Basic Sciences

Therapeutically Targeting Tumor Necrosis Factor-α/Sphingosine-1-Phosphate Signaling Corrects Myogenic Reactivity in Subarachnoid Hemorrhage

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Background and Purpose—Subarachnoid hemorrhage (SAH) is a complex stroke subtype characterized by an initial brain injury, followed by delayed cerebrovascular constriction and ischemia. Current therapeutic strategies nonselectively curtail exacerbated cerebrovascular constriction, which necessarily disrupts the essential and protective process of cerebral blood flow autoregulation. This study identifies a smooth muscle cell autocrine/paracrine signaling network that augments myogenic tone in a murine model of experimental SAH: it links tumor necrosis factor-α (TNFα), the cystic fibrosis transmembrane conductance regulator, and sphingosine-1-phosphate signaling.

Methods—Mouse olfactory cerebral resistance arteries were isolated, cannulated, and pressurized for in vitro vascular reactivity assessments. Cerebral blood flow was measured by speckle flowmetry and magnetic resonance imaging. Standard Western blot, immunohistochemical techniques, and neurobehavioral assessments were also used.

Results—We demonstrate that targeting TNFα and sphingosine-1-phosphate signaling in vivo has potential therapeutic application in SAH. Both interventions (1) eliminate the SAH-induced myogenic tone enhancement, but otherwise leave vascular reactivity intact; (2) ameliorate SAH-induced neuronal degeneration and apoptosis; and (3) improve neurobehavioral performance in mice with SAH. Furthermore, TNFα sequestration with etanercept normalizes cerebral perfusion in SAH.

Conclusions—Vascular smooth muscle cell TNFα and sphingosine-1-phosphate signaling significantly enhance cerebral artery tone in SAH; anti-TNFα and anti–sphingosine-1-phosphate treatment may significantly improve clinical outcome. (Stroke. 2015;46:2260-2270. DOI: 10.1161/STROKEAHA.114.006365.)

Key Words: inflammation ■ muscle, smooth, vascular ■ signal transduction ■ sphingosine kinase-1

Subarachnoid hemorrhage (SAH) affects ≈10 in 100000 people/y.1 Although SAH accounts for only 5% to 10% of all strokes, it is particularly devastating: half of all SAH patients die within 1 month and half of those who survive will require life-long assistance for daily living (ie, ≈75% of patients die or are seriously debilitated).2,3 The poor clinical outcome can be attributed to the biphasic course of the disease, characterized by an initial hemorrhagic stroke that is frequently followed by delayed cerebral ischemia (DCI) within 4 to 12 days.3,5 The latter is a primary therapeutic concern when treating SAH, because DCI causes at least half of the morbidity and mortality in SAH.2

The transition from the hemorrhagic insult to secondary ischemia is driven by prominent changes in cerebrovascular reactivity, which compromises cerebral autoregulation4,6 and evokes angiographic vasospasm (ie, radiographically identifiable arterial narrowing in the proximal cerebrovascular circulation).7,8 Conceptually, both events originate from augmented

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myogenic responsiveness, in direct response to the initial hemorrhage. Although angiographic vasospasm clearly associates with significant and local cerebral blood flow (CBF) reductions, perfusion deficits also frequently occur in regions and patients without localized vasospasm. A general augmentation of myogenic tone in the cerebrovascular microcirculation, therefore, may be a more meaningful contributor to clinically evident ischemic injury, because it causes broadly distributed perfusion deficits. Consequently, therapies that fail to restore cerebral autoregulation are unlikely to improve clinical outcome, even if they successfully curtail angiographic vasospasm.

Because blood flow autoregulation is required to maintain the structural and functional integrity of the brain, therapeutic interventions must specifically correct the SAH-induced myogenic tone enhancement. Unfortunately, most approaches nonselectively interfere with vasoconstriction and, unsurprisingly, do not consistently improve outcome.

The present investigation builds on mechanistic insight gained from an etiologically distinct model of heart failure, where myocardial infarction remotely stimulates the enhancement of proximal cerebral artery tone and concomitant disruption of CBF autoregulation. The underlying mechanism relies on vascular smooth muscle cell TNFα acting in an autocrine/paracrine manner to downregulate the cystic fibrosis transmembrane conductance regulator (CFTR). Because CFTR is a critical transporter involved in smooth muscle cell sphingosine-1-phosphate (S1P) degradation, its downregulation enhances S1P’s bioavailability and proconstrictive effects. We demonstrate the operation of this novel molecular mechanism in SAH and identify cerebrovascular TNFα and S1P signaling as potential therapeutic targets capable of correcting the enhancement of myogenic responsive-ness, without compromising general contractility.

Methods

This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (Publication No. 85-23, revised 1996). All animal care and experimental protocols were approved by the Institutional Animal Care and Use Committees at the University of Toronto, St. Michael’s Hospital (Toronto), and Tokushima University Medical School and were conducted in accordance with national animal protection laws.

Mice

Wild-type mice (C57BL/6 N) were purchased from Charles River Laboratories (Montreal, Canada); germ-line TNFα knockout mice (TNFα−/−) from Taconic Biosciences (Hudson, NY); and germ-line TNFα receptor 1 knockout mice from Jackson Laboratories (Bar Harbor, ME). Germ-line Sphk1 knockout mice (Sphk1−/−) were generously provided by Dr Richard L. Proia (Bethesda, MD). Inducible, smooth muscle cell–targeted TNFα knockout mice were generated by crossing floxed TNFα mice with mice expressing a recombinant Cre recombinase under the control of a smooth muscle–specific promoter. Specific details related to breeding and controls are available in the online-only Data Supplement. All mice were housed under a standard 14-hour:10-hour light–dark cycle, fed normal chow, and had access to water ad libitum.

Induction of SAH

We used a well-characterized model of experimental SAH. Each mouse was anaesthetized (isoflurane) and its head was fixed in a stereotactic frame; a 7-mm incision was made along the midline of the anterior scalp and 0.9-mm hole drilled into the skull 4.5 mm anterior to the bregma. A spinal needle was advanced to the chiasmatic cistern: 80 μL of arterial blood was injected into the intracranial space for 45 seconds. The scalp incision was closed. Buprenorphine (0.05 mg/kg; 0.5–1.0 mL volume) was administered twice per day (initiated immediately after the SAH surgical procedure).

