Contribution of 5-Hydroxytryptamine<sub>1B</sub> Receptors and 20-Hydroxyeicosatetraenoic Acid to Fall in Cerebral Blood Flow After Subarachnoid Hemorrhage

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Background and Purpose—This study examined the interaction between 5-hydroxytryptamine<sub>1B</sub> (5-HT<sub>1B</sub>) receptors and 20-hydroxyeicosatetraenoic acid (20-HETE) in contributing to the acute fall in regional cerebral blood flow (rCBF) after subarachnoid hemorrhage (SAH) in rats.

Methods—The effects of intracisternal injection of 0.3 mL of arterial blood, artificial cerebrospinal fluid, and 5-HT on rCBF and the levels of 20-HETE and 5-HT in cerebrospinal fluid were measured in rats pretreated with vehicle, a 5-HT<sub>1B</sub> receptor antagonist (isamoltane hemifumarate), or an inhibitor of the synthesis of 20-HETE (HET0016). The effects of HET0016 and isamoltane on the vasoconstrictor response and changes in [Ca<sup>2+</sup>] to 5-HT were also studied in middle cerebral arteries and vascular smooth muscle cells isolated from these vessels.

Results—20-HETE and 5-HT levels in cerebrospinal fluid rose from 172±10 to 629±44 ng/mL and from 6±4 to 1163±200 nmol/mL, respectively, after SAH. rCBF fell by 30% 10 minutes after SAH, and it remained at this level for the next 2 hours. Blockade of 5-HT<sub>1B</sub> receptors prevented the sustained fall in rCBF seen after SAH. Intracisternal injection of 5-HT mimicked SAH by increasing 20-HETE levels in cerebrospinal fluid to 475±94 ng/mL and reducing rCBF by 30%. Blockade of the synthesis of 20-HETE with HET0016 prevented the fall in rCBF produced by 5-HT. Isamoltane and HET0016 reduced the vasoconstrictor response of isolated MCA to 5-HT by >60% and diminished the rise in [Ca<sup>2+</sup>], produced by 5-HT in vascular smooth muscle cells isolated from these arteries.

Conclusions—These results suggest that the release of 5-HT after SAH activates 5-HT<sub>1B</sub> receptors and the synthesis of 20-HETE and that 20-HETE contributes to the acute fall in rCBF by potentiating the vasoconstrictor response of cerebral vessels to 5-HT. (Stroke. 2003;34:1269-1275.)

Key Words: arachidonic acids • cerebral blood flow • head injury • stroke • rats

Subarachnoid hemorrhage (SAH) has a high mortality rate of 32% to 67%,<sup>1</sup> and 61% of the deaths occur within the first 2 days after the initial bleed.<sup>2</sup> Significant ischemic injury of the brain has been documented in 75% of the patients who die within the first 24 hours after SAH,<sup>3</sup> but the cause of the cerebral hypoperfusion remains unknown. Studies in rats found no correlation between the elevation in intracranial pressure (ICP) after SAH and neurological outcome.<sup>4</sup> Studies in experimental animals also suggest that the acute fall in regional cerebral blood flow (rCBF) after SAH is due to constriction of the large cerebral arteries at the base of the brain.<sup>5</sup> Acute vasospasm of cerebral arteries has yet to be documented in patients with SAH; however, transcranial Doppler ultrasound measurements suggest that there is a high correlation between the level of cerebral perfusion within hours after SAH and the later degree of ischemic brain injury.<sup>6</sup>

Previous studies have suggested that the accumulation of blood in cerebrospinal fluid (CSF) alters the balance of the formation of vasoconstrictive and vasodilatory factors in the cerebral circulation.<sup>7</sup> For example, the levels of endothelin, thromboxane, and serotonin (5-hydroxytryptamine [5-HT]) in CSF are elevated immediately after SAH.<sup>8-10</sup> More recently, we reported that the levels of 20-hydroxyeicosatetraenoic acid (20-HETE) increase in CSF after SAH and that inhibitors of the synthesis<sup>11</sup> or actions<sup>12</sup> of 20-HETE can reverse the fall in rCBF after SAH in rats. However, the factors that trigger the rise in 20-HETE levels in CSF after SAH and the interaction between 20-HETE and other constricting factors in mediating the fall in rCBF after SAH are still unknown.

