) rabbit secondary antibody (1:1,000; GE Healthcare, Piscataway, NJ) followed by detection using Supersignal chemiluminescent reagents (Thermo Fisher Scientific, Waltham, MA) with a FluorChem FC2 (Protein Simple, Santa Clara, CA). Blots were stripped using Restore Western Blot Stripping Buffer (Thermo Fisher Scientific) and re-probed for beta actin (1:2,000; Sigma-Aldrich) and followed by HRP mouse secondary antibody (1:1,000; GE Healthcare) and re-imaged.

Statistical analysis

Human CSF eicosanoid data followed a non-normal distribution and are displayed either as whisker box plots with the line representing the median, the box representing the interquartile range and error bars of traditional Tukey whiskers (Fig. 1) or as median with error bars of interquartile range (Fig. 2,3). Error reported with median values in the text and tables is the absolute deviation around the median. Multiple comparisons between time points (Fig. 1) were made using the Kruskal-Wallis test with Dunn's Multiple Comparison test for post-hoc analysis. For assessing changes in CSF eicosanoid levels over time, we fitted mixed-effects regression models with random intercepts and slopes for natural log-transformed concentrations of 14, 15-EET and 20-HETE (STATA, College Station, TX).

Single comparisons were made using the Mann-Whitney U test (Fig. 2, 3). To determine the threshold CSF eicosanoid value for relative risk analysis a receiver-operator curve was generated and the value with the highest likelihood ration was chosen. Relative risk was calculated using Fisher's exact test. For animal studies, data are expressed as means ± SD. Protein and eicosanoid concentrations were compared using Student's t-test. For acute studies of MAP and CBF, as well as longitudinal studies of CBV, comparisons of sEHKO and WT mice were conducted using a two-way mixed effects ANOVA followed by Bonferroni post-hoc tests for pairwise comparisons. Hemorrhage grades were compared using a Mann-Whitney test. OMAG vessel diameters were compared within groups using a repeated measures ANOVA followed by a Tukey's multiple comparison test for pair-wise

comparisons. For diameter change comparisons between groups, we used a Kruskal-Wallis test with Dunn's multiple comparison test for pairwise comparison. A value of $p < 0.05$ was considered statistically significant. All statistical tests were made using Prism 5.0 (Graphpad).

Results

Time course of CSF 14, 15-EET and 20-HETE following SAH

We enrolled 34 SAH patients, who required external ventricular drainage on admission into the study along with 10 healthy control patients who received lumbar puncture. All but one SAH patient had a modified Fisher score of 3 or above (median of 4), indicating severe bleeding and high risk of developing DCI [26]. As shown in Figure 1 (and supplementary Fig 1), all eicosanoids measured were elevated in CSF from SAH patients compared to control CSF, where eicosanoids were undetectable. Median CSF values of 20-HETE were highest on days 1–2 (88.4 ± 74.4 pg/ml), which by days 13–14 decreased by more than 80% (17.3 ± 3.9 pg/ml, $p < 0.05$) (Fig 1a). This finding confirms other studies that measured 20-HETE in SAH patients CSF [27]. A similar pattern was also observed for 12-HETE (supplementary Fig 1). Conversely, median CSF values of 14, 15-EETs were low on days 1–2 (6.3 ± 4.3 pg/ml), which rose over the course of the study by more than 6 fold on days 13–14 (38.7 ± 22.9 pg/ml, $p < 0.05$). We confirmed that CSF values of 20-HETE decreased over time and 14, 15-EET values increased over time by fitting a mixed-effects regression model for repeated measures which was statistically significant ($P < 0.001$). A similar pattern was also found for CSF levels of other EETs regio-isomers 8,9-EETs and 11, 12-EETs (supplementary Fig 1). No discernable patterns were detected for 11-HETE or 15-HETE (supplementary Fig 1).

