Reversal of Delayed Vasospasm by an Inhibitor of the Synthesis of 20-HETE

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Running head: Role of 20HETE in delayed vasospasm in SAH

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ABSTRACT

This study characterized the time course of changes in cerebral blood flow (CBF) and vascular diameter in a dual hemorrhage model of subarachnoid hemorrhage (SAH) in rats and examined whether acute blockade of the synthesis of 20-hydroxyeicosatetraenoic acid (20-HETE) with TS-011, N-(3-Chloro-4-morpholin-4-yl) phenyl-N'-hydroxyimido formamide, can reverse delayed vasospasm in this model. Rats received an intracisternal (icv) injection of blood (0.4 ml) on day 0 and a second injection 2 days later. CBF was sequentially measured using laser-Doppler flowmetry and the diameters of the cerebral arteries were determined after filling the cerebral vasculature with a casting compound. CBF fell to 67% of control after the first icv injection of blood but returned to a value near control 24 hrs later. CBF again fell to 63% of control following a second icv injection of blood and remained 30% below control for 5 days. The fall in CBF following the second icv injection of blood was associated with a sustained 30% reduction in the diameters of the middle cerebral, posterior communicating and basilar arteries. Acute blockade of the synthesis of 20-HETE with TS-011 (0.1 mg/kg, i.v.), 5 days following the second SAH, increased the diameters of the cerebral arteries and CBF returned to control. These results indicate that the rats develop delayed vasospasm following induction of the dual hemorrhage model of SAH and that blockade of the synthesis of 20-HETE fully reverses cerebral vasospasm in this model. They also implicate 20-HETE in the development and maintenance of delayed cerebral vasospasm.

KEY WORDS: subarachnoid hemorrhage; delayed vasospasm; 20-HETE; cerebral injury
INTRODUCTION

The incidence of subarachnoid hemorrhage (SAH) in the US is 11 per 100,000 people per year. Despite improvements in the diagnosis and the surgical repair of ruptured aneurysms, the 30 day mortality rates for SAH and intraventricular hemorrhage still hover around 50% (range 32 - 67%) (4, 17). The majority of deaths (>60%) occur within the first 2 days and are associated with acute reductions in cerebral blood flow (CBF) and extensive ischemic injury to the brain (4, 6, 53). Previous studies have documented that there are biphasic changes in CBF following SAH in both man (30, 54) and experimental animals (29, 52). The acute phase of cerebral vasospasm lasts several hrs but CBF returns to control within a day. Over the next 4 - 7 days, about half the patients develop delayed cerebral vasospasm. One third of these patients die and one third suffer some sort of permanent neurological damage (6, 10, 17).

The mechanisms of delayed vasospasm remain to be established. Previous studies have indicated that delayed vasospasm is associated with activation of the protein kinase C (PKC) (22, 23, 55) and Rho/Rho-kinase (23, 55), diminished K⁺ channel activity (1, 46) and depolarization of vascular smooth muscle cells (15). The responses of cerebral arteries to endothelin, serotonin and other vasoconstrictors are elevated and there is a diminished response to nitric oxide (NO) (14, 15, 47, 48). The levels of endothelin (6, 44), thromboxane (7, 38), ATP (26, 58), isoprostane (43), glutamate (3), platelet-activating factor (PAF) (16) and serotonin (5-HT) (5, 42) in cerebrospinal fluid (CSF) increase following SAH and the development of cerebral vasospasm.
can be attenuated by blocking the synthesis of endothelin or by using Ras (57), Rho/Rho-kinase (23, 37), mitogen-activated protein kinase (MAPK) (20) and PKC (23, 37) inhibitors.

