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ORIGINAL RESEARCH

Reversal of Delayed Vasospasm by TS-011 in the Dual Hemorrhage Dog Model of Subarachnoid Hemorrhage

L. Hacein-Bey
D.R. Harder
H.T. Meier
P.N. Varelas
N. Miyata
K.K. Lauer
J.F. Cusick
R.J. Roman

PURPOSE: Arachidonic acid is avidly metabolized to a potent vasoconstrictor, 20-hydroxyeicosatetraenoic acid (20-HETE), in the cerebral circulation. 20-HETE has been reported to contribute to the acute fall in cerebral blood flow following subarachnoid hemorrhage (SAH), but its role in the development of delayed vasospasm is unknown. The present study examined whether delayed vasospasm is associated with elevations in 20-HETE in CSF in the dual hemorrhage model of SAH in dogs and if blockade of the synthesis of 20-HETE with *N*-(3-chloro-4-morpholin-4-yl)phenyl-*N*′-hydroxyimido formamide (TS-011) can reverse delayed vasospasm in this model.

MATERIALS AND METHODS: Delayed vasospasm was induced in 22 adult beagle dogs by dual injection of blood (0.5 mL/kg) into the cisterna magna on days 1 and 4. Sequential samples of CSF were collected before intracisternal injections of blood on days 1 and 4 and after the development of delayed vasospasm on day 7. Sequential angiograms were obtained before and after intracisternal injection of blood on days 1 and 4 and before and 1 hour after administration of TS-011 (1 mg/kg IV) on day 7.

RESULTS: The dogs consistently developed delayed vasospasm, and the diameter of the basilar artery fell to 68 ± 3% ($n = 15$), 3 days after the second intracisternal injection of blood. The levels of 20-HETE in CSF increased from 4 ± 2 to 39 ± 16 pg/mL. In 9 dogs with delayed vasospasm, acute blockade of the synthesis of 20-HETE with TS011 (1 mg/kg IV) significantly increased the diameter of the basilar artery by 39%. Chronic administration of TS-011 (1 mg/kg per day) attenuated the development of delayed vasospasm, and the diameter of the basilar artery fell by 17 ± 1% versus the 33 ± 3% decrease in diameter seen in control animals 3 days following the second injection of blood into the cisterna magna.

CONCLUSIONS: These results indicate that the development of delayed vasospasm in dogs is associated with an increase in 20-HETE levels in CSF, and acute blockade of the synthesis of 20-HETE with TS-011 reverses delayed vasospasm in this model.

Previous studies indicated that arachidonic acid is metabolized by cytochrome P450 (CYP) enzymes in cerebral arteries to 20-hydroxyeicosatetraenoic acid (20-HETE) and that this compound plays an important role in the regulation of cerebral vascular tone.[1-3] 20-HETE is a potent vasoconstrictor that depolarizes vascular smooth muscle (VSM) cells by inhibiting the open-state probability of Ca^{2+}–activated K^+ channels. The formation of 20-HETE is stimulated by angiotensin II, endothelin, and serotonin and is inhibited by nitric oxide (NO) and carbon monoxide. Blockade of the formation of 20-HETE attenuates the myogenic response of cerebral arteries,[1,5] autoregulation of cerebral blood flow (CBF),[1,4] and the vascular responses to both vasoconstrictors and dilators.[5,6] Recent studies have indicated that the levels of 20-HETE in the CSF fluid increase after subarachnoid hemorrhage (SAH) and that inhibitors of the synthesis or actions of 20-HETE prevent the acute fall of CBF in rats after SAH.[4,5] However, the role of 20-HETE in the development of delayed vasospasm is unknown. The present study examined whether the development of delayed vasospasm is associated with elevations in 20-HETE in CSF in the dual hemorrhage model of SAH in dogs and if blockade of the synthesis of 20-HETE with *N*-(3-chloro-4-morpholin-4-yl)phenyl-*N*′-hydroxyimido formamide (TS-011)[6] can attenuate or reverse the development of delayed vasospasm in this model.