CSF 14, 15-EET and 20-HETE correlate with DCI

We next sought to determine if CSF concentrations of 14, 15-EET and 20-HETE correlate with the development of DCI. To do this, we used the peak, nadir, and mean CSF concentrations for 14, 15-EET and 20-HETE over the course of the study for each patient (Table 1). Levels were correlated with DCI or mortality as primary outcomes, and with the disposition of the patient at time of discharge (i.e., discharged to home, inpatient care, expired, etc.) as the secondary outcome. We found no difference in 14, 15-EET or 20-HETE levels in patients who died compared to survivors. Peak and mean 14, 15-EET and 20-HETE levels were elevated in patients who experienced DCI versus those who did not. Nadir, mean and peak 20-HETE CSF concentrations were elevated in patients with a worse disposition compared to those discharged to home.

Before making further comparisons between the non-DCI and DCI groups, we excluded three patients from additional analysis. These patients were considered “neurologically devastated” and care was withdrawn, making assessment of DCI in these patients impossible. Admission characteristics of the remaining patients were similar between groups. Specifically, there was no difference in Hunt and Hess clinical score or modified Fisher score between non-DCI and DCI groups (Table 3).

We then compared the time course of 20-HETE and 14, 15-EET levels between DCI (n= 13) and non-DCI (n =18) patients (Fig. 2). We found the greatest differences occurring in the first four days for both 20-HETE (Fig. 2a) and 14, 15-EET (Fig. 2b) as well as the final 4 days for 14, 15-EET. A comparison of all values collected in the first four days between DCI and non-DCI patients revealed a significant increase in both 20-HETE (170.3 ± 114.0 pg/ml vs. 19.9 ± 9.0 pg/ml, $p < 0.01$, Fig 2c) and 14, 15-EET (14.8 ± 10.8 pg/ml vs. 4.9 ± 1.6 pg/ml, $p < 0.05$, Fig 2d) in the DCI vs. non-DCI group. There were no differences between groups in the final four days of the study in either 20-HETE or 14, 15-EET CSF levels.

CSF 14, 15-EET and 20-HETE as predictive biomarkers of DCI

Because the major difference in CSF levels between groups occurred early after admission, we tested the value of early 14, 15-EET and 20-HETE CSF concentrations (in the first 96 hours) as predictive biomarkers of DCI occurring later in the clinical course of the disease. We included only patients who had at least one CSF sample collected within the first 96 hours following SAH. For those patients with multiple CSF samples collected during this period, the highest value was used in the analysis. We compared 20-HETE and 14, 15-EET concentrations (Fig. 3a) between DCI (n =12) and non-DCI (n = 15) groups, and found that the DCI group had elevated levels of both 20-HETE (218.2 ± 101.1 pg/ml vs. 23.4 ± 8.9 pg/ml, $p < 0.01$) and 14, 15-EET (22.3 ± 15.3 pg/ml vs. 6.4 ± 2.4 pg/ml, $p < 0.05$). To determine the ideal threshold values for a predictive test for DCI that was both sensitive and specific, we calculated the receiver operator characteristics (ROC), which plot the range of sensitivity and specificity over several different threshold values (Fig. 3b). As a comparison with a clinically used test, we also included the peak Lindegaard Ratio (LR) values for each patient within the same time frame (96 hours). The LR is calculated as the ratio of blood flow velocity in the middle cerebral artery (MCA) to the velocity in the internal carotid artery using Transcranial Doppler Ultrasound. LR is routinely used to assess large vessel vasospasm in SAH patients.