The present study focused on the possible interactions between 5-HT and 20-HETE in mediating the fall in rCBF after SAH. This decision was based on previous observations that 5-HT levels are markedly elevated in CSF after SAH<sup>10,13</sup> and that 5-HT is one of the most powerful constrictors of cerebral arteries.<sup>14</sup> On the other hand, previous studies using 5-HT receptor antagonists, such as methylsergide, have failed to support a role for 5-HT in mediating the fall in rCBF.

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during SAH.15–17 More recent studies have revealed that the 5-HT_1B_ receptor appears to be the subtype that mediates the vasoconstrictor response of cerebral arteries.18,19 This forms the basis of the popular treatment of migraine with 5-HT_1B_ receptor agonists, such as sumatriptan. In some instances, the use of this class of drugs has been associated with the development of Call-Fleming syndrome, which mimics neurological deficits and cerebral vasospasm that accompany SAH.20 These findings suggest that 5-HT, acting via 5-HT_1B_ receptors in the vasculature, may play a role in the development of vasospasm following extravasation of blood into CSF after spontaneous SAH or associated with head injury. Since the 5-HT_1B_ receptor also activates phospholipase A_2,21 it is possible that 5-HT increases the production of 20-HETE in the brain. Thus, the present study examined the hypothesis that 20-HETE production and release are stimulated by increased levels of 5-HT after SAH and that 20-HETE increases the production of 5-HT in cerebral vessels mediated by activation of 5-HT_1B_ receptors.

Materials and Methods
Experiments were performed on male Sprague-Dawley rats (Harlan) weighing 270 to 350 g. The rats were housed in an animal care facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care International at the Medical College of Wisconsin. All protocols were reviewed and approved by the Animal Care Committee of the Medical College of Wisconsin. (Supplemental Materials and Methods are available online at http://stroke.ahajournals.org.)

Induction of SAH and Measurement of rCBF
Rats were anesthetized with ketamine (Ketajet, 20 mg/kg IM) and thiobutabarbital sodium (Inactin, 100 mg/kg IP) and surgically prepared for induction of SAH and measurement of rCBF by laser-Doppler flowmetry as previously described.11 Rats were randomly assigned to 7 treatment groups. After surgery and a 30-minute equilibration period, 0.1 mL of CSF was collected for measurement of baseline levels of 5-HT and 20-HETE. After an additional 30-minute recovery period, rCBF, MAP, temperature, and PCO_2 were recorded during a 15-minute control period. Group 1 (SAH; n = 7) received an infusion of 0.3 mL of autologous, unheparinized arterial blood in the cisterna magna at a rate of 30 μL/min over a 10-minute period. ICP, rCBF, MAP, temperature, and PCO_2 were monitored for 2 hours, and 0.1 mL of CSF was collected 2 hours after induction of SAH to measure levels of 5-HT and 20-HETE. Group 2 (artificial CSF [aCSF]; n = 11) received an infusion of 0.3 mL of aCSF in the cisterna magna instead of blood. Group 3 (5-HT_1B_ receptor antagonist + SAH; n = 9) was pretreated with a selective antagonist of 5-HT_1B_ receptors, 1-(2-(1-pyrrollyl)-phenoxy)-3-isopropylamino-2-propanol hydrochloride, isamoltane hemifumarate (isamoltane, 3 mg/kg SC), which has 27-fold selectivity to 5-HT_1B_ receptors over 5-HT_1A_ receptors.22 Group 4 (5-HT; n = 9) received an intracisternal infusion of 5-HT (30 nmol in 0.3 mL of aCSF) at a rate of 30 μL/min over a 10-minute period after the control period. Group 5 (HET0016 + 5-HT; n = 7) was given a selective inhibitor of the synthesis of 20-HETE, 20-hydroxyeicosatetraenoic acid (HET0016; 23 nmol in 0.3 mL of aCSF); HET0016; n = 6), the vasoconstrictor response to 5-HT was determined before and after addition of a selective inhibitor of the synthesis of 20-HETE, 20-hydroxyeicosatetraenoic acid (HET0016; 23 nmol in 0.3 mL of aCSF).23 In groups 4 and 5 (vehicle controls), the vasoconstrictor response to 5-HT was determined before and after the vehicles used for isamoltane (physiological saline solution [PSS]; n = 6) or HET0016 and WIT003 (ethanol; n = 6) were added to the bath.