Sham-operated animals underwent an identical surgical procedure, including spinal needle advancement, but were not injected with blood. The blood injected into SAH animals (termed hemorrhagic blood) was taken from a separate donor mouse (via cardiac puncture) immediately before injection and did not contain anticoagulants.

Isolation of Olfactory Arteries

Two days after SAH, animals were anesthetized and decapitated; brains were rapidly removed from the cranium and immersed in ice-cold sterile 3-(N-morpholino)propanesulfonic acid-buffered saline (MOPS). Olfactory artery segments (a first branch of the anterior cerebral artery; 0.8–1.0 mm in length) were carefully dissected from the surrounding connective tissue. The isolated vessel segments were cannulated on glass micropipettes, stretched to their in vivo lengths, and pressurized to 45 mm Hg.

Functional Assessments

All assessments were conducted in MOPS at 37°C with no perfusion and a transmural pressure of 45 mm Hg, with the exception of myogenic responses (see below). Vasomotor responses to a single phenylephrine dose (10 μmol/L) provided a viability assessment at the start of the experiment and immediately before passive diameter assessment (ie, diameter under Ca2+-free conditions). Vessels failing to constrict ≥25% to 10 μmol/L phenylephrine throughout the experimental process (ie, in response to the 2 single-dose assessments and during the phenylephrine dose–response assessment) were excluded.

Our preliminary experiments indicated that olfactory artery myogenic tone increases with successive measurements (data not shown), which is not an unprecedented observation. To maintain comparability across all data sets, myogenic tone was only assessed once per vessel (ie, an unpaired experimental design). All experiments were conducted at 2 days post-SAH induction, which our preliminary results indicated was the time point where the SAH-induced myogenic tone augmentation was largest (Figure I in the online-only Data Supplement).

Vasomotor responses (ie, in response to phenylephrine) used the calculation of tone (% of dia max)=[(diamax−diaactive)/diamax]×100, where diaactive represents the steady-state vessel diameter after the application of a given concentration of phenylephrine and diamax represents the maximal passive diameter at 45 mm Hg (measured under Ca2+-free conditions).

Myogenic tone was assessed by subjecting vessels to step-wise increases in transmural pressure (20 mm Hg increments) from 20 to 80 mm Hg. At each pressure step, vessel diameter (dia) was measured once a steady state was reached (3–5 minutes). Myogenic tone was calculated as the percent constriction in relation to the maximal passive diameter at each respective transmural pressure: tone (% of dia max)=[(dia−dia active)/dia active]×100, where dia active is the vessel diameter in MOPS containing Ca2+ and dia max is the diameter in Ca2+-free MOPS.

CBF Measurement

We used 2 methods to measure CBF: (1) a 2-dimensional blood flow-meter (Omegazone, laser speckle blood flow imager, Omegawave, Tokyo, Japan) and (2) flow-sensitive alternating inversion recovery magnetic resonance imaging (FAIR MRI). Mice were anesthetized with isoflurane (1.5%–2%) and thermostatically maintained. For the laser speckle approach, the skull was surgically exposed and irrigated with saline; a 780-nm semiconductor laser was used to illuminate the middle cerebral artery region for flow measurement. For the latter, MRI signals were acquired from vertical sections of the forebrain, midbrain, and hindbrain, which correspond to the
anterior, mixed, and posterior circulations. The FAIR technique isolates perfusion as an accelerated T1 signal relaxation. FAIR images were evaluated for designated regions of interest using standardized algorithms and image processing procedures (MIPAV; National Institutes of Health, Bethesda; http://mipav.cit.nih.gov/). Additional details pertaining to the MRI approach are provided in the online-only Data Supplement.

Behavioral Assessment
The modified Garcia score is an acute neurobehavioral assessment for SAH, consisting of 6 domains: spontaneous activity, spontaneous movement of all 4 limbs, forepaw outstretching, climbing, body proprioception, and response to vibrissae touch. Two blinded observers conducted the behavioral assessment 2 days after SAH. The maximum score is 18, indicative of normal mouse behavior.

Biochemical Assessments
We have previously published the procedures used for fluorescent immunolocalization, apoptosis assessment, and Western blotting. Western blots used vessel lysates prepared by pooling the arteries of the circle of Willis (including the anterior, middle, and posterior cerebral arteries, the anterior and posterior communicating arteries, and the cistern-localized section of the internal carotid and basilar arteries), with each lysate sample prepared from a single mouse. Specific details can be found in the online-only Data Supplement.

Statistics
Mice were randomized into groups, including treatment interventions, before Sham/SAH surgery; there was no mortality associated with the SAH surgery. Neurobehavioral tests and histological cell counting (apoptosis/neurodegeneration) were conducted under blinded conditions; all other assessments did not require blinding. With the exception of ordinal data (ie, neurobehavioral scores), all data are expressed as mean±SEM, where n is the number of vessels or assessments. Comparison of 2 groups used an unpaired Student t test; in situations where experimental groups were compared more than once, an ANOVA was conducted followed by Bonferroni-corrected t tests. Ordinal data are expressed as medians with interquartile range; these data were statistically compared with a Kruskal–Wallis nonparametric ANOVA, followed by a Student–Newman–Keuls post hoc test. Differences were considered significant at P<0.05; this study is powered for basic science conclusions.

