Soluble Epoxide Hydrolase in Hydrocephalus, Cerebral Edema, and Vascular Inflammation After Subarachnoid Hemorrhage

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Background and Purpose—Acute communicating hydrocephalus and cerebral edema are common and serious complications of subarachnoid hemorrhage (SAH), whose causes are poorly understood. Using a mouse model of SAH, we determined whether soluble epoxide hydrolase (sEH) gene deletion protects against SAH-induced hydrocephalus and edema by increasing levels of vasoprotective eicosanoids and suppressing vascular inflammation.

Methods—SAH was induced via endovascular puncture in wild-type and sEH knockout mice. Hydrocephalus and tissue edema were assessed by T2*-weighted magnetic resonance imaging. Endothelial activation was assessed in vivo using T2*-weighted magnetic resonance imaging after intravenous administration of iron oxide particles linked to anti–vascular cell adhesion molecule-1 antibody 24 hours after SAH. Behavioral outcome was assessed at 96 hours after SAH with the open field and accelerated rotarod tests.

Results—SAH induced an acute sustained communicating hydrocephalus within 6 hours of endovascular puncture in both wild-type and sEH knockout mice. This was followed by tissue edema, which peaked at 24 hours after SAH and was limited to white matter fiber tracts. sEH knockout mice had reduced edema, less vascular cell adhesion molecule-1 uptake, and improved outcome compared with wild-type mice.

Conclusions—Genetic deletion of sEH reduces vascular inflammation and edema and improves outcome after SAH. sEH inhibition may serve as a novel therapy for SAH. (Stroke. 2015;46:1916-1922. DOI: 10.1161/STROKEAHA.114.008560.)

Key Words: communicating hydrocephalus ■ edema ■ subarachnoid hemorrhage ■ vascular cell adhesion molecule-1

Acute communicating hydrocephalus and global cerebral edema are common life-threatening complications of subarachnoid hemorrhage (SAH), which occur in 20% of patients1–3 and are independent risk factors for poor outcome.1,4 Although both represent a dysfunction in water handling within the cranium,5 their causes are likely different and possibly unrelated. Current treatments for hydrocephalus and cerebral edema are largely supportive and do not target the underlying pathologies, especially for hydrocephalus, which leaves some patients requiring permanent ventricular shunts caused by unremitting disease.6,7 A better understanding of the mechanisms underlying these complications is needed to identify viable therapeutic targets.

Mouse models of SAH have been used to study mechanisms of cerebral edema8,9 but do so without acknowledging the potential contribution of hydrocephalus to brain water content.10 To date, there are no studies describing hydrocephalus in mouse models of SAH. In the current study, we use high-field magnetic resonance imaging (MRI) to study the timing, severity, and localization of acute communicating hydrocephalus, as well as cerebral edema, occurring simultaneously in the mouse endovascular puncture model of SAH.

Vasogenic edema is caused by extravasation of ions and proteins through a disrupted blood–brain barrier and is often preceded by activation of the vascular inflammatory cascade.11 Within endothelial cells, nuclear translocation of nuclear factor-kB is an essential step in the expression of endothelial proinflammatory adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1).12 Epoxyeicosatrienoic acids (EETs) are eicosanoids formed by cytochrome P450 enzymes in brain glia and endothelium,13 which oppose VCAM-1 expression by blocking nuclear factor-kB translocation.14 We have previously demonstrated that mice with elevated levels of EETs caused by
genetic deletion of their metabolizing enzyme soluble epoxide hydrolase (sEH knockout [sEHKO] mice) are protected from experimental cerebral ischemia\textsuperscript{15} and delayed microvascular dysfunction\textsuperscript{16} after experimental SAH. Furthermore, we have shown that patients with genetic polymorphisms that reduce sEH activity have improved outcomes after SAH.\textsuperscript{17} We hypothesized that the beneficial effects of EETs also modulate acute inflammation and edema formation after SAH.

Methods
An extended version of methods can be found in the online-only Data Supplement.