Recent studies have drawn attention to the role of 20-hydroxyeicosatetraenoic acid (20-HETE) in the development of cerebral vasospasm. 20-HETE is a potent vasoconstrictor that is produced by the metabolism of arachidonic acid by cytochrome P4504A (CYP) enzymes in cerebral arteries (11, 12, 25). The vasoconstrictor response to 20-HETE mimics the changes in cerebral vascular tone associated with cerebral vasospasm. 20-HETE activates PKC (24, 36), Ras, tyrosine kinase, MAPK and Rho/Rho-kinase pathways (32-35, 40, 50). It promotes calcium entry by depolarizing (25) cerebral arteries secondary to blockade of the large conductance $K_{Ca}$ channel (24, 49). 20-HETE also increases $Ca^{2+}$ influx by activating L-type $Ca^{2+}$ channels in the cerebral vasculature (12). The concentration of 20-HETE in CSF increases markedly following SAH, and inhibitors of the synthesis (5, 18) or actions (59) of 20-HETE prevent the acute fall in CBF following SAH in rats. However, the role of 20-HETE in the development of delayed vasospasm remains to be explored.

Delayed vasospasm has typically been studied in dogs or monkeys using a dual hemorrhage model of SAH (29). These models faithfully reproduce the time course of the changes in the diameter of cerebral arteries following SAH in man, however, CBF has not been well characterized and dogs and monkeys do not develop neurological deficits (29). The single injection model of SAH has been widely used for the study of acute vasospasm in rats. However, since CBF returns to control within 24 hrs many investigators have concluded that rats are not a
suitable model system for the study of delayed vasospasm (9, 29). However, this perception is changing since more recent studies have suggested that a sustained reduction in the diameter of cerebral arteries can be elicited by following second icv injection of blood in rats (28, 51, 56). Thus, the purpose of the present study is to characterize the time course of changes in CBF and the diameter of cerebral arteries using a dual hemorrhage model of SAH in rats to confirm that they develop delayed vasospasm and to determine whether acute blockade of the synthesis of 20-HETE with a selective inhibitor of the synthesis of 20-HETE, N-(3-Chloro-4-morpholin-4-yl) phenyl-N'-hydroxyimido formamide (TS-011) (31) reverses the delayed vasospasm in this model.

METHODS

Experiments were performed on 83 male Sprague-Dawley rats weighing 300-400 g. The rats were housed in an AAALAC accredited animal care facility at the Medical College of Wisconsin and they had free access to food and water throughout the study. 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 of the American Physiological Society.

Surgical preparation for chronic monitoring of cerebral blood flow. The rats were anesthetized with 2% isoflurane (Abbott, Abbott Park, IL) and placed in a stereotaxic apparatus (Stoelting, Wood Dale, IL). A 3X3 mm area of the left and right parietal bones overlying the
irrigation area of the middle cerebral artery (MCA), 2 mm posterior and 6 mm lateral to the Bregma, were thinned with a hand-held drill until the superficial pial vessels were visible. A 10 mm length of polyethylene tubing with the ends heat flared was affixed over the cranial windows with Vetbond adhesive (3M Corp, Minneapolis, MN) to serve as positioning guide for the laser-Doppler flowmeter probes. The guides were further fixed to the skull with dental acrylic cement and the scalp incisions were closed around the probe guides with 4-0 silk suture. After surgery, the rats received enrofloxacin (10 mg/kg i.m., Bayer, Pittsburg, PA) and buprenorphine (0.1 mg/kg s.c., Reckitt Benckiser, Richmond, VA) to prevent infection and relieve pain. The rats were given five days to recover from surgery before CBF was measured. This recovery period was necessary since baseline CBF flow was elevated for several days following chronic cranial window surgery due to local inflammation.