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From the Division of Neuroradiology, Department of Radiology (L.H.-B., H.T.M.) and the Departments of Neurological Surgery (L.H.-B., P.N.V., J.F.C.), Neurology (P.N.V.), Physiology (D.R.H., R.J.R.), and Anesthesiology (K.K.L.), Medical College of Wisconsin, Milwaukee, Wis; and the Medicinal Research Laboratories (N.M.), Taisho Pharmaceutical Co., Saitama, Japan.

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Please address correspondence to: Lotfi Hacein-Bey, MD, Neuroradiology and Interventional Neuroradiology, Departments of Radiology, Neurosurgery, and Neurology, Loyola University Medical Center, 2160 S First Ave, Maywood, IL 60153.

Materials and Methods

General. Experiments were performed on 22 beagle dogs weighing between 8 and 14 kg. The dogs were pair-housed in a standard kennel in the Veterinary Medical Unit at the Milwaukee VA Medical Center (VAMC). The facility is accredited by the Association of Accreditation and Assessment of Laboratory Animal Care. The dogs were fed once a day and had free access to tap water. All experimental procedures were approved by the Animal Care and Use Committee of the Medical College of Wisconsin and conformed to the *Guide for the Care and Use of Laboratory Animals*.

Experimental Protocol. All angiographic studies were performed in the Experimental Animal Angiography Laboratory of the VAMC under sterile conditions by using a mobile C-arm imaging system (OEC 9800 Plus, GE Medical Systems, Waukesha, Wis). Before obtaining angiograms, we premedicated the dogs with an IM injection of butorphenol (0.4 mg/kg), diazepam (0.25 mg/kg), and atropine (0.05 mg/kg). Anesthesia was induced by using propofol (3 mg/kg) and maintained with isoflurane. Body temperature was controlled at 99 ± 1°F with a heating pad. O_2 saturation was continuously monitored

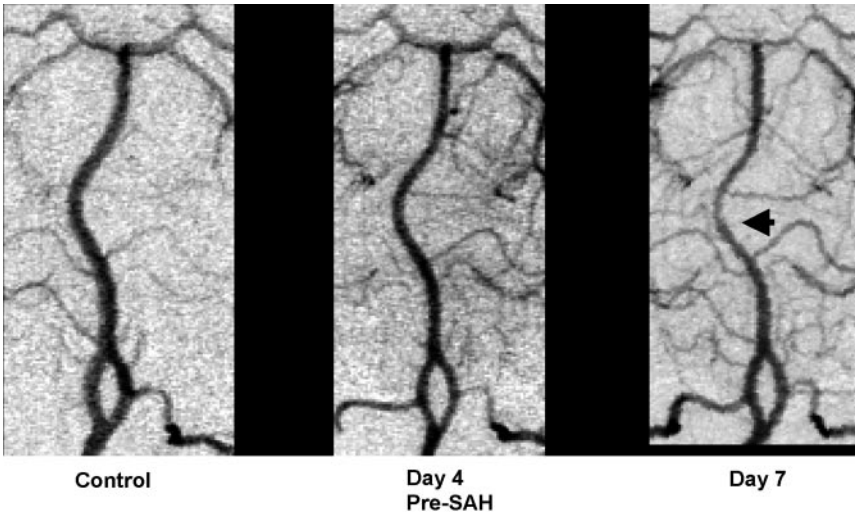

Fig 1. Representative sequential angiograms obtained in a dog after induction of the dual hemorrhage model of SAH. The left panel presents the control angiogram before induction of SAH. The middle panel depicts the diameter of the basilar artery 3 days after the first cisternal injection of blood. The right panel depicts the appearance of the basilar artery 3 days after the second cisternal injection of blood. The arrow indicates the narrowest point in the basilar artery, which shows vasospasm where sequential measurements were made.

and kept >95%. Ventilation was regulated to maintain end-tidal PCO_2 between 35 and 45 mm Hg, and the dogs received an intravenous infusion of a lactated Ringer's solution (10 mL/kg per hour) to replace fluid losses.