Animals
All experiments were approved by the institutional animal care and use committee of Oregon Health and Science University. Adult (8–12 weeks) male wild-type (WT) C57BL/6J mice obtained from Jackson Laboratories and homozygous sEHKO mice in the C57BL/6J background were used.\textsuperscript{15}

Endovascular Puncture
SAH was induced in mice using the endovascular perforation technique as previously described.\textsuperscript{18} Briefly, a nylon suture was introduced into the internal carotid artery and advanced into the Circle of Willis to induce a hemorrhage. In sham-operated animals, the suture was advanced without arterial perforation.

Physiological Monitoring
In a subset of nonsurvival surgeries, animals were monitored invasively for intracranial pressure (ICP), mean arterial pressure, and cerebral blood flow with laser Doppler flowmetry for 30 minutes after SAH.

VCAM-1–Bound Microparticles of Iron Oxide
We conjugated monoclonal rat antimouse CD106 (VCAM-1; 1510-01 Southern Biotech) or mouse IgG1 (0102-01 Southern Biotech) to Dynabeads MyOne Tosylactivated microparticles of iron oxides (MPIOs; Invitrogen) per manufacturer’s instructions. MPIOs were used at a final concentration of 5 mg MPIO/mL in PBS plus 0.1% BSA and 0.05% Tween 20 at 37°C.

MRI
MRI used a Bruker-Biospin 11.75T small animal MR system with Paravision 4.0. To quantify brain size, ventricle size and white matter edema, a T\textsubscript{2}-weighted image set was obtained at baseline, 6 hours, 12 hours, 24 hours, and 72 hours after SAH or sham surgery. To quantify nanoparticle uptake 24 hours after SAH or sham surgery, 3-dimensional T\textsubscript{2}-weighted images were obtained without injection of MPIOs, then 80 minutes after injection with VCAM-1 MPIO or IgG MPIO at 4.5 mg Fe/kg body weight. All image processing was done using tools from FSL\textsuperscript{19–21} and Jim (Xinapse Systems).

Histology
Fixed and embedded brains from mice 72 hours after SAH were cut into 8-μm sections and stained with hematoxylin & eosin. Images were obtained on a BX40 microscope (Olympus).

Behavioral Assessment
A subset of animals were survived for 96 hours after surgery and tested for behavioral deficit. To test general locomotor activity, mice were placed in an open-field apparatus (Columbus instruments). To test sensorimotor function, mice were placed on the accelerated rotarod (Columbus instruments).

Statistics
Group data are expressed as mean±SEM unless otherwise stated. Laser Doppler flowmetry, ICP, mean arterial pressure, ventricular volume, brain size, brain edema, and behavioral performance were compared between groups using a 2-way ANOVA with repeated measures where appropriate. Vaculization data, and VCAM-1 MPIO uptake were compared using 1-way ANOVA. The Holm–Sidak post hoc test was used for all pairwise comparisons.

Results
SAH Induces Immediate and Sustained Rise in ICP
Endovascular puncture caused blood to fill the basal cisterns and subarachnoid space of the mouse within minutes. T\textsubscript{2}-weighted MRI scans within 30 minutes showed blood had flowed retrogradely as far as the fourth ventricle (Figure 1A; note the change in T\textsubscript{2} signal in cerebrospinal fluid (CSF) spaces from white to black). We monitored ICP, mean arterial pressure, and cerebral blood flow in a cohort of WT and sEHKO mice for 30 minutes after vessel puncture. At the moment of hemorrhage, ICP spiked as cerebral blood flow decreased (Figure 1B). Arterial pressure also increased. The ICP waveform changed after hemorrhage with increased pressure fluctuations during systole, indicating reduced CSF compliance (Figure 1B, inset). In both sEHKO and WT mice, ICP rose considerably at the time of hemorrhage (WT, 60.2±9.9 mm Hg versus sEHKO, 69.2±6.0 mm Hg) and returned to an elevated set point by 30 minutes (WT, 33.0±3.2 mm Hg versus sEHKO, 30.4±1.1 mm Hg). Laser Doppler flowmetry also decreased substantially at the time of SAH (WT, 55.2±19.2% versus sEHKO, 37.3±6.8% of baseline) and returned to a point below baseline by 30 minutes (WT, 80.9±28.2% baseline versus sEHKO, 77.9±20.7% baseline). Baseline mean arterial pressure seemed lower in sEHKO mice but was not statistically significant (WT, 74.3±1.0 mm Hg versus sEHKO, 60.8±2.3 mm Hg) and rose to similar levels after hemorrhage (WT, 83.6±8.6 mm Hg versus sEHKO, 85.4±6.8 mm Hg) before returning back toward baseline by 30 minutes (WT, 74.2±1.8 mm Hg versus sEHKO, 70.2±2.1 mm Hg). There were no significant differences between WT and sEHKO mice at any time point.