Results
SAH Increases Myogenic Tone and Vascular Wall TNFα Expression
SAH augments olfactory artery myogenic tone (Figure 1A), without enhancing overall vascular contractility, as evidenced by unaltered phenylephrine responses (Figure 1B). Myogenic tone does not increase when saline is injected into

Figure 1. Subarachnoid hemorrhage (SAH) and tumor necrosis factor-α (TNFα) augment myogenic tone in olfactory arteries. SAH evokes (A) significant myogenic tone augmentation in olfactory arteries, without altering (B) phenylephrine (PE) responses. Passive diameters were not different (sham: 120±3 μm, SAH: 123±4 μm; n=6; P=NS). C, Brain slices containing olfactory artery cross-sections were costained with 4′,6-diamidino-2-phenylindole (DAPI; to identify cell nuclei; blue) and conjugated antibodies against TNFα (green) and smooth muscle α-actin (red). SAH increases TNFα expression throughout the vessel wall; although endothelial TNFα expression is evident, the increase is predominantly localized to the smooth muscle layer (yellow overlay). TNFα application in vitro (50 nM/L) evokes (D) myogenic tone augmentation, without affecting (E) PE responses. Passive diameters were not different (control, 128±5 μm; n=6 and TNFα, 124±5 μm; n=5; P=NS). *P<0.05 for unpaired comparisons.
Figure 2. Smooth muscle cell tumor necrosis factor-α (TNFα) is required for subarachnoid hemorrhage (SAH)-mediated myogenic tone enhancement. A, SAH increases olfactory artery myogenic tone only at the 60 mmHg transmural pressure in TNFα knockout mice (TNF−/−); B, phenylephrine (PE) responses are not altered. Passive diameters were not different (TNF−/− sham, 117±4 μm; n=6; and TNF−/− SAH, 113±5 μm; n=6; P=NS). Neither (C) myogenic tone nor (D) PE responses are altered by SAH in TNFα receptor 1 knockout mice (TNFR1−/−); passive diameters were not different (TNFR1−/− sham, 125±7 μm; TNFR1−/− SAH, 125±6 μm; n=5; P=NS). E, Wild-type (WT) mice treated with TNF−/− hemorrhagic blood (open circles; SAH WT+TNF−/−blood) possess myogenic tone levels that are similar to that of WT mice treated with WT blood (upward triangles; data set duplicated from Figure 1A). Accordingly, TNF−/− mice treated with WT hemorrhagic blood (closed circles; SAH TNF−/−+WT blood) possess myogenic tone levels similar to that of WT sham-operated mice (downward triangles; data set duplicated from Figure 1A). Directly comparing the 2 manipulations, myogenic tone is statistically higher at 40 and 60 mmHg in the SAH WT+TNF−/− blood group than in the SAH TNF−/−+WT blood group.

Genetic Manipulations Implicate Smooth Muscle TNFα as Causative

Germ-line deletion of genes encoding either TNFα or TNFα receptor 1 attenuates/prevents the SAH-stimulated increase in myogenic tone (Figure 2). We next determined the relevant source of TNFα. Injecting TNFα−/− hemorrhagic blood into wild-type mice induces a robust increase in myogenic tone that is not different from wild-type SAH (Figure 2E, open circles). Conversely, injecting wild-type hemorrhagic blood into TNFα−/− mice does not result in altered myogenic tone (ie, comparable with the wild-type sham; Figure 2E, closed circles). These results indicate that tissue-derived TNFα drives the increase of myogenic tone in SAH. Furthermore, smooth muscle–specific TNFα deletion fully blocks the SAH-mediated enhancement of myogenic tone (Figure 2G); controls confirm the specificity of the system and exclude nonspecific effects of the inducible knockout approach (Figure 2G). None of the manipulations alters PE responsiveness (Figure 2).

TNFα Inhibition Eliminates the SAH-Mediated Myogenic Tone Augmentation In Vitro and In Vivo

Sequestering TNFα (10 mg/mL etanercept; Figure 3A) or preventing TNFα release (via inhibition of TNFα-converting enzyme; 50 μmol/L TNFα proteinase inhibitor treatment; Figure 3C) in vitro reverses the SAH-stimulated myogenic tone augmentation (ie, myogenic tone is not different from sham controls). In vivo, systemic etanercept treatment (20 mg/kg IP per day; treatment initiated immediately after SAH; 2 injections total) prevents the SAH-stimulated increase in myogenic tone (Figure 3E). Myogenic tone is also attenuated after delayed (24 hours post-SAH induction) intrathecal etanercept administration (30 μg; Figure 3G). None of the interventions alters PE responsiveness (Figure 3).
Tumor necrosis factor-α (TNFα) Downregulates the CFTR in SAH

Our previous work demonstrates that TNFα-stimulated CFTR downregulation correlates with augmented cerebral artery myogenic tone.15 We observe a similar correlation in SAH: cerebral artery CFTR protein expression is downregulated (Figure 4A) and 3 interventions targeting TNFα, including (1) in vivo etanercept treatment (Figure 4A), (2) germ-line TNFα deletion (Figure 4B), and (3) smooth muscle cell–specific TNFα deletion (Figure 4C), abolish this downregulation. These observations indicate a close correlation between CFTR expression/activity and myogenic tone. Accordingly, inhibition of CFTR activity in vitro (100 mmol/L CFTR(inh)-172; 30-minute incubation of isolated arteries from naïve mice) enhances myogenic tone (Figure 4D). Although the CFTR inhibition shifts the basal tone, it does not alter PE responsiveness (Figure 4E).

SIP Signaling Mediates Myogenic Tone Augmentation in SAH

Consistent with our previous observations in posterior cerebral arteries,16 TNFα fails to increase myogenic tone in olfactory arteries isolated from Sphk1−/− mice (Figure III in the online-only Data Supplement). However, SAH significantly increases myogenic tone in arteries isolated from Sphk1−/− mice (between 40 and 80 mm Hg), although the magnitude of the augmentation is clearly attenuated (compared with wild-type mice; Figure 5A). Injecting wild-type hemorrhagic blood into Sphk1−/− mice yields a virtually identical enhancement (Figure IV in the online-only Data Supplement), thereby excluding the involvement of blood-borne Sphk1 activity.

SIP receptor antagonism (1 μmol/L JTE013) in vitro fully reverses the SAH-stimulated myogenic tone augmentation (ie, myogenic tone was not different from sham controls; Figure 5C). Accordingly, systemic JTE013 treatment in vivo (3 mg/kg IP per day; vehicle 7.5% DMSO+3% fatty acid–free bovine serum albumin; treatment initiated immediately after SAH; 3 injections total) prevents the SAH-stimulated increase in myogenic tone (Figure 5E). None of the interventions alters PE responsiveness (Figure 5).