A skin incision was made to expose the femoral artery and a 5F catheter (Cook, Bloomington, Ind) was advanced into the vertebral artery. One to two milliliters of iohexol (Omnipaque, 300 mg I/mL) was injected while angiograms of the posterior cerebral vasculature were obtained by using digital subtraction angiography. After obtaining a baseline angiogram, we collected 2 mL of CSF from the cisterna magna for measurement of 20-HETE levels, and 1.25 mL of arterial blood was injected. Following this procedure, the catheter was removed, the skin incisions were closed by using surgical tissue glue and a 6–0 polypropylene (Prolene; Ethicon/Johnson & Johnson, Warren, NJ) suture, anesthesia was withdrawn, and the animals were allowed to recover. Useful baseline angiograms were obtained from 18 of 19 dogs studied.

On day 4, 3 days after the first injection of blood in the cisterna magna, the dogs were reanesthetized and 2 mL of CSF was withdrawn from the cisterna magna for measurement of 20-HETE, and 1.25 mL of arterial blood was injected. Angiograms were obtained from 7 of 19 dogs to document the degree of vasospasm after the first injection.

On day 7, 3 days after the second injection of blood into the cisterna magna, all of the dogs were reanesthetized and a 2-mL sample of CSF was collected for measurement of 20-HETE. Another angiogram was then obtained in 15 of the 19 animals. Unfortunately, 4 dogs had to be euthanized before obtaining the angiogram on day 7 because they developed severe delayed neurologic deficits. After documenting the development of delayed vasospasm, some of dogs (9/15) were given an intravenous bolus injection (1 mg/kg) of TS-011, a newly described selective inhibitor of the synthesis of 20-HETE.[6] One hour later, a final angiogram was obtained, and the animals were then euthanized with pentobarbital sodium (Beuthanasia D, 100 mg/kg IV).

Studies were also performed in 3 additional dogs to determine if chronic treatment with TS-011 could attenuate the development of delayed vasospasm. These animals were subjected to the dual hemorrhage model of SAH and were given injections of TS-011 (1 mg/kg, SC) twice a day. Sequential angiograms were obtained before and after intracisternal injection of blood on days 1 and 4 and on day 7.

Assessment of Vascular Diameter. Great care was taken during the angiographic procedures to ensure that the distance from the x-ray source was standardized for all studies. In addition, a 23-gauge needle of known diameter was placed in the field to serve as an internal diameter standard on all angiograms. The angiographic images were transferred digitally to an image-processing software (Adobe Photoshop, Adobe Systems, San Jose, Calif), and each of the images obtained from the same dog was cropped to standardize the magnification. We first identified the narrowest point on the delayed vasospasm angiogram. Then diameter measurements were made at this point on all of the angiograms obtained from the same animal by using the Metamorph Imaging System (Molecular Devices, Sunnyvale, Calif). The diameter data were expressed as a percentage of the baseline diameter measured in the same animal.