Protocol 1: Characterization of the time course of changes in CBF in the dual hemorrhage model of SAH in rats. These experiments were performed using rats prepared for chronic monitoring of CBF as described above. After a 5 day recovery period, rats were anesthetized with isoflurane (2.0%). A microrenathane catheter was chronically implanted in the femoral artery for collection of blood and measurement of blood pressure. The rat was positioned in a stereotaxic apparatus. Body temperature was maintained at 37° C with a heating pad. A small skin incision was made at the base of the skull to expose the atlanto-occipital membrane, and a 27-gauge needle attached to a microrenathane catheter was inserted into the cisterna magna for withdrawal of cerebrospinal fluid (CSF) and injection of arterial blood or saline. Baseline
CBF was monitored over the left and right hemispheres using a dual channel laser-Doppler flowmeter (Perimed model 5000, Stockholm, Sweden) during a 30-min control period. Then 0.2 ml of cerebrospinal fluid was withdrawn from the cisterna magna, and 0.4 ml of arterial blood (n=15) or saline (n=6) was slowly infused into the cisterna magna at a rate of 40 µl/min for 10 min. CSF was withdrawn and the blood was infused rather than given as a bolus injection to avoid a large spike in intracranial pressure. This modified procedure allowed us to introduce a very large blood clot into the subarachnoid space, which resulted in a more consistent vasospasm. After the injection, the needle in the cisterna magna was removed, the skin incision was closed and the rat was tilted in a 20° head down position for 30 min. The mean value of CBF 30 min after the injection of blood or saline into the cisterna magna was recorded as the value of CBF after acute SAH. After measuring CBF, the femoral artery catheter was filled with heparinized saline (500 units/ml), tucked under the skin, and the skin incision was closed. The rats were given enrofloxacin (10 mg/kg, i.m.) and buprenorphine (0.1 mg/kg, s.c.) to prevent infection and pain. Two days later, the rats were reanesthetised with isoflurane (2.0%) and the procedure was repeated. The rats were also anesthetized 1, 3 and 5 days after the second intracisternal (icv) injection and CBF remeasured. CBF was expressed as a percentage of the control value measured on day 0.

Protocol 2: Measurement of cerebral vascular diameters at various times following the dual hemorrhage model of SAH in rats. These experiments were performed in 5 groups of rats surgically prepared for induction of the dual hemorrhage model of SAH. At various times after
the induction of SAH, the cerebral circulation was perfusion fixed and filled with a silicone rubber compound for measurement of vascular diameters. Group 1-control (n=6): the cerebral circulation was filled prior to the induction of SAH on day 0. Group 2-acute SAH (n=6): the cerebral circulation was filled 30 min following the induction of acute SAH on day 0. Group 3-delayed vasospasm, day 3 (n=6): the cerebral circulation was filled one day following the second icv injection of blood. Group 4-delayed vasospasm, day 7 (n=6): the cerebral circulation was filled 5 days following the second icv injection of blood. Group 5-vehicle control, day 7 (n=6): these rats received icv injection of saline on days 0 and 2 and the cerebral circulation was filled 5 days following the second icv injection.

At the appropriate times following the induction of SAH, the rats were anesthetized with isoflurane (2.0%), and the right and left carotid arteries were cannulated with polyethylene tubing (PE-50). The cerebral circulation was flushed with 30 ml of a heparinized (20 units/ml) physiological saline solution (PSS) containing (in mM) 119.0 NaCl, 4.7 KCl, 1.6 CaCl2, 1.17 MgSO4, 1.18 NaH2PO4, 12.0 NaHCO3, 0.03 EDTA, 10.0 glucose and 10.0 HEPES (pH 7.4) that was perfused via the carotid arteries at pressure of 110 mmHg followed by perfusion fixation with another 30 ml of PSS containing 4% of paraformaldehyde. After fixation, the cerebral vessels were filled at 110 mmHg with 12 ml of a silicone rubber casting material (Microfil MV-122, FlowTek, Bounder, CO) that was diluted 1:4 with the diluent supplied by the manufacturer. The casting material was allowed to cure for 4 hrs. The brain was then removed and placed in cold PSS. The diameter of the filled cerebral arteries was measured using a video
system composed of stereomicroscope (Carl Zeiss, Germany), a video camera (COHU-4815, COHU Electronics, Poway, CA) and a video measuring system (VIA-100, Boeckeler Instrument, AZ) to prevent shrinkage. The diameter of the basilar artery (BA) was measured 400 µm above the junction of the vertebral arteries, just below the origin of the anterior inferior cerebellar arteries, and 400 µm below the origin of the posterior cerebral arteries. The diameters of right and left middle cerebral arteries (MCAs) and posterior communicating arteries (PCAs) were measured 400 µm distal to the PCA–MCA bifurcation and 400 µm proximal to the PCA–MCA bifurcation. The minimum diameter measured for each of these arteries was recorded.