Disrupting TNFα Signaling Restores CBF in SAH

As our in vitro data predicts, SAH significantly decreases CBF (Figure 6); the FAIR MRI approach localizes the CBF reduction primarily to the anterior microcirculation (ie, forebrain; Figure V in the online-only Data Supplement). Although both laser speckle flowmetry (Figure 6A) and FAIR MRI (Figure 6B) readily detect the CBF reduction, the 2 methodologies differ in terms of magnitude (24% versus 56% reduction, respectively). Because FAIR-MRI provides a valid and quantitative CBF measure,26,27 we deem these results to more accurately represent the true magnitude of the CBF reduction. Systemic etanercept treatment (20 mg/kg IP per day; treatment initiated immediately after SAH; 2 injections total) eliminates the SAH-mediated perfusion deficit (Figure 6A and 6B), which correlates with the normalization of cerebral artery myogenic tone (Figure 3E). Of note, no perfusion

Figure 3. Tumor necrosis factor-α (TNFα) inhibition eliminates the subarachnoid hemorrhage (SAH)–mediated myogenic tone augmentation. A, Myogenic tone and (B) phenylephrine (PE) responses in olfactory arteries isolated from sham and SAH mice are similar after in vitro etanercept treatment (ETN; 10 ng/mL); passive diameters were not different (sham+ETN, 128±2 μm; n=4 and SAH+ETN, 118±7 μm; n=6; P=NS). C, Myogenic tone and (D) PE responses are also similar after in vitro TNFα proteinase inhibitor treatment (TAPI; 50 μmol/L); again, passive diameters were not different (sham+TAPI, 127±5 μm; n=5 and SAH+TAPI, 120±3 μm; n=5; P=NS). Systemic ETN (20 mg/kg IP; initiated immediately after SAH induction) prevents the SAH-induced myogenic tone augmentation; (E) PE responses and passive diameters (sham+vehicle intraperitoneally [IP], 127±7 μm; n=4; SAH+vehicle IP, 130±1 μm; n=5; sham+ETN IP, 124±7 μm; n=5; SAH+ETN IP, 127±4 μm; n=8; P=NS) were similar across all groups. Delayed intrathecal (i.t.) ETN (30 μg; initiated 24 hours after SAH induction) attenuates the SAH-induced myogenic tone augmentation; (F) PE responses and passive diameters (sham+vehicle i.t., 121±5 μm; n=8 and SAH+ETNi .t., 127±3 μm; n=6; P=NS) were not different. *P<0.05 for single unpaired comparisons in A to D, G, and H and for multiple unpaired comparisons in E and F.
deficit is evident when saline is injected into the intracranial space, confirming independence from nonspecific effects related to the fluid injection (Figure 6A).

**Disrupting Either TNFα or S1P Signaling Attenuates Neuronal Apoptosis and Improves Neurological Outcome in SAH**

Neuronal cell apoptosis, measured by caspase-3 expression (Figure 6D) and Fluoro-Jade staining (Figure 6E), is significantly increased in SAH; etanercept treatment abolishes these SAH-stimulated effects. Germ-line TNFα deletion does not significantly reduce caspase-3 expression in SAH (Figure 6D); however, it eliminates the increased Fluoro-Jade staining evoked by SAH (Figure 6E). In smooth muscle cell–targeted TNFα knockout mice, no increase in caspase-3 expression is observed in response to SAH; Fluoro-Jade is elevated in SAH mice containing the floxed allele, but not in tamoxifen-treated animals (Figure VI in the online-only Data Supplement).

The improved cerebral perfusion and neuronal injury in etanercept-treated mice correlates with higher neurobehavioral scores (Figure 6F). Specifically, SAH mice scored lower than sham-operated mice on the modified Garcia behavior test. Etanercept treatment significantly increased the neurobehavioral scores; although they improved, the scores remained significantly lower than those for sham-operated mice.

Antagonizing S1P signaling (3 mg/kg IP per day JTE013), which we propose lies downstream of TNFα, also reduces SAH-induced neuronal cell apoptosis (caspase-3 expression; Figure 6G) and Fluoro-Jade staining (Figure 6H). As observed for etanercept, the JTE013-mediated attenuation of neuronal injury correlates with an improved modified Garcia behavioral score (which was restored to the sham level; Figure 6I).

**Discussion**

Our investigation demonstrates that SAH induces profound changes to myogenic reactivity before angiographic vasospasm and DCI. We provide the mechanistic basis for these changes and identify potential therapeutic targets.

The myogenic response is the dominant mechanism mediating CBF autoregulation: it continuously matches vascular resistance (via diameter changes) to the prevalent transmural pressure, thereby maintaining constant cerebral perfusion over a wide range of pressures. Impaired cerebral autoregulation is now recognized as an independent predictor of both DCI and negative outcome. Thus, failing to restore cerebral autoregulation in SAH will limit a treatment’s efficacy, even if angiographic vasospasm can be successfully curtailed. This aspect may underlie the failure of antivasospastic medications in certain clinical trials.

There is broad consensus that SAH changes cerebrovascular function by inducing an inflammatory reaction within the vascular wall. Although several cytokines may be involved, TNFα has garnered special attention because (1) cerebrospinal and interstitial fluid TNFα levels rise and peak between 4 and 10 days post-SAH (ie, a time-frame consistent with a role in DCI); (2) elevated TNFα levels...
Subarachnoid hemorrhage (SAH) increases myogenic tone via sphingosine-1-phosphate (S1P) signaling. A, Subarachnoid hemorrhage augments myogenic tone in olfactory arteries isolated from sphingosine kinase 1 knockout mice (Sphk1−/−); however, the magnitude of the increase is significantly less than in wild-type mice (wild-type sham [downward triangles] and SAH [upward triangles] traces are copied from Figure 1A). B, SAH does not affect phenylephrine (PE) responses in arteries from Sphk1−/− mice; passive diameters were not different (Sphk1−/− sham, 130±4 μm; n=5 and Sphk1−/− SAH, 126±3 μm; n=7; P=NS). C, Myogenic tone and (D) PE responses in arteries isolated from sham and SAH mice are similar after in vitro S1P receptor antagonism (JTE013; 1 μmol/L); passive diameters were not different (sham + JTE013, 134±4 μm; n=4 and SAH + JTE013, 126±4 μm; n=5; P=NS). Systemic JTE013 treatment (3 mg/kg IP per day injection, initiated immediately after SAH induction; 3 injections total) prevents (E) the SAH-induced myogenic tone augmentation; (F) PE responses and passive diameters (sham + vehicle intraperitoneally [IP], 129±5 μm; n=5; SAH + vehicle IP, 123±6 μm; n=5; sham + JTE013 IP, 127±4 μm; n=5; SAH + JTE013 IP, 123±6 μm; n=5; P=NS) were similar across all groups. *P<0.05 relative to the respective sham control; (A) +P<0.05 compared with wild-type SAH responses (upward triangles; data copied from Figure 1A).