Liquid Chromatography/Mass Spectroscopy Measurement of 20-HETE Levels in CSF. The CSF samples (1 mL) were diluted with 1 mL of water and 2 ng of an internal standard 5Z, 14Z, 20-hydroxydienoic acid was added to each sample. The samples were extracted with 6 mL of chloroform-methanol (2:1) and dried under nitrogen. The samples were reconstituted in chloroform and loaded onto a normal-phase Sep-Pak (Waters, Milford, Mass) column. The column was washed with 2 mL of water and 1 mL of hexane and then eluted with 1 mL of cyclohexane–ethyl acetate (50:50, vol/vol). The sample was dried, reconstituted in 50 μL of methanol and water (50:50), and cleaned by using an on-line reverse-phase high-performance liquid chromatography (HPLC)–trapping column. The HETEs in the samples were separated at a flow rate of 150 μL/min with an isocratic step gradient on an 18C-RP 2 $\times$ 250 mm microbore HPLC column (3 μm particle size; BetaBasic18, Thermo Hypersil-Keystone, Shelton, Conn) by using a mobile phase, consisting of acetonitrile-water–acetic acid (57:43:0.1) for 20 minutes to resolve the HETEs, followed by acetonitrile-water–acetic acid (75:25:0.01) for 15 minutes to elute the internal standard. Samples were ionized by using negative ion electrospray, and the peaks were eluted with m/z 319 (HETEs and epoxyeicosatrienoic acids [EETs]) or 323 (internal standard) and were isolated and monitored in the selective ion mass spectroscopy mode by using an Agilent LSD ion trap mass spectrometer (Agilent Technologies 1100, Boulder, Colo). The ratio of ion abundances in the peaks of interest (HETEs and EETs, m/z 319) versus that in the internal standard (20-HETE, m/z 323) was determined and compared with a standard curve generated with each set of samples for a range of 0.01–2 ng of 20-HETE.

Results

Representative angiograms illustrating the development of delayed vasospasm in the present study are presented in Fig 1, and the summary data are presented in Fig 2. The diameter of the cerebral arteries decreased to 85.2 $\pm$ 3.5%, 3 days after the first cisternal injection of blood. A more severe reduction in the diameter of the basilar artery was seen on day 7, 3 days after the second cisternal injection of blood.

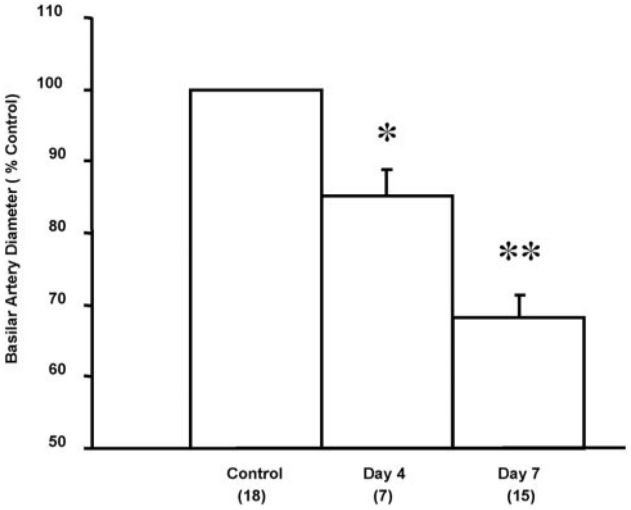

Fig 2. Graph shows the time course of changes in the diameter of the basilar artery after induction of the dual hemorrhage model of SAH in dogs. The numbers in parentheses indicate the number of dogs studied at each time point. The asterisk indicates a significant difference in the diameter of the basilar artery at day 4 (*) and day 7 (**) versus the control diameter measured before the induction of SAH.

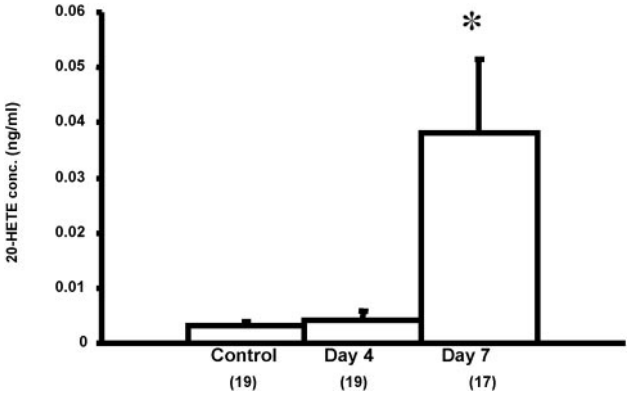

Fig 3. Graph shows the time course of changes in the concentration of 20-HETE in CSF following induction of the dual hemorrhage model of SAH in dogs. The numbers in parentheses indicate the number of dogs studied at each time point. The asterisk indicates a significant difference versus the corresponding control value measured before the induction of SAH.