Protocol 3: Effect of TS-011 on the delayed vasospasm in the dual hemorrhage model of SAH in rats. These experiments examined the ability of an inhibitor of the synthesis of 20-HETE, TS-011, \((N-(3-Chloro-4-morpholin-4-yl) phenyl-N\'-hydroxyimido formamide)\) to reverse delayed cerebral vasospasm in rats. The rats were surgically prepared for chronic measurement of CBF and induction of the dual hemorrhage model of SAH. CBF was measured on day 0 and 7, 5 days following the second icv injection of blood. After measuring basal CBF and MAP on day 7, the rats received a bolus i.v. injection of vehicle (11% of sulfobutylether β-cyclodextrin in 300 mM mannitol, n=6) or TS-011 (0.1 mg/kg, n=6) and the CBF and mean arterial pressure (MAP) was followed for additional 3 hrs. At the end of each experiment, the cerebral circulation was perfusion fixed with 4% of paraformaldehyde and filled with a silicone rubber casting material to allow for the measurement of the diameter of the cerebral arteries.
**Protocol 4: Effects of TS-011 on formation of 20-HETE in cerebral arteries in vitro and in the brain in vivo.** Experiments were performed to confirm the effectiveness of TS-011 as an inhibitor of the synthesis of 20-HETE in cerebral arteries. Middle cerebral and basilar arteries were microdissected from the brains of four rats and divided into two samples. These samples were incubated for 1 hr at 37°C in 1 ml of a 10 mM potassium phosphate buffer containing 40 µM AA, 1 mM NADPH and 100 nM TS-011 (n=3) or vehicle (n=3). The reactions were stopped by acidification with 1M formic acid, extracted with ethyl acetate and dried. The reactions were resuspended in 50% methanol and water and the products were separated and measured using LC/MS on an Agilient 1100 ion trap mass spectrometer as previously described (8).

Additional experiments were performed to determine whether TS-011 effectively inhibits the synthesis of 20-HETE in the brain following in vivo administration. Rats were anesthetized with isoflurane and given i.v. injection of TS-011 (0.1 mg/kg, n=4) or vehicle (n=4). Ninety minutes later, the brains of these animals were collected and homogenized in 2 ml of a 10 mM potassium buffer (pH 7.7) containing (in mM) 250 sucrose, 1 EDTA, and 0.1 phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 3,000 g for 15 min, and the supernatant was centrifuged at 11,000 g for 15 min followed by 100,000 g for 1 hr. The microsomal pellets were resuspended in 100 mM potassium buffer (pH 7.25) containing 30% glycerol, 1 mM dithiothreitol, and 0.1 mM PMSF. The microsomes (0.5 mg protein) were incubated for 60 min at 37°C in 1 ml of a 0.1M potassium phosphate buffer containing 40 µM arachidonic acid (AA), 1 mM NADPH, 10 mM sodium isocitrate and 0.16 unites/ml isocitrate.
dehydrogenase. The samples were extracted with ethyl acetate and the production of 20-HETE determined by LC/MS as previously described (8).

**Statistical Analysis.** Mean values ± S.E. are presented. CBF was expressed as a percentage of the baseline value measured on day 0, prior to the icv injection of blood or saline. The significance of changes in CBF within groups was evaluated using an ANOVA for repeated measures followed by Dunnett’s test. The significance of differences in the diameter of cerebral arteries between groups was evaluated using an ANOVA followed by a Duncan’s multiple-range test or an unpaired t test. A $P < 0.05$ was considered significant.

**RESULTS**

**Protocol 1: Time course of changes in CBF following the dual hemorrhage model of SAH in rats.** The results of these experiments are presented in Fig. 1. CBF was not significantly altered during the experiment in control rats that received icv injections of saline alone. In contrast, CBF fell to $67.2 ± 3.1$ % of control, 30 min after the injection of blood into the cisterna magna. CBF fully recovered to a value near control 24 hrs later. CBF again fell significantly to $63.8 ± 2.8$ % of control, 30 min following the second icv injection of blood. Thereafter, CBF remained 30 % below control when measured 1, 3 or 5 days later.