Given the location of the therapeutic target (ie, to reach vascular smooth muscle cells, the therapeutic must cross the blood–brain barrier), it is clinically relevant to question whether the blood–brain barrier could interfere with an intervention. Etanercept is a large molecule and consequently does not cross an intact blood–brain barrier.58 Yet, systemically delivered etanercept successfully normalizes several critical endpoints in the present study, including vascular reactivity, CBF, and neuronal injury. We have not directly demonstrated that etanercept crosses blood–brain barrier; however, etanercept’s clear therapeutic effects suggest that the endothelial layer becomes permeable. Indeed, SAH is known to cause substantial blood–brain barrier disruption39 that permits the diffusion of large proteins.40 In support of this premise, systemic (Figure 3E) and intrathecal (Figure 3G) etanercept have similar effects on the enhanced myogenic tone in SAH: given that smooth muscle cell TNFα augments myogenic tone in SAH (Figure 2G), it is therefore reasonable to presume that both systemic and intrathecal etanercept sequester smooth muscle cell TNFα.

In humans, etanercept is administered subcutaneously and requires ≈2 days to reach effective plasma levels41: this pharmacokinetic profile may be sufficient, given that the earliest onset of angiographic vasospasm and DCI is at least 3 days after SAH, with peak incidence at 7 to 8 days. The fact that SAH patients frequently undergo craniotomy procedures to repair ruptured aneurysms or to have a ventricular catheter placed (for monitoring intracranial pressure) makes the intrathecal route a viable option for local etanercept delivery. From a perspective of clinically managing the patient, the intrathecal administration tested in our study may carry several advantages, including (1) minimal systemic side effects, (2) opportunity to use smaller doses (by at least 50x, as evidenced here; Figure 3G), and most importantly, (3) superior pharmacokinetic profile and hence rapid onset of the effect. Intrathecal administration could effectively bridge the time from the initial surgical intervention until plasma etanercept levels (via subcutaneous injection) have reached effective concentrations.
Importantly, etanercept’s functional effects were restricted to the SAH-mediated enhancement of myogenic tone: (1) the etanercept-mediated reduction of myogenic tone in SAH did not fall beyond the sham level, (2) etanercept had no effect in sham mice, and (3) unaffected PE responsiveness indicated that general contractility was not compromised. These features support the premise that targeting TNFα signaling in SAH will be superior to conventional therapies that blunt vasoconstriction and autoregulation.

In vivo, SAH markedly reduces CBF, induces significant neuronal injury (as measured by markers of apoptosis and neuronal degeneration), and decreases neurobehavioral performance (Figure 6). Systemic etanercept treatment ameliorated all of these deficiencies (Figure 6), implicating TNFα
as causative. This raises the question as to whether the neuronal injury results from direct TNFα actions on neurons or from TNFα-dependent reductions in blood supply (ie, ischemia resulting from altered autoregulation). Unfortunately, our attempts to separate the vascular and nonvascular TNFα effects using smooth muscle cell–targeted TNFα knockout mice (Figure VI in the online-only Data Supplement) were confounded by tamoxifen’s documented protective effect against neurodegeneration (Fluoro-Jade; Figure VI in the online-only Data Supplement). As a result, SAH did not increase caspase-3–positive staining in these animals (Figure VI in the online-only Data Supplement), a discrepancy that coincided with a higher baseline in smooth muscle–specific TNFα knockout sham mice (compared with Figure 6). These results, therefore, do not allow us to directly attribute the neuronal injury to altered vascular reactivity/autoregulation; consequently, we cannot exclude that etanercept has beneficial, nonvascular effects in addition to perfusion correction.

Nevertheless, etanercept’s beneficial effects on vascular reactivity, cerebral perfusion, neuronal injury, and neurobehavioral performance provide a strong rationale for initiating an anti-TNFα treatment strategy immediately after the onset of SAH. From the vascular perspective, the immediate normalization of myogenic reactivity presumably removes the functional basis for the later development of angiographic vasospasm, DCI, and stroke. In fact, the present investigation may provide the mechanistic basis for observations that infliximab prevents SAH-induced basilar artery vasospasm.6

Consistent with our previous observations,15,16,44 the molecular mechanism linking TNFα to enhanced myogenic tone is the S1P signaling pathway. In principle, TNFα can modify S1P signaling by 2 distinct mechanisms: (1) directly activating Sphk144,45 and (2) inhibiting S1P degradation, via genomic and nongenomic effects on CFTR expression/function.15 We propose that TNFα acutely augments myogenic tone by enhancing S1P synthesis.24 In the more chronic setting of SAH, this response is complemented by a reduction in S1P degradation, via the downregulation of CFTR (Figure 4).15 Thus, the augmented myogenic tone observed at 2 days after SAH results from a combination of increased S1P synthesis and reduced S1P degradation.

Genetically deleting Sphk1 putatively eliminates the SAH/TNFα-stimulated increase in S1P synthesis, as evidenced by the lack of TNFα effect in Sphk1−/− arteries in vitro (Figure III in the online-only Data Supplement). However, because Sphk1 gene deletion does not substantially reduce tissue S1P levels,17 a second isoform, Sphk2, can at least partially compensate for the loss of Sphk1. Isoform localization resolves this apparent conundrum: TNFα-stimulated Sphk1 localizes to the plasma membrane,46 whereas Sphk2 is primarily nuclear.47 Signal compartmentalization is critical for S1P’s myogenic functions48 and thus, the localization difference renders Sphk2 incapable of transducing the TNFα signal in the same manner as Sphk1.