Acute Communicating Hydrocephalus Forms Rapidly and Persists After SAH
Before and at several time points after (6, 12, 24, and 72 hours) inducing SAH, we imaged the animals using T\textsubscript{2}-weighted MRI. Within 6 hours of SAH, we imaged the animals using T\textsubscript{2}-weighted MRI. Within 6 hours of SAH, ventricular volume had increased substantially (Figure 2A). Signs of mass effect in the form of central sulcus effacement were also evident at 6 hours (Figure 2A, arrowheads). To ascertain whether the hydrocephalus was obstructive or communicating in nature, we looked for signs of uneven enlargement of the ventricular system or large blood clots within the cerebral aqueduct that could block CSF flow. In all scans, the enlargement of the ventricular system was uniform from the anterior horns of the lateral ventricles to the cisterna magna. In addition, we found enlargement of the CSF spaces outside of the ventricular system including the intrathecal space around the spinal cord and the subarachnoid space separating the cortex from midbrain structures (Figure 2A, arrows). WT and sEHKO mice had a similar increase in ventricular volume at each time points beginning as early as 6 hours.
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(1918, 1987 ± 4.9% baseline versus sEHKO, 185.4 ± 17.5% baseline) and persisting the length of the study at 72 hours (WT, 206.2 ± 13.2% baseline versus sEHKO, 208.2 ± 23.9% baseline). Total brain volume also increased in both groups beginning at 6 hours (WT, 0.67 ± 0.5% versus sEHKO, 1.7 ± 0.8% change from baseline) persisting to 72 hours (WT, 3.0 ± 0.5 versus sEHKO, 2.2 ± 0.6 change from baseline). Brain tissue volume (total brain volume–ventricular volume) did not increase significantly during the study (Figure I in the online-only Data Supplement). There were no differences between WT and sEHKO mice at any time point.

sEHKO Mice Have Reduced Periventricular White Matter Edema After SAH

Edema, visualized by hyperintensities on T2-weighted images, began to form specifically in the periventricular white matter of SAH mice within 6 hours then peaked at 24 hours (Figure 3A). The white matter structures affected included the corpus callosum and the dorsal hippocampal commissures (Figure 3A, arrows). Histological sections of the corpus callosum showed substantial vacuolization of the white matter in WT mice. sEHKO mice had significantly less edema at 24 hours (WT, 3.1 ± 0.5% brain volume versus sEHKO, 1.7 ± 0.4% brain volume; *P* < 0.05) and 72 hours (WT, 2.7 ± 0.5% brain volume versus sEHKO, 0.7 ± 0.2% brain volume; *P* < 0.05) compared with WT mice (Figure 3B). Vacuolization found in histological sections of the corpus callosum was also significantly reduced in sEHKO mice than in WT mice (WT, 1.6 ± 0.4 a.u. versus sEHKO, 1.2 ± 0.09 a.u.; *P* < 0.05). Further histological analysis of the white matter identified subtle evidence of axonal injury within the corpus callosum of SAH mice and variable expression of microglial marker Iba-1 (Figures II and III in the online-only Data Supplement).

sEHKO Mice Have Reduced Expression VCAM-1 After SAH

As a measure of vascular inflammation in vivo, we studied the expression of VCAM-1 on brain endothelium using VCAM-1 MPIOs, which have been well validated in previous studies to