**Protocol 2: Time course of changes in the diameter of cerebral arteries following the dual hemorrhage model of SAH in rats.** The typical appearances of the cerebral circulation following acute SAH, prior to the second injection of blood on day 2, and 5 days following the
second hemorrhage is presented in Figs. 2 and 3. Blood was found to surround the MCA, PCA and BA, 30 min following icv injection of blood on day 0 or 2 (Fig. 2B) and the diameter of the BA, MCA and PCA exhibited obvious vasospasm (Fig. 2B and 3C and D). However, the injected blood was nearly cleared, 24 hrs following the first injection of blood (Fig. 2C) and the diameter of these vessels returned toward control. The blood was completely cleared within one day following the second icv injection of blood (Fig. 2D), but the diameter of the MCA, PCA and BA remained constricted for at least 5 days (Fig. 3E and 3F). A summary of the vessel diameter data is presented in Fig. 4. Baseline diameters of the MCA, PCA and BA in control rats averaged 201.0 ± 7.3, 238.1 ± 9.0 and 225.5 ± 8.7 µm, respectively. The diameters of MCA, PCA and BA fell to 73, 66 and 78 % of control, 30 min following the first icv injection of blood. The diameters of MCA, PCA and BA measured on day 3 and 7, 1 and 5 days following the second icv injection of blood remained significantly lower than control (Fig. 4).

Protocol 3: Effects of TS-011 on delayed vasospasm in the dual hemorrhage model of SAH in rats. The effects of inhibition of the synthesis of 20-HETE with TS-011 on CBF in the dual hemorrhage model are presented in Fig. 5. Administration of TS-011 fully reversed the fall in CBF in rats with delayed vasospasm on day 7. CBF recovered to control within 120 min after a bolus i.v. injection of TS-011 (Fig. 5A). TS-011 had no effect on MAP in these animals (Fig. 5B).

We also examined the effects of TS-011 on the diameter of cerebral arteries. A representative appearance of the cerebral circulation following administration of vehicle or TS-
011 in rats with delayed vasospasm is presented in Fig. 6A and a summary of the diameter data is presented in Fig 6B. The diameter of the MCA, PCA and BA on day 7 returned to values not different from control after administration of TS-011 to rats subjected to the dual hemorrhage model of SAH.

Protocol 4: Effects of TS-011 on the formation of 20-HETE by cerebral arteries in vitro and in the brain in vivo. The effects of TS-011 on the metabolism of AA by isolated MCA are presented in Fig 7. MCA incubated with AA in vitro produced peaks detected by LC/MS with m/z of 319 that co-elute with 20-HETE, 15-, 12- and 5-HETE and 14, 15- EET. Addition of TS-011 (100 nM) to the incubations reduced the formation of 20-HETE by 80% (n=3) without affecting the formation of 15-HETE or 12-HETE or EETs.

We also verified that the dose of TS-011 (0.1 mg/kg, i.v.) used in these studies was sufficient to inhibit the formation of 20-HETE in vivo (Fig. 8). The baseline production of 20-HETE by microsomes prepared from the brains of rats treated with vehicle averaged 6.0 ± 3.1 pmol/min/mg protein. The production of 20-HETE was significantly reduced by 91% (n = 4) in rats treated with TS-011, while the formation of other products of AA, 12-HETE, 11, 12- and 14, 15-EET and 11, 12-DiHETE was not significantly altered.

**DISCUSSION**

The present study characterized the time course of changes in vascular diameter and CBF in a modified dual hemorrhage model of SAH in rats and examined the contribution of 20-HETE
in the development of delayed vasospasm in this model using a selective inhibitor of the synthesis of 20-HETE, TS-011. We found that that acute blockade of the synthesis of 20-HETE reversed vasospasm in this model, thereby suggesting that 20-HETE plays a critical role in the development and maintenance of delayed cerebral vasospasm.