In our proposed scheme, genetically deleting Sphk1 eliminates the SAH/TNFα-stimulated increase in S1P synthesis. However, because tissue S1P levels do not substantially drop after Sphk1 deletion,17 the downregulation of CFTR (Figure 4) would continue to enhance S1P bioavailability within the vascular wall.15 This explains why Sphk1 deletion was only partially effective in blocking the SAH-mediated increase in myogenic tone (Figure 5A) and implies that specifically targeting Sphk1 in SAH may not yield optimal therapeutic results.

Our model predicts that breaking the signaling chain at the receptor level (ie, downstream of S1P synthesis) would be more effective than targeting Sphk1. Indeed, S1P receptor antagonism (JTE013) fully prevented (in vivo)/reversed (in vitro) the SAH-mediated increase in myogenic tone (Figure 5). This is the first evidence that systemically administered S1P receptor antagonists may have clinical applicability for correcting vascular dysfunction. Like etanercept, JTE013 (1) did not reduce SAH-enhanced myogenic tone beyond the sham level, (2) had no effect in sham mice, and (3) left PE responsiveness intact (Figure 5), all of which support the conclusion that JTE013’s effects were restricted to the SAH-mediated enhancement of myogenic tone. As observed for etanercept, the normalized myogenic function in JTE013-treated SAH mice strongly correlates with attenuated neuronal injury and improved behavioral scores (Figure 6). Thus, our investigation adds a second viable therapeutic option for SAH that does not compromise vasoconstriction or autoregulation.

There are 2 important caveats to this conclusion. First, although JTE013 is a purported to be S1P2 receptor subtype selective, emerging evidence suggests that it can antagonize other S1P receptors16,49,50. We therefore urge cautious interpretation with respect to attributing JTE013’s effects to a particular S1P receptor subtype. Second, because S1P signaling directs a diverse array of biological processes in virtually every cell type,51 we cannot exclude the possibility that S1P receptor antagonism has deleterious effects on end points not assessed in the present investigation.

Our mechanistic scheme predicts that reducing CFTR expression will enhance myogenic tone,15 a premise substantiated by the observation that inhibiting CFTR activity augments myogenic tone (Figure 4). Because disrupting TNFα in vivo fully normalizes myogenic tone in SAH (Figures 2 and 3), these interventions must also normalize CFTR expression for our scheme to hold. Indeed, systemic etanercept treatment, germ-line TNFα knockout, and smooth muscle cell–targeted TNFα deletion all prevented the SAH-stimulated CFTR downregulation (Figure 4).

In summary, this investigation provides compelling evidence that SAH modifies 3 serially arranged cell signaling components (TNFα, CFTR, and S1P), forming a smooth muscle cell–based autocrine/paracrine network that profoundly alters cerebrovascular mechanosensitivity. All 3 identified components have treatment compounds clinically available (TNFα, etanercept and infliximab) or in late-stage clinical trials (CFTR, corrector/potentiator compounds; S1P, receptor antagonists). Our investigation demonstrates the therapeutic potential of anti-TNFα and anti-S1P receptor treatment and strongly promotes the assessment of clinical treatments against these targets immediately.

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Disclosures

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Therapeutically Targeting Tumor Necrosis Factor-α/Sphingosine-1-Phosphate Signaling Corrects Myogenic Reactivity in Subarachnoid Hemorrhage

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SUPPLEMENTARY MATERIAL

SUPPLEMENTARY METHODS

Reagents
JTE013 was purchased from Tocris Bioscience (Ellisville, USA) and etanercept (Embrel®) from Amgen Canada (Mississauga, Canada). Fatty acid free bovine serum albumin and protease inhibitor cocktail tablets (Complete®; used in western blot lysis buffers) and were purchased from Roche (Mississauga, Canada). Unless otherwise specified, all other reagents were purchased from Sigma-Aldrich (Oakville, Canada). MOPS-buffered salt solution contained [mmol/L]: NaCl 145, KCl 4.7, CaCl2 3.0, MgSO4 •7H2O 1.17, Na2HPO4 •2H2O 1.2, pyruvate 2.0, EDTA 0.02, MOPS (3-morpholinopropanesulfonic acid) 3.0, and glucose 5.0.

Generation of smooth muscle cell-targeted TNFα knockout mice and related controls
A mouse strain with a “floxed” TNFα gene (i.e., the TNFα gene is flanked by LoxP sites; TNFα^floxflox) was crossed with a transgenic mouse strain expressing a chimeric Cre recombinase (Cre-ERT²) under the control of the smooth muscle myosin heavy chain promoter (of note, the Cre transgene is located on the Y chromosome). The resulting progeny, which were heterozygous at the TNFα allele, were crossed. Breeding pairs homozygous for the floxed (TNFα^floxflox) or wild-type TNFα allele (TNFα^wt/wt) were selected from the resulting second-generation progeny (all male progeny express the Cre allele).

Only male mice were used in experiments (i.e., all experimental mice express the Cre transgene). The recombinase activity of the Cre-ERT² is controlled by a tamoxifen ligand-binding domain (ER²), which allows for temporal control of Cre recombinase activity. Activation of Cre recombinase induces the excision of the target gene, resulting in complete targeted deletion within 5 days. Tamoxifen was administered for 5 consecutive days (1mg intraperitoneal injections per day) one week prior to the induction of subarachnoid hemorrhage (SAH). TNFα^floxflox mice that were not tamoxifen treated and TNFα^wt/wt treated with tamoxifen (TNFα^wt/wt + TAM) served as controls for the transgene and tamoxifen treatment, respectively.