Figure 1. Subarachnoid hemorrhage (SAH) causes similar changes to physiology in both wild-type (WT) and soluble epoxide hydrolase knockout (sEHKO) mice. A, Representative gross images (left) and T2-weighted MRI (right) of naïve (top) and SAH (bottom) mice 30 minutes after induction. Blood within cerebrospinal fluid space causes the T2-weighted signal to change from white to black (arrows). B, Representative tracing of intracranial pressure (ICP) with view of waveform (inset), laser Doppler flowmetry (LDF), and mean arterial pressure (MAP) in a WT SAH mouse during SAH. C, Average changes in ICP, LDF, and MAP in WT (*n=5*) and sEHKO (*n=5*) mice after SAH. There were no significant differences between groups. ABP indicates arterial blood pressure; BL, baseline; and MRI, magnetic resonance imaging.
detect endothelial VCAM-1 expression in vivo. Twenty-four hours after SAH or sham surgery, we injected the labeled particles into the vasculature and imaged the animals via T2*-weighted MRI. VCAM-1 MPIO uptake showed as hypointensities on the scans and was greatest in the vasculature along the midline and surrounding the midbrain of the mice (Figure 4A). Immunolabeling of brain tissue sections from SAH mice show that in areas with high MPIO uptake, VCAM-1 primarily localized in the large caliber cerebral veins with relative sparing of the microvasculature (Figure IV.

Figure 2. Subarachnoid hemorrhage (SAH) induces acute communicating hydrocephalus in both wild-type (WT) and soluble epoxide hydrolase knockout (sEHKO) mice. A, Representative T2*-weighted MRI images at baseline (top) and 6 hours after SAH (bottom). Expansion of the cerebrospinal fluid space occurs at all levels including lateral ventricles (lv), third ventricle (3v), cerebral aqueduct (aq), fourth ventricle (4v), cisterna magna (cm), and the subarachnoid space (arrows). Effacement of the central sulcus is also apparent at 6 hours after SAH (arrowheads). B, Ventricular volume changes WT (n=10), sEHKO (n=7), SAH animals and sham (n=5). There is no significant difference between WT and sEHKO SAH mice. C, Total brain volume change in WT (n=10), sEHKO (n=10), SAH animals and sham (n=5). There is no significant difference between WT and sEHKO SAH mice. BL indicates baseline; and MRI, magnetic resonance imaging.

Figure 3. Soluble epoxide hydrolase knockout (sEHKO) mice have less edema than wild-type (WT) mice. A, Representative T2*-weighted MRI images in sham (top) WT subarachnoid hemorrhage (SAH; middle), and sEHKO SAH (bottom) at 24 hours after SAH. Edema forms in the specifically within the white matter of the corpus callosum and dorsal hippocampal commissures (arrows). Histological sections (right) of the corpus callosum 72 hours after SAH show vacuolization within the white matter of SAH mice. B, Periventricular white matter (PVWM) edema formation as a percentage of brain volume in WT (n=10), sEHKO (n=7), and sham (n=5) mice. sEHKO mice have less edema at 24 and 72 hours after SAH (*P<0.05). C, Vacuolization determined by changes in mean pixel intensity of the corpus callosum in WT SAH (n=6), sEHKO SAH (n=6), and sham (n=4) mice 72 hours after SAH. sEHKO mice have less vacuolization than WT mice (*P<0.05). BL indicates baseline; and MRI, magnetic resonance imaging.
We found little uptake in the IgG-tagged MPIO mice and the sham-operated mice. After injection (80 minutes), bound VCAM-1 MPIO caused a negative shift in the voxel intensity histogram when compared with preinjection scans (Figure 4B). sEHKO mice had reduced overall VCAM-1 uptake in the whole brain compared with WT mice (WT, 9.3±1.9×10³ voxels versus sEHKO 3.7±1.3×10³ voxels; *P*<0.05; Figure 4C).

**Discussion**

The present study shows that acute communicating hydrocephalus and cerebral edema occur simultaneously in the mouse endovascular puncture model of SAH. The onset of hydrocephalus was rapid and sustained, occurring within 6 hours of SAH and remaining present for at least 72 hours. Cerebral edema formed primarily in the white matter fiber tracts and followed a time course that was distinct from that of the hydrocephalus. Specifically, edema formation was gradual, peaked at 24 hours and began to recede by 72 hours after SAH, whereas hydrocephalus persisted. Mice with genetic deletion of sEH, which have elevated basal levels of EETs,
had a similar severity of hydrocephalus and brain swelling compared with WT mice, but significantly less edema formation within the white matter. We tested 1 potential mechanism of this edema formation, vascular inflammation indicated by expression of VCAM-1, which was reduced in sEHKO mice compared with WT. Finally, sEHKO mice have improved behavioral outcome after SAH.