Measurement of 20-HETE and 5-HT
CSF samples were collected from the cisterna magna, transferred to methanol-rinsed 0.5-mL glass autoanalyzer vials, frozen in liquid nitrogen, and stored at −80°C until assayed. 20-HETE concentration in CSF was measured with a fluorescent high-performance liquid chromatography (HPLC) assay previously described in detail.24 The levels of 5-HT in the samples were analyzed by HPLC as previously described.25

In Vitro Studies in Isolated Middle Cerebral Artery
Rat middle cerebral arteries (MCAs) were microdissected, mounted on glass micropipettes, and pressurized to 80 mm Hg in a perfusion chamber as previously described.26 After a 45-minute equilibration period, the response to increasing concentrations (10⁻⁸ to 10⁻⁴ mol/L) of 5-HT was determined. The vessels received 1 of 5 treatments, and then the response to 5-HT was determined again. In group 1 (5-HT_1B_ antagonist; n = 7), isamoltane (10⁻⁷ mol/L) was added to the bath after the control period. In group 2 (HET0016; n = 6), the vasoconstrictor response to 5-HT in the presence of the 20-HETE inhibitor HET0016 (10⁻⁶ mol/L) was determined. In group 3 (HET0016+WIT003; n = 5), we examined whether 20-HETE potentiates the contractile response to 5-HT. These vessels were pretreated with HET0016 (10⁻⁶ mol/L), and the vasoconstrictor response to 5-HT was determined before and after addition of 20-HETE agonist, 20-hydroxyeicosatetraenoic acid (HET0016; 10⁻⁵ mol/L).27 In groups 4 and 5 (vehicle controls), the vasoconstrictor response to 5-HT was determined before and after the vehicles used for isamoltane (physiological saline solution [PSS]; n = 6) or HET0016 and WIT003 (ethanol; n = 6) were added to the bath.

[Ca²⁺] Responses to 5-HT in Vascular Smooth Muscle Cells Isolated From Rat MCA
Vascular smooth muscle (VSM) cells were isolated from the microdissected MCA as previously described.28 The cells were loaded with 4 μmol/L fura-2-acetoxymethyl ester (fura 2-AM) at room temperature for 45 minutes and superfused with PSS at 37°C for 30 minutes. Fluorescence intensity ratios were measured with an InCyt C for 30 °C until assayed. 20-HETE concentration in CSF was measured with a fluorescent high-performance liquid chromatography (HPLC) assay previously described in detail.24 The levels of 5-HT in the samples were analyzed by HPLC as previously described.25

Statistical Analysis
Values are expressed as mean±SEM. The significance of changes in rCBF and vessel diameters was evaluated with an ANOVA for repeated measures followed by the Duncan multiple range test. The significance of differences in 5-HT and 20-HETE levels in CSF and Ca²⁺ measurements in isolated VSM cells was evaluated by paired Student’s t test. P<0.05 was considered significant.

Results
Measurement of 20-HETE and 5-HT Levels in CSF
Baseline levels of 20-HETE in CSF were <30 ng/mL (Figure 1A). Thinning of the parietal bone for measurement of rCBF increased 20-HETE levels to 172±10 ng/mL, and this value was not significantly different from the level of 197±39 ng/mL found in the control rats 2 hours after injection of aCSF into the cisterna magna. Intracisternal injection of blood increased 20-HETE levels by 2.8-fold. Pretreatment of the rats with isamoltane reduced 20-HETE levels in CSF after SAH to levels that were not significantly different from those measured in control rats that received intracisternal injection of aCSF alone. Injection of 5-HT into the cisterna...
magna increased 20-HETE levels in CSF by 2.1-fold. Pretreatment of the rats with HET0016 reduced the levels of 20-HETE in CSF after injection of 5-HT by 60%.