Fluorescent Immunohistochemistry
At 2 days post-SAH induction, animals were anesthetized with isoflurane; their brains were perfused with phosphate buffered saline (PBS) and then perfusion fixed with phosphate-buffered paraformaldehyde (4%; pH 7.4), both via the ascending aorta. Brains were immediately dissected and cut into three coronal sections (anterior, middle and posterior): the anterior section contains the olfactory artery; the posterior section was used for staining controls and neuronal apoptosis assessments. The brain sections were post-fixed in 4% paraformaldehyde (pH 7.4) for 48 hours at 4°C and then incubated in 10% sucrose (3 hours), followed by 30% sucrose overnight (at 4°C) for cyroprotection. The brain sections were then embedded in OCT (Sakura Finetek USA; Torrance, USA) on isopentane and dry ice. Cryostat sections (5μm thick coronal slices) were collected on “Tissue Path Superfrost Plus Gold” slides (Fisher Scientific; Whitby, Canada) and stored at -80°C until used.

All samples were pre-treated with 10-minute proteinase (20μg/ml; Promega; Madison, USA), followed by a standard 60-minute blocking procedure with either 10% normal donkey (Jackson ImmunoResearch Laboratories; West Grove, USA) in PBS or 10% goat serum (Invitrogen Life Technologies; Burlington, Canada) in PBS containing 1% bovine serum albumin.

Anterior brain slices containing the olfactory artery were incubated with goat anti-mouse TNFα polyclonal antibody (1:200 dilution; R&D systems; Minneapolis, USA; cat# AF410NA). After washing, the slices were simultaneously incubated with (i) Alexa Fluor 488 donkey anti-goat IgG (Invitrogen; 1:600; secondary conjugate for TNFα antibody) and (ii) Cy3-conjugated mouse anti-smooth muscle α-actin monoclonal antibody (clone 1A4; 1:6000 dilution; Sigma). All antibodies
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were diluted in Can Get Signal® immunoreaction enhancer solution (Toyobo; Osaka, Japan) and used an incubation time of 1 hour at room temperature. Specimens were then washed, treated with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen), washed and mounted with CC Mount™ (Sigma).

Posterior brain slices used for cortical assessments were incubated with monoclonal rabbit anti-human active cleaved caspase-3 (clone C92-605; 1:1000 dilution; BD Biosciences Canada; Mississauga Canada) and conjugated with Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen). Both antibodies were diluted in Can Get Signal® immunoreaction enhancer solution and used an incubation time of 1 hour at room temperature. Specimens were washed and mounted with CC Mount™.

Digital immunofluorescence images were acquired using either an Olympus BX50 fluorescence microscope (brain slices), or a Zeiss LSM 710 confocal laser-scanning microscope in conjunction with Zeiss ZEN 2010 software (vessel images). Overlays were constructed with freely-available ImageJ 1.44p software (National Institutes of Health, USA).

**Fluoro-Jade staining**

Brain slices were serially incubated with 1% NaOH / 80% ethanol (5 minutes), 70% ethanol (2 minutes), distilled water (2 minutes) and 0.06% potassium permanganate (10 minutes). After washing with deionized water, brain slices were stained with 0.0004% Fluoro-jade B (Histo-Chem Inc., Jefferson, USA) in 0.1% acetic acid (15 minutes). The samples were then washed with deionized water, dried and cleared by immersion in xylene for 1 minute. Slides were mounted using DPX mounting medium (Sigma). Digital fluorescence images were acquired using an Olympus BX50 fluorescence microscope.

**Assessment of brain damage by cell counting:**

As we 3,4 and others 5 have previously described, caspase-3 and Fluoro-Jade positive cells were counted in four fixed cortical regions in a single coronal brain slice 1mm posterior to the bregma (400x magnification); the cortical fields are situated in the left parietal, left temporal, right parietal and right temporal lobes. For each animal, the number of positive cells was averaged, yielding a mean positive cell count/field of view, which was then used for statistical analyses. Two independent assessors completed the counting under blinded conditions.

**Western Blotting**

Cerebral arteries in the basal cistern (the anterior, middle and posterior cerebral arteries, the anterior and posterior communicating arteries and the cistern-localized section of the internal carotid and basilar arteries; each sample contains arteries from 1 mouse) were isolated, pooled and ground in tissue in lysis buffer containing 25mM Tris (pH6.8), 1% SDS, 10% glycerol, 1mM EDTA, 0.7M β-mercaptoethanol and 25µg/ml protease inhibitor cocktail. The lysates were subjected to freeze-thaw cycles using liquid nitrogen, heated to 65°C for 10 minutes and then centrifuged (30 minutes at 15,000g; 4°C) to remove insoluble material. Protein levels were quantified spectrophotometrically using Bradford reagent.

We have previously described a standard polyacrylamide gel electrophoresis procedure that separates and quantifies CFTR 6. Briefly, equal amounts of protein were loaded onto 5% acrylamide gels, separated electrophoretically and transferred onto PVDF membranes. The blotted protein levels were quantified densitometrically (using freely-available “Image J” software from the National Institutes of Health, USA) with the rapidly reversible Ponceau S protein stain. After washing the Ponceau stain with distilled water, the membranes were blocked for 60 minutes in 5% non-fat skim milk (in phosphate-buffered saline containing 1% Tween 20 (PBST); 137mM NaCl, 2.7mM KCl, 10mM Na2HPO4, 1.76mM K2HPO4; pH 7.4) and sequentially incubated with rabbit polyclonal anti-CFTR (clone H182; Santa Cruz Biotechnology, Dallas, USA; 1:1000 dilution in 2% bovine serum albumin in PBST),
conjugated with peroxidase-labeled donkey anti-rabbit IgG antibody (GE Healthcare; Piscataway, USA; 1:40,000 dilution in 2% bovine serum albumin in PBST) and detected with an “ETA C” enhanced chemiluminescence substrate (Cyanagen; distributed by VPQ Scientific, Toronto, Canada) and X-ray film. Developed films were evaluated densitometrically. Antibody specificity has been previously validated using cultured smooth muscle cells isolated from CFTR knockout mice.⁶