We have identified additional features of the endovascular puncture model that mimics human disease seen clinically. Specifically, a substantial number of SAH patients present with acute hydrocephalus at admission, and our model replicates this rapid time scale, a feature not found in other rodent models of hydrocephalus. Although we see the hydrocephalus lasting at least 72 hours in our model, we do not know the time frame at which hydrocephalus resolves, so whether our model represents the 10% to 20% of those chronically hydrocephalic patients that require implantation of semipermanent shunts to manage unrelenting disease remains to be determined. Edema formation in our model shares similarities with the human condition as well. Although human brains have a substantially larger percentage of white matter than mice, the phenomenon that edema forms preferentially within white matter, sparing the gray matter, holds true in this model.

The anti-inflammatory effects of EETs are well documented in mice and inform us about the cause of hydrocephalus and edema after SAH. First, the rate of formation, severity, and persistence of hydrocephalus are not altered in sEHKO mice, which have elevated levels of basal EETs. This supports our hypothesis that hydrocephalus is likely mechanical in nature and not inflammatory. We have previously shown that CSF flow is blocked within minutes of SAH by fibrin deposition within the CSF pathways and is likely sufficient to induce acute communicating hydrocephalus after SAH. In addition, although hydrocephalus is unchanged in the sEHKO mice, edema is significantly reduced in association with the reduction in vascular inflammation as measured by reduced VCAM-1 expression in the cerebrovasculature. VCAM-1 is a cell adhesion molecule expressed by damaged or activated endothelium and important to the transmigration of infiltrating inflammatory cells. Elevated levels of VCAM-1 have been detected in the plasma and CSF of SAH patients. Its activation is generally accepted to be a part of the brain inflammatory response and has been associated with breakdown of the blood–brain barrier in other models of disease. In this context, we interpret our finding to mean that the inflammatory response to SAH is responsible, in part, for cerebral edema after SAH and that modulation of sEH to increase levels of EETs is protective.

Important considerations in the interpretation of our data are the multiple complications other than edema and hydrocephalus previously documented in this model, including elevated ICP and acute and delayed cortical hypoperfusion. Therefore, the improvement in behavior may result from improvement in any of these parameters, independent of hydrocephalus. Furthermore, one must consider the wide range of beneficial effects of EETs in the brain, which include their anti-inflammatory, antithrombotic, cytoprotective, and vasodilator properties. Thus, it is likely that the benefits of sEH deletion are multifactorial. Overall, these findings complement our previous work demonstrating improved outcomes after SAH in both humans and mice when sEH activity is altered and support the hypothesis that sEH inhibition may be a viable therapeutic strategy in SAH.

In conclusion, the endovascular puncture model in the mouse is useful to study the cause of acute communicating hydrocephalus and cerebral edema after SAH. Genetic deletion of sEH reduces vascular inflammation and edema formation in this model and improves outcome. This finding adds to the body of literature supporting further investigation of sEH inhibition as a therapeutic target in SAH.

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Disclosures

None.

References


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SUPPLEMENTAL MATERIAL

Soluble Epoxide Hydrolase in Hydrocephalus, Cerebral Edema and Vascular Inflammation after Subarachnoid Hemorrhage.
Supplemental Methods:

Animals

Experimental animal procedures performed in this study conform to the guidelines of the US National Institutes of Health, and the animal protocol was approved by the Institutional Animal Care and Use Committee of Oregon Health & Science University, Portland, OR, USA. 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 abnormalities. Genotype was confirmed by PCR, as previously described. 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.