Previous studies have documented that there are biphasic changes in the diameter of cerebral arteries in man following SAH (30, 54). The acute phase lasts several hours. However, over the next 4 - 7 days, half the patients develop delayed vasospasm that is refractory to treatment with vasodilators or calcium channel blockers (52, 54). Delayed vasospasm has typically been studied angiographically in dogs or monkeys using a dual hemorrhage model of SAH (29). The diameter of the basilar artery of dogs acutely falls by 30% following the injection of blood into the cisterna magna (21, 52). It then returns to control within 24 - 48 hrs, but a delayed vasospasm develops following a second icv injection of blood. While the dual hemorrhage model of SAH in dogs and monkeys reproduces the time course of the changes in the diameter of cerebral arteries following SAH in man, CBF has not been well characterized in these models, and dogs and monkeys do not develop neurological deficits (29). In addition, studies performed in these large animal models are very expensive and this limits the amount of mechanistic work that can be done. The development of a small animal model of delayed vasospasm could offer many advantages. Indeed, many investigators have switched to rats to study the acute fall in CBF following SAH that is associated with a constriction of the MCAs, PCAs and BA (3, 9, 13, 19). However, CBF and the diameters of the cerebral arteries return to
control within 48 hrs in rats following SAH. This observation has lead most investigators to conclude that rats are not a suitable model for the study of delayed vasospasm (9, 29). However, more recent studies have suggested that the diameter of cerebral arteries is reduced for several days after a second icv injection of blood in rats as is seen in larger animal models (28, 51, 56). These observations led us to characterize the changes in CBF and vascular diameter in rats subjected to a dual hemorrhage model of SAH to see if rats develop delayed vasospasm.

The present results confirm previous findings that CBF acutely falls following SAH in rats and this is associated with a reduction of 30 – 40% in the diameter of MCA, PCA and BA. We also confirmed that the blood is rapidly cleared from CSF following SAH and CBF returns to values with 90% of control 24 hrs later. However, after a second icv injection of blood, there is a 30% fall in CBF along with sustained constriction of MCA, PCA and BA for 5 days. Most of the rats in the present study also exhibited neurological deficits such as weakness in the front paws. Overall, the present findings indicate that rats subjected to dual hemorrhage model of SAH develop biphasic changes in CBF and the diameter of the cerebral arteries that follow the same time course (peaks on days 5 - 7) and is of the same magnitude (30 - 40%) as that seen in man or in the dual hemorrhage models of SAH in dogs or monkeys (29, 30, 52-54, 57).

**Role of 20-HETE in delayed vasospasm in rats.** Previous studies have indicated that 20-HETE plays an important role in the development of acute vasospasm following SAH in rats (5, 18). A more recent study has indicated that the levels of 20-HETE cerebrospinal fluid (CSF) also increase in patients with SAH (39). However, the role of 20-HETE in the development of
delayed vasospasm is unknown. To determine if 20-HETE contributes to the increase in cerebral vascular tone in delayed vasospasm, we studied the effects of TS-011 a new and very selective inhibitor of the synthesis of 20-HETE (31) on CBF and cerebral vascular diameter in the dual hemorrhage model of SAH in rats. We found that TS-011 fully returned CBF to control without affecting mean arterial pressure and this was associated with an increase in the diameter of the MCA, PCA and BA. In further studies, cerebral arteries microdissected from the brains of rats synthesize 20-HETE when incubated with AA in vitro and that TS-011 inhibits the formation of this substance. We also found that microsomes prepared from the brains of rats synthesize 20-HETE and that pretreatment of rats in vivo with TS-011 (0.1 mg/kg) selectively inhibited the synthesis of 20-HETE. These observations suggest that upregulation of the synthesis of 20-HETE may contribute to the development of delayed vasospasm. A role for 20-HETE in the development of delayed vasospasm is consistent with previous observations that inhibitors of Ras, Rho/Rho kinase, MAPK or PKC attenuate the development of delayed cerebral vasospasm (20, 23, 37, 57) since 20-HETE promotes depolarization and contraction of cerebral arteries by activating these same second messenger pathways (2, 24, 50, 59).