The levels of 20-HETE in CSF increased from 4 ± 2 to 39 ± 16 pg/mL with the onset of delayed vasospasm, 3 days after the second injection of blood into the cisterna magna (Fig 3). The levels of 20-HETE in the CSF were significantly correlated with the magnitude of the reduction in the diameter of the basilar artery following SAH (Fig 4). In 9 dogs with delayed vasospasm, acute blockade of the synthesis of 20-HETE with TS-011 (1 mg/kg IV) significantly increased the diameter of the basilar artery by 39% (Fig 5). Chronic administration of TS-011 (1 mg/kg per day) attenuated the development of delayed vasospasm (Fig 6), and the diameter of the basilar artery fell by only 17 ± 1% in the TS-011 dogs ($n = 3$) versus the 33 ± 3% reduction in diameter seen in control dogs ($n = 15$), 3 days after the second injection of blood into the cisterna magna.

Discussion

The present study examined whether the development of delayed vasospasm in the dual hemorrhage model of SAH in dogs[7,8] is associated with elevations in 20-HETE levels in CSF

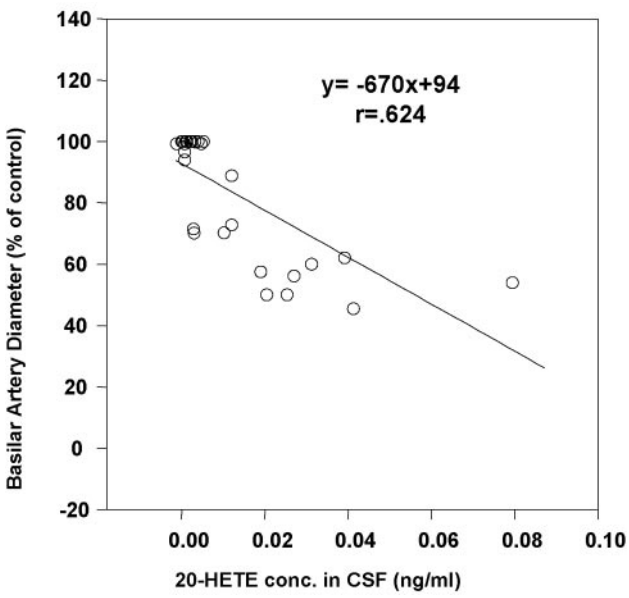

Fig 4. Graph shows the relationship between changes in the diameter of the basilar artery versus the concentration (conc.) of 20-HETE in CSF at various time points after induction of the dual hemorrhage model of SAH in dogs.

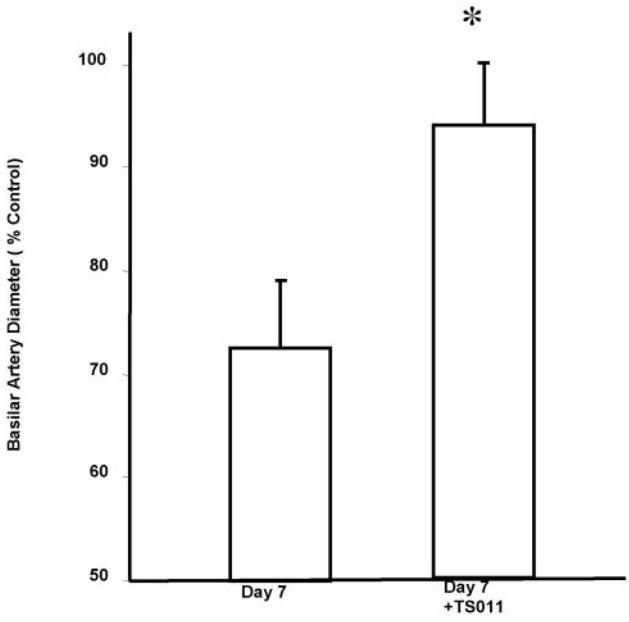

Fig 5. Graph shows the effects of acute administration of TS-011 (1 mg/kg IV) on the diameter of the basilar artery of dogs, with documented evidence of delayed vasospasm after induction of the dual hemorrhage model of SAH. Paired angiograms were obtained in 9 dogs before and 1 hour after administration of TS-011. The asterisk indicates a significant difference in the diameter of the basilar artery versus that of the corresponding control value measured before administration of TS-011.