Endovascular Puncture

SAH was induced in mice using the endovascular perforation technique as previously described. Briefly, mice were anesthetized with isoflurane (1.5 to 2% in O2-enriched air by face mask), and maintained at 37±0.5°C rectal temperature using warm water pads. Two small laser-Doppler probes (Moor Instruments) were affixed bilaterally over the dorsolateral parietal cortex to monitor cerebrocortical perfusion and confirm vascular rupture. To induce hemorrhage, a nylon suture (5-0) was introduced into the internal carotid artery via the external carotid artery and advanced ~10mm beyond the carotid bifurcation and into the Circle of Willis. The suture was then advanced slightly further to induce a hemorrhage, and then removed. The common carotid artery was maintained patent at all times to maximize flow to the ruptured artery immediately following arterial perforation. After endovascular puncture, the filament was removed, the wound closed and the animal was allowed to recover. Mortality throughout the study was less than 10% and most often occurred immediately following SAH induction. Only mice which survived the initial surgery were included in this study. In sham operated animals, the suture was advanced into the internal carotid artery and then removed without arterial perforation.

Physiological Monitoring

In a subset of non-survival surgeries, animals were monitored for intra-cranial pressure (ICP), systemic arterial blood pressure (BP) and cerebral blood flow (CBF) for thirty minutes following induction of the hemorrhage. For ICP measurements, a small burr hole (<0.5mm) was made into the left parietal bone and a micro-tip catheter transducer (Millar) 0.33 mm in diameter was placed into the parenchyma. The transducer was connected to a PCU-200 ICP monitor (Millar). For blood pressure recording, the femoral artery was catheterized and connected to a BP-1 monitor (World precision Instruments). For cerebral blood flow monitoring, a single laser-Doppler probe (Moor Instruments) was placed over the right parietal bone. All three monitoring devices were connected to a digi-data 1440 digital to analog converter (molecular devices) and recorded using pCLAMP (molecular devices). After all devices were connected, SAH was
induced as described above and the mouse was monitored for thirty minutes. All probes were then removed and the mice were deeply anesthetized with isoflurane, then cardiac perfused with cold heparinized saline and the brain examined to confirm hemorrhage.

**Vascular Cell Adhesion Molecule-1 (VCAM-1)-bound micro particles of iron oxide (MPIO)**

We conjugated monoclonal rat anti-mouse CD106 (VCAM-1; 1510-01 Southern Biotech) with a monoclonal mouse IgG1 (0102-01 Southern Biotech) covalently bound to Dynabeads MyOne Tosylactivated MPIOs (Invitrogen) per manufacturer’s instructions. Briefly, MPIOs were washed with sodium borate buffer (0.1 M, pH 9.5) and combined with antibodies (40μg antibody/1mg MPIO) and 3 M ammonium sulfate to give a final concentration of 6mg/ml MPIO. The solution was incubated on a rotating platform at 37 °C overnight. We then collected MPIO by using a magnet and discarded the supernatant. We added PBS plus 0.5% BSA and 0.05% Tween 20 (pH 7.4) as a blocking agent and incubated MPIO at 37 °C overnight again. We washed MPIO with PBS plus 0.1% BSA and 0.05% Tween-20 before storing at a final concentration of 5mg MPIO per ml in PBS plus 0.1% BSA and 0.05% Tween 20 at 4 °C.

**MRI**

MR imaging employed a Bruker-Biospin 11.75T small animal MR system with a Paravision 4.0 software platform, 9cm inner diameter gradient set (750 mT/m), and a mouse head (20 mm ID) quadrature RF transceiver coil (M2M Imaging Corp.) The mice were positioned with their heads immobilized in a specially designed head holder with adjustable ear pieces. Body temperature was monitored and maintained at 37°C using a warm air temperature control system (SA instruments). Mice were initially anesthetized with a ketamine/xylazine mixture (15 mg xylazine/kg, 100 mg ketamine/kg) and isoflurane (0.5–2%) in 100% oxygen was administered and adjusted while monitoring respiration.