The finding that blockade of the synthesis of 20-HETE with TS-011 fully reversed the fall in CBF and reduction in the diameter of the cerebral arteries in rats with delayed vasospasm does not preclude an important role for other mediators in response. Indeed, previous investigators have shown that the levels of endothelin (6, 44), thromboxane (7, 38), ATP (26, 58), isoprostane (43), glutamate (3), platelet-activating factor (PAF) (16) and serotonin (5-HT)
(5, 42) all increase in cerebrospinal fluid (CSF) following SAH and the degree of cerebral vasospasm can be attenuated by blocking the synthesis or actions of most of these mediators. Others have reported that elevations in superoxide radicals (19) and a fall in the bioavailability of NO (48) contribute to the development of cerebral vasospasm. The most likely explanation is that many of the vasoactive mediators released by clotting blood likely trigger the development of vasospasm, but that many of these pathways converge on a common pathway leading to elevated production of 20-HETE in cerebral arteries which potentiates the vasoconstrictor actions of these compounds by depolarizing vascular smooth muscle by blocking the K$_{Ca}$ channel. The results of previous studies indicating that 20-HETE contributes to the vasoconstrictor responses to endothelin, AII, serotonin, vasopressin, norepinephrine, inhibition of the synthesis of NO and ATP support this possibility (2, 5, 41, 59).

The mechanism responsible for the upregulation of the formation and or actions of 20-HETE following SAH remain to be determined. The expression of inducible nitric oxide synthase (iNOS) is elevated in cerebral arteries after SAH (56). Moreover, heme oxygenase-1 (HO-1), which metabolizes the heme to iron, biliverdin and carbon monoxide (CO), is also induced in the brain following SAH (27). Both NO and CO avidly bind to heme in CYP enzymes and inhibit the formation of 20-HETE (49). Indeed, induction of the formation of NO and CO may contribute to the rapid recovery of CBF following SAH by activating cGMP-dependent vasodilator pathways and by inhibiting of the formation of the vasoconstrictor, 20-HETE. Besides blocking the formation of 20-HETE, NO is known to upregulate the expression of
CYP4A enzymes (45). CO might have a similar effect. Upregulation of the expression CYP4A enzymes and the local formation of 20-HETE in cerebral arteries following SAH might contribute to the development of delayed vasospasm after the clotted blood is cleared from the CSF and the levels of NO and CO return to control.

In summary, the present indicate that rats subjected to the dual hemorrhage model of SAH exhibit biphasic changes in CBF and the diameter of cerebral arteries that closely mimic the time course and magnitude of the response previously reported in man (30, 54), and the dual hemorrhage dog and monkey models of SAH (29, 52). Acute blockade of the synthesis of 20-HETE fully reversed cerebral vasospasm in this model. These results suggest that 20-HETE plays a critical role in the development and maintenance of delayed cerebral vasospasm.
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**FIGURE LEGENDS**

**Fig. 1.** Time course of changes in cerebral blood flow (CBF) in the dual hemorrhage model of SAH in rats. 0.4 ml of blood (closed circles, n=15) or saline (open triangles, n=6) was injected into the cisterna magna on days 0 and 2. Values are means ± S.E. * $P < 0.05$ versus the corresponding value in rats treated with saline; † $P < 0.05$ versus the corresponding control value on day 0.

**Fig. 2.** Appearance of the cerebral circulation at various times following the dual hemorrhage model of SAH in rats. Panel A: control. Panel B: acute SAH, 30 min following the first intracisternal (icv) injection of blood. Panel C: one day following the first icv injection of blood. Panel D: one day following the second icv injection of blood.