and if blockade of the synthesis of 20-HETE with TS-011[6] can reverse delayed vasospasm in this model. The dogs in the present study consistently developed delayed vasospasm after induction of the dual hemorrhage model of SAH. On average, they exhibited a 30%–35% decrease in the diameter of the basilar artery, 3 days after a second injection of blood into the cisterna magna, and the development of vasospasm in these animals was associated with a parallel rise in the concentration of 20-HETE in CSF as measured by liquid chromatography/mass spectroscopy. There is a strong correlation between the

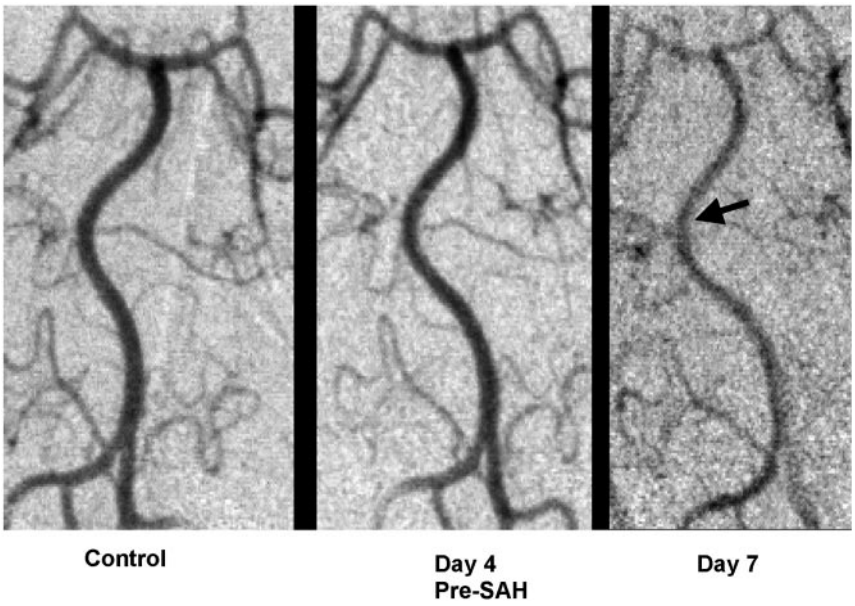

Fig 6. Representative sequential angiograms obtained in a dog after induction of the dual hemorrhage model of subarachnoid hemorrhage that was chronically treated with TS-011 (1 mg/kg twice a day) from initiation of SAH. The left panel presents the control angiogram before induction of SAH. The middle panel depicts the diameter of the basilar artery 3 days after the first cisternal injection of blood. The right panel depicts the appearance of the basilar artery 3 days after the second cisternal injection of blood. The arrow indicates the narrowest point in the basilar artery, which is showing vasospasm where sequential measurements were made.

increase in the levels of 20-HETE in CSF and the degree of delayed vasospasm that developed in individual animals. Additional evidence suggesting that the elevation in 20-HETE levels in CSF contributes to the delayed vasospasm was obtained by using TS-011, which is the most selective inhibitor of the synthesis of 20-HETE that is currently available.[5,6] TS-011 reversed delayed vasospasm in 9 dogs with documented angiographic evidence of delayed vasospasm, and the diameter of the basilar artery returned to a value not different from that of the controls 1 hour after intravenous bolus administration of this agent. In further studies, we also found that chronic treatment with TS-011 attenuated the development of delayed vasospasm in this model.