The area under the curve (AUC) in Fig 3b is a rough estimate of the “clinical usefulness” of a given test. 20-HETE had the highest AUC (0.93) followed by 14, 15-EET (0.79). The AUC of the LR (0.58) fell closest to the zero discrimination line indicating limited clinical usefulness. Using the sensitivity and specificity calculated from the ROC, we selected the threshold values with the highest likelihood ratios (sensitivity/1-specificity) for 20-HETE (148.6 pg/ml, likelihood ratio = 11.3) and 14, 15-EET (23.9 pg/ml, likelihood ratio = 7.5) to assess relative risk of DCI (Fig. 3c). We included additional metrics for comparison including Hunt & Hess clinical grading score greater than or equal to 3, which has been reported to be associated with DCI. We also included early peak LR greater than 3 (days 1–4) and late peak LR greater than 3 (days 7–10), the latter considered part of the diagnostic criteria for DCI during most “at-risk” time points following SAH. Neither H&H ≥ 3 nor LR > 3 at any time point was significantly associated with increased relative risk of DCI. However, CSF 20-HETE levels above 148.9 pg/ml were associated with a greater than five-fold increase in the risk of DCI (5.1, 95% CI 1.8–14.5, $p < 0.01$). CSF 14, 15-EET levels above 23.9 pg/ml were also associated with a significant increase in the risk of DCI (2.9, 95% CI 1.4–5.6, $p < 0.05$).

14, 15-EET is elevated in sEHKO mice

While a significant body of work exists documenting the negative effects of 20-HETE in SAH and other diseases, it is counterintuitive that elevated levels of 14, 15-EETs would confer greater risk of DCI. Previous work has shown 14, 15-EET to be protective in both cerebral and cardiac ischemia, in part due to its vasodilator and anti-inflammatory properties. To study the effects of elevated 14, 15-EET on DCI, we studied mice with sEH gene deletion (sEH knockout, sEHKO). The enzyme sEH hydrolyzes 14, 15-EET molecule into its vicinal diol 14, 15-DHET. Western blot analysis of sEH in whole brain showed no expression in the brains of sEHKO compared to WT mice (Fig 4a). Consequently, these mice had elevated basal levels of 14, 15-EETs (Fig. 4b, $p < 0.05$) and lower levels of 14, 15-DHET (Fig. 4c, $p < 0.05$) in brain tissue. As a negative control, we measured the levels of 20-HETE, which is not known to interact with sEH, and found no difference in basal brain 20-HETE between sEHKO and WT mice (Fig. 4d).

We then subjected the mice to the endovascular perforation model of SAH which involves perforation of the Circle of Willis with a stiff filament inserted into the internal carotid artery at the neck. To insure that WT and sEHKO mice received a similar insult, we measured changes in laser Doppler flux (Fig. 5a) over the MCA ipsilateral to the hemorrhage site as well as mean arterial blood pressure (Fig. 5b). We found no differences in the initial response to SAH between groups. We additionally measured the hemorrhage grade based on the amount of blood in the brainstem and basal cisterns and found no differences between in WT and sEHKO in severity and extent of hemorrhage (Fig 5c).

Early decrease in arterial vessel diameters after SAH

Optical Microangiography (OMAG) is an interferometry-based imaging technique, where scans show both perfusion and 3-D morphological information of mouse cortical surface vasculature through an intact skull. OMAG scans can be repeated non-invasively many times to measure changes in the same vessels over time. Using OMAG, we first looked for signs of vasospasm by measuring the diameters of terminal branches of the MCA in sham, WT SAH and sEHKO SAH mice (Fig 6a). Diameters of MCA surface branches within the scanned area ranged from 35 to 120 μm at baseline. Induction of SAH caused a decrease in vessel diameters in both WT and sEHKO mice within 24h which persisted for up to 72h (Fig 6b, $p < 0.05$ both days). There were no further changes in vessel diameters between 24h and 72h after SAH, nor were there differences in the degree of vessel diameter change between WT and sEHKO mice at any time point after SAH (Fig 6c).

sEHKO mice are protected from delayed decreases in microvascular perfusion after SAH

Since OMAG scans are based upon the Doppler shift caused by flow of RBCs, changes in velocity or flux can be determined in parallel with vessel diameter. This information was used to look at relative changes in cortical perfusion in the same animals over time (Fig 7a). Although surface vessel diameters decreased 24h after SAH, microvascular cortical tissue perfusion was preserved in these same animals at this time point. However, 72h after SAH, WT mice experienced a significant drop in mean pixel intensity ($-16.93 \pm 5.6\%$ from baseline, $p < 0.05$), indicating a decrease in microvascular perfusion compared to baseline, whereas sEHKO mice had no change in perfusion between 24 and 72 hours after SAH.