**Fig. 3.** Representative appearance of the middle cerebral artery (MCA), posterior communicating artery (PCA) and basilar artery (BA) in the dual hemorrhage model in rats. Panels A and B: control. Panels C and D: acute vasospasm, 30 min following the first intracisternal (icv) injection of blood on day 0. Panels E and F: delayed vasospasm, 5 days following the second icv injection of blood in the dual hemorrhage of SAH in rats.
**Fig. 4.** Time course of changes in the diameter of the middle cerebral artery (MCA), posterior communicating artery (PCA) and basilar artery (BA) in the dual hemorrhage model of SAH in rats. 0.4 ml of blood (closed circles, n=6) or saline (open triangles, n=6) was injected into the cisterna magna on days 0 and 2. Values are means ± S.E. * $P < 0.05$ versus corresponding control value; † $P < 0.05$ versus the corresponding value in rats treated with saline.

**Fig. 5.** Effects of TS-011 on cerebral blood flow (CBF) in the dual hemorrhage model of SAH in rats. Panel A presents the time course of changes in CBF after bolus i.v. injection of TS-011 (closed circles, n=6) or vehicle (open circles, n=6) on day 7, five days following the second injection of blood into the cisterna magna of rats. Panel B presents the time course of changes in mean arterial pressure (MAP) in these same animals. Values are means ± S.E. * $P < 0.05$ versus the corresponding value in vehicle-treated rats.

**Fig. 6.** Effects of TS-011 on the diameter of cerebral arteries in the dual hemorrhage model of SAH in rats. Panel A presents the appearance of the MCA and PCA after bolus intravenous injection of TS-011 or vehicle on day 7, five days following the second injection of blood into the cisterna magna of rats. Panel B presents the summary data on the diameter of MCA and PCA after administration of TS-011 (n=6) or vehicle (n=6) on day 7, five days following the second injection of blood into the cisterna magna of rats. The control diameter data (control, n=6) and the data from the SAH treated group on day 7 (SAH(D7), n=6) was replotted from the data
presented in Fig. 4 to facilitate comparisons. Values are means ± S.E. * $P < 0.05$ versus the corresponding control value. † $P < 0.05$ versus the corresponding value in vehicle-treated rats.

**Fig. 7.** Effects of TS-011 on the production of 20-HETE by rat middle cerebral and basilar arteries *in vitro.* The figure presents a representative LC/MS chromatogram illustrating the metabolism of arachidonic acid by middle cerebral and basilar arteries of rats incubated in the presence and absence of TS-011 (100 nM). TS-011 inhibited the formation of 20-HETE (m/z of 319) that elutes with a retention time of 16 min but it did not reduce the synthesis of 15-, 12- or 5-HETE or 14, 15-EET.

**Fig. 8.** Panel A shows a representative LC/MS chromatogram illustrating the metabolism of arachidonic acid by microsomes of prepared from the brains of rats treated with TS-011 (0.1 mg/kg, *i.v.*) or vehicle *in vivo.* TS-011 inhibited the formation of 20-HETE (m/z of 319) that elutes with a retention time of 16 min but it did not reduce the synthesis of 15-, 12- or 5-HETE EETs or DiHETEs. Panel B compares the production of 20-HETE and EETs by microsomes prepared from brain of rats pretreated with TS011 (0.1 mg/kg, *i.v.*) or vehicle. Mean values ± SE from 4 rats per group are presented. * indicates a significant difference from the corresponding control value.
Figure 1
Figure 3
Figure 4
Figure 5

A  

- TS011 (n=6)
- Vehicle (n=6)

CBF (% of control)

Time (min)

(B)  

MAP (mmHg)

Time (min)
Figure 6
Figure 7
**Figure 8**

A  

- **Signal Intensity**  
- **Time (min)**  
- **20-HETE**  
- **19-HETE**  
- **12-HETE**  
- **11,12-EET**  
- **14,15-EET**  
- **11,12-DiHETE**  
- **15-HETE**  
- **14,15-EET**  
- **11,12-EET**  
- **15-HETE**  
- **20-HETE**  
- **19-HETE**  
- **Internal Standard**  

B  

- **Vehicle (n = 4)**  
- **TS-011 (0.1 mg/kg, iv)**

**HETEs, EETs & DiHETE Production (% of control)**

- **20-HETE**  
- **12-HETE**  
- **11,12-EET**  
- **14,15-EET**  
- **11,12-DiHETE**