To quantify brain size, ventricle size and white matter edema, a coronal 25 slice T2 weighted image set (Paravision spin echo RARE, 256×256 matrix, 98 μm in-plane resolution, 0.5 mm slice width, TR 4000 msec, TE_{effective} 32 msec, RARE factor 8, 1 average) was obtained at baseline, 6h, 12h, 24h and 72h after SAH or sham surgery to assess brain anatomy. Images were processed using Jim (Xinapse Systems). The analysis employed images between +3.00 mm bregma and -6.00 mm bregma. Images were masked to remove non-brain tissue voxels. Brain volume was quantified as percent change from baseline scans to normalize for variations in brain volume between animals. To measure ventricular volume and edema, images were thresholded to the intensity of grey matter to isolate hyperintensities representing ventricles and edematous tissue. Areas of edema were defined as areas of hyperintensity that were located within brain parenchyma. The total volume of voxels identified as ventricles and edematous tissue were quantified. To normalize for variation in baseline ventricular volume, percent changes from baseline scans were calculated. To normalize for variation in brain size, edema volume was calculated as percent of total brain volume.

To quantify nanoparticle uptake 25h after SAH or sham surgery, we employed a 3D T2* weighted sequence (Paravision 3D FLASH, TE 9ms, FOV 3.5 x 1.75 x1.75 cm, 256x128x128 matrix, 137μm isotropic resolution, TR 25 msec, FA 15°, acquisition time 6.8min). Mice were
first imaged without injection of MPIOs. Mice were then injected with VCAM-1 MPIO or IgG MPIO at 4.5 mg Fe/kg body weight and rescanned at the 80 minute time point, which was chosen based upon previous work\textsuperscript{3,4}. All image processing was done using tools from FSL\textsuperscript{5-7} and Jim. Images were masked to remove non-brain voxels. The resulting brain extracted images were scaled and normalized to the intensity of the upper 70th percentile of nonzero voxels. Hypointense voxels were quantified using a fixed intensity threshold on the normalized images. The number of hypointense voxels at baseline was then subtracted from the number of hypointense voxels post-iron to obtain a measure of the extent of signal decrease due to iron uptake.

**Histology**

72h after SAH, mice were deeply anesthetized with isoflurane (5%) and perfused transcardially first with cold heparinized saline followed by 4% paraformaldehyde as a fixative. Brains were then incubated in 4% paraformaldehyde for 48h at 4ºC before being embedded in paraffin. Brains were then cut into 8um sections and stained with hematoxilin & eosin. Images were collected on a BX40 microscope (Olympus) using a micropublisher 5.0 camera (Qimaging). Images of the corpus callosum were obtained and converted to greyscale then thresholded. Vacuolization at the corpus callosum was quantified by taking the mean pixel intensity of the thresholded images.

In an additional cohort of animals 24h after SAH, brains were fixed as above, dehydrated and cleared (using Prosoft and Propar, Anatech Ltd, Battle Creek, MI) for paraffin embedding. Six micron thick sections were stained at two levels in the brain: mid-striatum and mid-hippocampus. Sections were stained with eriochrome for myelin, and immunohistochemistry for neurofilaments, myelin basic protein (MBP), and Iba-1 with a chromogenic marker and for CD31/VCAM double labeling with fluorescence.

For neurofilaments, MBP and Iba-1 deparaffinized sections were heated in 0.5 mM sodium citrate buffer at pH 6.0 in a steamer for 30 minutes for antigen retrieval. All rinses were with Tris-buffered saline (TBS), pH 7.6 with 0.1% Triton X-100. Sections were incubated with 3% normal goat serum in phosphate buffered saline with 1% BSA and 0.1% Triton (blocking serum) for 20 minutes at room temperature, followed by an avidin/biotin blocking step. Sections were incubated with the primary antibody (mouse monoclonal anti-neurofilament, 1:3000, Abcam ab24574; rabbit polyclonal anti-MBP, 1:12000, Milipore AB980; rabbit polyclonal anti-Iba-1, 1:1500, Wako 019-19741) diluted in the blocking serum, overnight at 4 ºC. The secondary antibody, biotinylated goat anti-rabbit IgG (diluted 1:200 in normal goat serum with 0.1% Triton, Vector BA-1000) or biotinylated anti-mouse Ig (Vector M.O.M. BMK-2202), was applied to the tissue for 30 minutes at room temperature, and sections were incubated with avidin-biotin-peroxidase complex ( Vectastain Elite kit, Vector PK6100) for 30 min. The color reaction was visualized with diaminobenzidine (DAB) and the sections were lightly counterstained with Mayer’s hematoxylin. The sections were dehydrated, cleared and coverslipped with Permount, and observed with a light microscope. Percent area per high powered field was calculated by a blinded researcher using color deconvolution and threshold features in ImageJ.
For CD31/VCAM double labeling, deparaffinized sections were incubated in 0.5M Tris buffer, pH10 for 30 min. Sections were incubated simultaneously in the primary antibodies (rabbit polyclonal anti-CD31, 1:320, Abcam 28364; goat anti-VCAM, 1:40, R&D systems AF643). The secondary antibodies were biotinylated donkey anti-rabbit IgG1:100 (Jackson 711-065-152) followed by Cy3 labeled streptavidin 1:800 (GE Healthcare, #PA43001.) for CD31 and Alexa Fluor 488 conjugated donkey anti-goat IgG (Life Technologies A11055) for VCAM. These sections were viewed with A1R+ confocal microscope (Nikon).