**Magnetic Resonance Imaging-based measurement of cerebral perfusion:**

As described in our previous investigation,⁷ the FAIR technique⁸ is a magnetic resonance imaging (MRI) approach that evaluates brain perfusion with a 7 Tesla micro-MRI system (BioSpec 70/30 USR, Bruker BioSpin, Ettlingen, Germany), including the B-GA12 gradient insert, 72mm inner diameter linear volume resonator for RF transmission, and anteriorly placed head coil for RF reception. FAIR isolates perfusion as an accelerated T₁ signal relaxation following slice-selective compared to non-selective inversion preparation, as per the following equation: \( \text{CBF} = \lambda \left( \frac{1}{T_{1, \text{ss}}} - \frac{1}{T_{1, \text{ns}}} \right) \) (ml/(100g*min)), where ‘ss’ and ‘ns’ denote slice-selective and non-selective measurements and \( \lambda \) is the blood-brain partition coefficient, defined as the ratio between water concentration per g brain tissue and per ml blood. This coefficient is approximately 90ml/100g in mice.⁹

FAIR optimization was a single-shot EPI technique with preceding slice-selective or non-selective adiabatic inversion pulse. Parameters included echo time of 12.5ms, repetition time of 17s, 18 inversion times ranging from 25 to 6825ms in 400ms increments, 3mm slice-selective inversion slab, 18x18mm field-of-view with 72x72 matrix for 250µm in-plane resolution, 1mm slice thickness, and 10min 12s data acquisition time. Acquisitions included vertical sections in the fore-, mid- and hind-brain regions, which correspond to the anterior, mixed and posterior circulations.

FAIR images were evaluated by manual prescription (MIPAV, NIH, Bethesda, MD; http://mipav.cit.nih.gov) of sub-hemispheric regions-of-interest (ROIs), termed ‘global’, and local ROIs corresponding to cortical and sub-cortical parenchyma in forebrain sections. ROIs were drawn directly on T₁-weighted signal images to enable manual correction for intra-scan motion. ROIs were registered with parametric CBF maps to verify absence of bias from high perfusion vessels and meninges. T₁ regressions and CBF calculations were performed using Matlab (Mathworks, Natick, MA). Significance was defined by 1-tailed Student’s t-test (P<0.05).
**SUPPLEMENTARY FIGURE I**

(A) Time-response assessment for myogenic tone in subarachnoid hemorrhage

(A) Subarachnoid hemorrhage (SAH) augments myogenic tone to the greatest extent at 2 days post-SAH induction (compared to 1 day and 5 days post-SAH induction). (B) Phenylephrine responses are not altered at any time point. Passive diameters were not different (sham: 128±5µm, n=6; SAH Day 1: 128±7µm, n=6; SAH Day 2: 123±4µm, n=6; SAH Day 5: 117±5µm, n=4; P=N.S.). * denotes P<0.05 relative to naïve control.
Comparison of sham procedures that include and do not include saline injection

(A) Myogenic tone and (B) phenylephrine responses were not different in sham procedures that included saline injection (80µl injected into chiasmatic cistern; analogous to SAH procedure) from sham procedure that did not include saline injection. Passive diameters were not different (sham: 120±3µm, n=6; sham+saline: 121±8µm, n=5; P=N.S.).
**SUPPLEMENTARY MATERIAL**

**SUPPLEMENTARY FIGURE III**

\[\text{TNF} \alpha \text{ does not increase myogenic tone in olfactory arteries isolated from Sphk1}^{/-} \text{ mice}\]

Traces for wild-type control and wild-type TNF\(\alpha\)-treated olfactory arteries are copied from Figure 1 for comparison purposes. (A) Myogenic tone in TNF\(\alpha\)-treated olfactory arteries isolated from Sphk1\(^{-/-}\) mice was not different from wild-type untreated controls. (B) Phenylephrine responses were not different. Passive diameter for Sphk1\(^{-/-}\) + TNF\(\alpha\) was 139±6\(\mu\)m, n=5. * denotes P<0.05 compared to from all other groups; + denotes P<0.05 relative to WT control.
Blood-borne Sphk1 activity does not contribute to the enhanced myogenic tone in SAH

(A) Subarachnoid hemorrhage increased olfactory artery tone in Sphk1−/− mice, regardless of whether the “hemorrhagic blood” was taken from wild-type or Sphk1−/− mice and the extent of the increase was similar. (B) Phenylephrine responses were similar for all groups. Passive diameters were not different (Sphk1−/− sham: 130±4 µm, n=5; Sphk1−/− + Sphk1−/− Blood: 126±7 µm, n=6; Sphk1−/− + WT Blood: 126±3 µm, n=7; P=N.S.). * denotes P<0.05 relative to the sham control.
**SUPPLEMENTARY FIGURE V**

**A**

Subarachnoid hemorrhage reduces cerebral perfusion primarily in the anterior microcirculation.

**(A)** Shown are representative FAIR-Magnetic Resonance Imaging perfusion maps of the fore- (left column), mid- (center column) and hind- (right column) brain regions from mice with a sham operation (top row) or subarachnoid hemorrhage (SAH; bottom row). **(B)** SAH significantly reduced cerebral blood flow in the forebrain region (anterior microcirculation), but not in the mid- or hind-brain regions (sham n=9, SAH n=8). Note: forebrain measures are reproduced from Figure 6B. * denotes P<0.05 relative to the sham control.

**Subarachnoid hemorrhage reduces cerebral perfusion primarily in the anterior microcirculation**

**B**

![Graph showing cerebral blood flow](image-url)
**Apoptosis markers following SAH in smooth muscle cell-targeted TNFα knockout mice**

(A) Displayed are representative images of cortical cells stained for cleaved caspase-3 expression (top) and with Fluoro-Jade (bottom). (B) Subarachnoid hemorrhage (SAH) did not increase the number of caspase-3-positive cells, relative to the sham control. (C) SAH increased the number of Fluoro-Jade positive cells in mice containing the floxed TNFα allele (SAH TNF α flox/flox). Tamoxifen treatment prevented this increase in both smooth muscle-targeted knockout mice and wild-type controls. Number of fields assessed for each group: sham n=3; SAH TNF α flox/flox n=4; SAH TNF α flox/flox+TAM n=5; SAH TNF wt/wt+TAM n=5. Note that all TNF flox/flox and TNF wt/wt mice express Cre recombinase (Cre-ER<sup>12</sup>) under the control of the smooth muscle myosin heavy chain promoter. * denotes P<0.05 relative to the sham control.
SUPPLEMENTARY MATERIAL

SUPPLEMENT REFERENCES