Since signal intensity reflects RBC velocity, which is slower in capillary beds, we were able to broadly differentiate between high-velocity, high-throughput vessels (such as large surface arteries or veins) and microvascular flow beds based on pixel intensity histograms of our scans. As shown in Figure 7c, we found that low-intensity signals corresponding to microvascular flow (Fig 7c shaded box) made up the bulk of our scan information and were shifted lower in WT SAH but not sEHKO mice at 72h. This observation suggests that sEHKO mice are protected from delayed reduction in microvascular perfusion after SAH.

Discussion

In the current study we provide both clinical and laboratory evidence for the role of P450 eicosanoids in the pathogenesis of DCI after aneurysmal SAH. We found that both 14, 15-EET and 20-HETE were elevated in CSF of aneurysmal SAH patients at high risk for DCI up to 14 days following admission. The degree to which these eicosanoids were elevated predicted DCI with higher sensitivity and specificity than traditional predictors, such as admission modified Fisher score, Hunt & Hess score, and early or delayed trans-cranial Doppler. We found that sEHKO mice, which have elevated 14, 15-EET, were protected from delayed reductions in perfusion seen in WT mice, and protection was specific to the microcirculation. To our knowledge this is the first study to identify elevated levels of P450 eicosanoids other than 20-HETE in the CSF of SAH patients, and to provide evidence in support of a protective role for EETs.

There are several important findings in the clinical arm of this study. First, the greatest differences in CSF eicosanoid levels between DCI and non-DCI patients occurred within the first 96h after admission. This is promising in that an early CSF eicosanoid test, which would be the most useful time point for clinical decision making, may be valuable for stratifying risk of DCI. This finding should guide future studies testing eicosanoid biomarkers to focus on early time points following SAH. Additionally, other eicosanoids are elevated after SAH besides 20-HETE and 14, 15-EET, including 11-HETE, 12-HETE, 15-HETE, 8,9-EET and 11, 12-EET. One explanation is that these eicosanoids are only present in the CSF due to the extravasated blood itself, as plasma contains measurable levels of all of these eicosanoids [28]. However, this is unlikely given that levels of EETs increase from day 1 to day 14, while 20-HETE decreases, suggesting that active production and secretion into the CSF is occurring. It is more likely that these eicosanoids are part of the ever-growing list of neuro-inflammatory mediators that are produced by cells residing inside the brain in response to injury [29]. In support of this idea, we have previously shown that brief, transient ischemia is sufficient to induce sustained up-regulation of the EETs synthetic enzyme P450 2C11 in rat brain tissue [30]. Similar work has shown brain tissue increases in 20-HETE synthetic enzymes, CYP4A/4F, for as long as 10 days following ischemia [31]. While 20-HETE has pro-inflammatory and vasoconstricting effects, which likely contribute to and exacerbate DCI, 14, 15-EET is a vasodilator and anti-inflammatory, and likely increases as an endogenous protective response against DCI. This is supported by our findings that individuals harboring a mutation in the gene encoding sEH, which could lead to elevated basal levels of 14, 15-EETs, have improved outcomes after SAH compared to those harboring the common polymorphism [32]. Overall, this view suggests there exists a balance between protective and injurious eicosanoids in regulating the response to injury.