Previous studies have documented that the presence of a blood clot alone in the subarachnoid space is sufficient to induce vasospasm.[9,10] The presence of clotting blood in the subarachnoid space triggers the acute fall in CBF and vasospasm after SAH,[4,5] and this is associated with the release of vasoconstrictor mediators such as endothelin, angiotensin II, serotonin, and vasopressin and a reduction of NO, which is scavenged by free hemoglobin. Recent studies have indicated that the concentration of 20-HETE in the CSF increases in 2 hours after induction of SAH in rats[11-13] and that blockade of the formation of 20-HETE could prevent and even reverse the acute fall in blood flow seen in this model.[4] These findings are consistent with the results of the present study indicating that the onset of delayed vasospasm in dogs following induction of the dual hemorrhage model of SAH is also associated with a significant increase in 20-HETE in CSF and can be reversed by administration of an inhibitor of the synthesis of 20-HETE.

The cell types in the brain responsible for the increased levels of 20-HETE in CSF following SAH remain to be determined. Previous studies have indicated that the formation of 20-HETE by polymorphonuclear leukocytes and vascular smooth muscle cells is stimulated by a number of factors released by clotting blood, including endothelin, angiotensin II and vasopressin.[4,5,15] There is also an increased turnover of fatty acids, including arachidonic acid, which is the substrate for the production of 20-HETE; and elevated concentrations of thromboxane and other vasoconstrictor metabolites of arachidonic acid have been reported in the CSF after SAH.[1,4]

20-HETE is a potent constrictor of cerebral arteries that depolarizes vascular smooth muscle cells through inhibition of K^+ channel activity. The results of the present study demonstrate that experimental inhibition of 20-HETE increases the diameter of cerebral arteries after the induction of delayed vasospasm in dogs. There is also convincing experimental evidence that other inhibitors of the synthesis of 20-HETE, like the enzyme inhibitors 17-octadecanoic and HET0016[4,16] or other putative antagonists of the vasoconstrictor actions of 20-HETE such as WIT003 and ABSA,[5] prevent the acute fall in cerebral blood flow and vasospasm following SAH in rats. Interestingly, we found that inhibition of the formation of 20-HETE with TS-011 resulted in vascular diameter improvement regardless of the timing of intravenous administration. Another study using CBF measurements with 20-HETE inhibition in rats after SAH also demonstrated that pretreatment or later therapeutic administration of 20-HETE inhibitors had a beneficial effect in preventing the acute fall of CBF in rats.[4] These results suggest that the development of delayed vasospasm may be associated with upregulation of the CYP4A enzymes responsible for the formation of 20-HETE in cerebral arteries and the brain.[17-24]

The clinical significance of vasospasm translates into significant morbidity, with reported 30-day mortality rates that range between 32% and 67% for cerebral aneurysm rupture–related vasospasm.[25] Vasospasm may also result from trauma and other causes, and it is well appreciated that a large proportion of patients die within the first 2 days of their initial hemorrhage, many before they seek medical attention or can receive effective treatment.[26-28] A large proportion of early deaths following SAH are related to an acute fall in CBF and early vasospasm because significant ischemic injuries have been noted in patients who die within the first 24 hours of SAH from cerebral aneurysm rupture.[29] Effective pharmacologic inhibition of cerebral vasospasm would have a significant positive impact in cerebrovascular medicine.

Conclusion

In this study, acute inhibition of 20-HETE with TS-011 increased the diameter of the basilar artery in dogs with angiographically demonstrated delayed cerebral vasospasm. Chronic administration of an inhibitor of the synthesis of 20-HETE, initiated at the onset of hemorrhage, attenuated the

development of delayed vasospasm in the dual hemorrhage model of delayed vasospasm in dogs. These results suggest that inhibitors of the synthesis of vasoconstrictor actions of 20-HETE hold promise in the treatment of neurovascular damage related to SAH.

Acknowledgment

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