**Behavioral Assessment**

Mice were allowed to recover for 96h after SAH or sham surgery before they were tested for behavioral deficit. To test general locomotor activity, mice were first placed in an open field apparatus (Columbus instruments) and allowed to roam freely for ten minutes. Total distance travelled was recorded. On the same day, to test sensorimotor function, mice were placed on the accelerated rotarod (Columbus instruments) with speeds increasing by one rpm every three seconds in three consecutive “time to fall” trials. Mice were tested again on the rotarod in the same manner for two additional days to reach a total of nine “time to fall” trials. Time to fall was then averaged over the nine trials for each mouse.

**Randomization, Blinding, and Exclusion**

All animals were randomized to sham or SAH at the time of surgery by being randomly selected from a group of five littermates. All imaging and behavioral data was collected by operators blinded to the treatment groups. All quantification of imaging data was performed under conditions universally applied across genotype or treatment groups using automated analysis tools where possible. Animals were excluded from the study if they did not survive the initial SAH surgery. Mortality at the time of surgery was ~10% across all SAH groups. 2 additional animals were excluded from VCAM-1 analysis due to technical difficulties involving IV injection.

**Statistics**

Group data are expressed as mean ± sem unless otherwise stated. LDF, ICP, MAP, ventricular volume, brain size, and edema percentage were compared between groups using a repeated measures two-way ANOVA with Holm-Sidak post-hoc tests. Vacuolization data was log transformed and compared using one way ANOVA with Holm-Sidak post-hoc tests. VCAM-1 uptake was compared using one way ANOVA with Holm-Sidak post-hoc tests. Finally, behavioral comparisons were made using two-way ANOVA with Holm-Sidak post-hoc tests.
Supplemental Figure I. **Tissue volumes do not increase after SAH.** Tissue volume was calculated by subtracting total brain volume (figure 2) from ventricular volume at each time point. There was no significant increase in tissue volume at any time point in any group. WT (n=10, sEHKO (n=10) SAH animals and sham (n=5).
Supplemental Figure II. SAH induces axonal injury in mice. A,B. Neurofilament and myelin stains of the corpus callosum at 24 hr after SAH in mice. A. Neurofilament stain shows occasional fragmented and dilated axons (arrows). B. Myelin stain from the same region shows myelin breakdown (arrows). Scale bar = 30um.
Supplemental Figure III. SAH induces variable changes in microglial activation in WT mice. A. Representative images of Iba-1 staining in the corpus callosum 24h after SAH in WT SAH, sEHKO SAH, and Sham mice. B.) Percent area per high power field with positive stain in Sham (n=4), WT SAH (n=4) and sEHKO SAH (n=4) mice. Each value is a mean of 3 measurements per brain. Scale bar is 50um.
Supplemental Figure IV

Supplemental Figure IV. VCAM immunoreactivity in large cerebral veins 24h after SAH. A. T2* weighted image of mouse 24h after SAH and 80 min after VCAM-1 MPIO injection. B. CD31 immunolabeling (red) within the white box indicated in A. C. VCAM-1 immunolabeling (green) in the same area. D. Merge of B and C. * represents microvessels and arrow represents large vessels. Blue is nuclear DNA. Scale bar is 100um.
References