Injury mechanisms that lead to elevated levels of these eicosanoids in the *early* time-points after SAH are collectively referred to as “early brain injury” [33]. Early pathogenetic mechanisms after SAH include global cerebral ischemia, blood brain barrier disruption, inflammation and hydrocephalus, among others. The occurrence of some of these early pathologies is associated with the development of DCI. Specifically, early ischemia in the first sixty hours of SAH, size of the initial hemorrhage, and neurologic status at admission all have been shown to predict DCI [34] [35]. We demonstrate in our study that elevated levels of these eicosanoids can also predict DCI, providing further support for the idea that early brain injury is an important determinant of DCI. It is worth noting that 94% of the patients enrolled in this study were classified as “high-risk” for DCI based upon assessment of modified Fisher score on admission. This means that an early CSF eicosanoid test has the potential to stratify DCI risk beyond conventional tests and allow the identification of those most likely to develop DCI. It is important to note that patients were prospectively enrolled in this study, but analysis of CSF eicosanoids and selection of test thresholds were retrospective. Confirming the predictive value and potential clinical utility of early eicosanoid testing to quantify EBI and predict DCI requires confirmation of our results in a blinded prospective study on a different patient population.

Another interesting finding from this study is that 14, 15-EET levels, while elevated above controls at the onset of SAH, rise even further in the late stages of recovery. While this rise in EETs over time coincides with the timeframe for likely DCI, we are hesitant to make any conclusions about cause and effect on clinical observation alone. Our data from the experimental model of SAH support our hypothesis that the rise in EETs may play a protective role against DCI. Further, because our previous work has shown that EETs synthesis is driven by ischemia [30], we speculate that this elevation could be in response to a secondary brain insult such as DCI. However, EETs levels rise late in both the non-DCI and DCI groups, suggesting that if this is indicative of a secondary insult, that insult is clinically silent in a large subset of these patients. Clinically silent ischemia is not uncommon in SAH, and multiple studies find that 10–23% of new infarcts attributable to DCI are clinically silent [36] [37]. Since 94% of the patients in our cohort have large SAH as determined by CT we do not know what the time course of CSF 14, 15-EETs would be for an individual with a less severe SAH. A study cohort that includes “low-risk” SAH patients and sensitive markers of ischemia is needed to test whether 14, 15-EET is a sensitive marker for asymptomatic and symptomatic DCI.

Our work in the mouse model of SAH provides an important insight into the mechanism of DCI. We found that pial branches of the MCA constrict early in the mouse, but do not lead to loss of microvascular perfusion. This is reminiscent of recent work questioning the relationship between proximal large-vessel vasospasm and DCI [38]. When delayed microvascular perfusion reductions do develop in WT mice, they are not accompanied by additional reductions in pial artery vessel diameter. This suggests that proximal vessel vasospasm and microvascular dysfunction do not share the same mechanisms. One characteristic of the microvasculature that sets it apart from larger vessels is a greater sensitivity to endothelial derived hyperpolarizing factors (EDHF), which 14, 15-EET behaves similarly to in the cerebral circulation [12]. As arterial branches diminish in size

along the vascular tree, their sensitivity to EDHF increases [6]. This is one possible explanation for our finding that sEHKO mice, which have elevated 14, 15-EET, have the same reduction in pial MCA artery diameter as WT mice yet are protected from the delayed microvascular dysfunction 72h after SAH. Other mechanisms of microvascular dysfunction that could be affected by elevated 14, 15-EETs include the formation of microthrombosis which is an increasingly understood contributor to microvascular dysfunction. Thrombi are generated within the inflamed microvasculature and become trapped within constricted arterioles [39] leading to blockade of poorly collateralized capillary beds and ischemia. EETs have a potent anti-inflammatory effect through inhibition of NF- κ B [40] and direct effects on thrombosis [41] both of which could reduce the formation of microthrombi. Other mechanisms of dysfunction such as pericyte dysfunction and swelling of astrocytic end-feet [42] could be affected by EETs which are capable of directly protecting endothelial (16), neuronal [43], and glial cells from death [44].

In conclusion, our data indicate that early CSF monitoring of P450 eicosanoids after SAH may be a useful tool in predicting the incidence of DCI. P450 eicosanoids have differential effects on DCI. Whereas 20-HETE has been shown to be detrimental, 14, 15-EET may be protective. In experimental SAH, the microvascular component of DCI is sensitive to enhanced 14, 15-EET due to genetic deletion of sEH. The findings suggest that strategies aimed at enhancing EETs synthesis and inhibiting their metabolism may protect against the development of DCI in SAH patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Eicosanoid levels following SAH. Concentration of A) 20-HETE and B) 14, 15-EET in picograms per milliliter of CSF in an age/sex matched control group (Ctrl n =10) and in SAH patients (n = 34) up to fourteen days following SAH. * = $p < 0.05$ compared to days 1–2.

Figure 2.

20-HETE and 14, 15-EET are elevated early in SAH patients who go on to experience DCI. (A,B) Time course of CSF 20-HETE (A) and 14, 15-EET (B) levels in patients with DCI and without DCI. (C,D) Comparison of samples gathered in the first 4 days after admission and the last four days between DCI and non DCI patients. Both CSF 20-HETE (C) and 14, 15-EET (D) levels are elevated early but not late after SAH in patients who go on to develop DCI. * = $p < 0.05$.

Figure 3.

Peak 96h eicosanoid levels predict DCI (A) Peak single sample CSF 20-HETE and 14, 15-EET levels within the first 96h after admission in DCI (n = 12) and non-DCI (n = 15) patients. * = $p < 0.05$ (B) Receiver operator characteristic (ROC) for predicting DCI using CSF 20-HETE, 14, 15-EET, or transcranial Doppler Lindegaard ratio (LR) measured within the first 96 h. Both 20-HETE and 14, 15-EET have an AUC well above zero discrimination. (C) Relative risk of DCI is elevated in patients with CSF 20-HETE or 14, 15-EET above threshold. Thresholds were chosen based on data from ROC. Included for comparison are admission Hunt & Hess (H&H) score above 3, early peak LR above 3 (first 96 h), and late peak LR (days 7–10) above 3. Data are expressed as relative risk with 95% CI error bars. * = $p < 0.05$.

Figure 4.

14, 15-EETs are elevated in sEHKO mice. (A) Representative western blot of sEH protein showing no expression in sEHKO mice. (B) Concentration of 14, 15-EETs measured by LC-MS/MS is elevated in whole brain of sEHKO mice relative to WT mice. (C) Whole brain 14, 15-DHET, the product of sEH hydrolysis, is lower in sEHKO mice compared to WT. (D) Whole brain 20-HETE is unchanged in sEHKO mice. $n = 10$ per group. $*$ = $p < 0.05$. ns = no significance.

Figure 5.

WT and sEHKO mice respond identically to SAH. (A) Laser doppler flowmetry of the ipsilateral MCA in WT (n = 8) and sEHKO (n = 8) mice in the first 30 minutes following SAH. (B.) Mean arterial pressure in WT (n = 4) and sEHKO (n = 4) in the same time frame. (C.) Hemorrhage grade measured by blood in the brainstem and basal cisterns in WT (n = 8) and sEHKO (n = 8).

Figure 6.

Arterial diameter changes following SAH: A.) Representative images of vessel diameter analysis using OMAG scans and IMARIS software in a WT mouse at baseline, 24h and 72h following SAH. Average diameters were measured in each segment separated by a branch point along the middle cerebral artery up to the point of penetration into the cortex. Scale bar = 50 μm. B.) Frequency distribution of vessel diameters within groups, over time for sham, wild-type SAH mice (WT), and sEHKO SAH mice (sEHKO). SAH causes modest vessel diameter narrowing in both sEHKO and WT mice at 24h and up to 72h after SAH. Data are expressed in percent frequency of absolute vessel diameters within 10μm bins. * = different from baseline $p < 0.05$. C.) Frequency distribution of changes in vessel diameter between groups for the 24h and 72h time points. Vessel diameters decrease in a similar manner in both WT and sEHKO mice at 24h and 72h. Data are expressed in percent frequency of percent change from baseline in 5% bins. * = different from sham $p < 0.05$. $n = 198, 98$, and 154 vessel segments for WT, sEHKO and sham respectively.

Figure 7.

Microvascular perfusion changes following SAH: A.) OMAG scans are sensitive to blood velocity and RBC flux; high throughput vessels (arrowhead) will have a higher intensity than low throughput microvasculature (asterisk). Representative images of OMAG scans at baseline, 24h and 72h following SAH in WT (upper) and sEHKO (lower) cortical surface vasculature showing a decrease in perfusion from baseline scans in WT but not sEHKO mice 72h after SAH B.) Quantification of mean pixel intensity in sham (n = 6), WT (n = 6), and sEHKO (n = 4) mice following SAH. * = different from WT, $p < 0.05$. C.) Average pixel intensity histograms fitted with a double gaussian model. Low intensity (< 150 shaded in grey) microvasculature makes up the bulk of the histogram. Arrow in WT histogram indicates a shift in low intensity pixels 72h after SAH in WT mice representing a decrease in microvascular perfusion that does not occur in sEHKO mice.

Table 1

Admission Characteristics

		Ctrl	SAH
	N	10	34
Demographics			
Average Age, years		63.0	56.8
Male, n (%)		3(30.0)	10(29.4)
Aneurysm Location, n (%)			
ACOM		-	10 (29.4)
ICA/MCA		-	13(38.2)
PCOM		-	8(23.5)
Vertebrobasilar		-	3(8.8)
Admission Status, n (%)			
Hunt Hess Score	3	-	23 (67.6)
WFNS	4	-	19 (55.9)
Fisher Score	3	-	32 (94.1)

Table 2

CSF Eicosanoid Levels and Outcome

	Nadir Levels			Mean Levels			Peak Levels		
	20-HETE	14, 15-EET		20-HETE	14, 15-EET		20-HETE	14, 15-EET	
Mortality									
Alive (27)	13.2 ± 9.2	4.0 ± 4.0		43.0 ± 26.2	11.7 ± 7.2		56.3 ± 41.9	20.8 ± 14.3	
Dead (7)	18.2 ± 4.7	4.0 ± 0.0		43.5 ± 21.8	5.8 ± 1.8		67.2 ± 42.4	8.8 ± 4.8	
Disposition									
Home (16)	9.4 ± 8.1**	4.0 ± 2.4		19.4 ± 8.7***	12.1 ± 7.2		40.9 ± 24.3**	23.6 ± 16.2	
SNF, Rehab, Dead (18)	69.7 ± 38.6	4.0 ± 0.0		69.7 ± 38.6	9.3 ± 5.3		133.1 ± 88.5	15.4 ± 11.4	
Delayed Cerebral Ischemia									
No DCI (21)	13.2 ± 5.5	4.0 ± 0.75		28.5 ± 14.4***	7.2 ± 3.2*		42.6 ± 21.5***	10.8 ± 6.8*	
DCI (13)	18.2 ± 4.7	4.0 ± 4.0		95.5 ± 40.8	20.9 ± 12.1		221.0 ± 121.4	46.5 ± 31.7	

Table 3

No DCI vs. DCI Admission Characteristics

		No DCI	DCI
	N	18	13
Demographics			
Average Age, years		57.4	55.2
Male, n (%)		5 (27)	2 (15.4)
Aneurysm Location, n (%)			
ACOM		7 (38.9)	3 (23.1)
ICA/MCA		4 (22.2)	7 (53.8)
PCOM		5 (27.8)	2 (15.0)
Vertebrobasilar		2 (11.1)	1 (8.0)
Admission Status, n (%)			
Hunt Hess Score	3	9 (50.0)	11 (85.0)
WFNS	4	7 (38.9)	9 (69.2)
Fisher Score	3	17 (94.4)	12 (92.0)