Baseline levels of 5-HT in CSF were 10 nmol/mL (Figure 1B). In control experiments, 5-HT levels remained unchanged 2 hours after injection of aCSF. In contrast, intracisternal injection of blood into the cisterna magna significantly increased the 5-HT levels to 1163 ± 200 nmol/mL in untreated rats and to 746 ± 184 nmol/mL in rats pretreated with isamoltane. Two hours after direct injection of 5-HT into the cisterna magna, 5-HT levels in CSF also remained elevated, in a manner similar to that seen in rats after SAH, and averaged 303 ± 90 nmol/mL.

**Effects of SAH on rCBF**

rCBF remained unchanged during the 2-hour course of the experiment (Figure 2A) in control rats in which 0.3 mL aCSF was injected into the cisterna magna. Since there was no difference in rCBF data in the rats that received the vehicle for isamoltane plus SAH versus SAH alone, the data from these 2 groups were combined and presented together. In this combined group, rCBF fell to 34% of control during the intracisternal infusion of blood and remained at levels averaging 72 ± 6% of control during the remainder of the experiment. In rats pretreated with isamoltane, rCBF fell by 46% during the infusion of blood but returned to control immediately after the infusion and remained at this level throughout the experiment.

**Effects of Intracisternal Injection of 5-HT on rCBF**

Intracisternal injection of 30 nmol 5-HT into the cisterna magna decreased rCBF to 35% of control during the injection, and it rebounded to 90% of control after the infusion was stopped (Figure 2B). Thirty to 120 minutes after administration of 5-HT, rCBF averaged 71 ± 6% of control, a value that was similar to that seen in the rats in which blood was injected into CSF. Pretreatment of rats with HET0016 had no effect on the initial fall in rCBF seen during infusion of 5-HT, but it prevented the sustained fall in rCBF seen 30 to 120 minutes after injection of 5-HT. There were no significant differences in rCBF measured in rats that received vehicle for...
HET0016 and 5-HT group, and therefore the data from these 2 groups were combined and presented together.

**Effects of SAH and 5-HT on MAP, ICP, and Cerebral Perfusion Pressure**

In all groups, MAP tended to decline slightly over the course of the experiment. However, it remained well above the rCBF autoregulatory range and averaged between 100 and 122 mm Hg in all groups throughout the experiment; there was no difference in MAP measured between the groups at any time during the experiment (Figure 3A). ICP rose to 62 ± 11 mm Hg, and cerebral perfusion pressure (CPP) fell to values below the autoregulatory range immediately after induction of SAH in rats treated with vehicle or isamoltane. However, ICP rapidly returned to levels only 10 mm Hg above control, and CPP rose to a value >80 mm Hg during the remainder of the experiment (Figure 3B and 3C). In rats that received intracisternal injection of aCSF, 5-HT, or 5-HT plus HET0016, ICP rose to 30 ± 3 mm Hg and CPP fell to approximately 80 mm Hg immediately after the injection, but both values rapidly returned to control in all groups (Figure 3B and 3C).

**Effects of Isamoltane, HET0016, and WIT003 on Vasoconstrictor Responses to 5-HT in Isolated MCA**

The results of these experiments are presented in Figure 4. The baseline inner diameter of MCA averaged 100 ± 10 μm. 5-HT reduced the diameter of MCA in a concentration-dependent manner. Blockade of 5-HT1B receptors with isamoltane or the synthesis of 20-HETE with HET0016 attenuated but did not eliminate the vasoconstrictor response to 5-HT, by approximately 60%. Addition of a 20-HETE agonist, WIT003, partially restored the vasoconstrictor response to 5-HT to 80% of control in vessels in which the synthesis of 20-HETE was blocked.

**Effects of Isamoltane and HET0016 on [Ca2+]i in VSM Cells Stimulated With 5-HT**

The results of these experiments are presented in Figure 5. Baseline levels of [Ca2+]i were similar in VSM cells isolated from the MCA that were pretreated with isamoltane, HET0016, and vehicle. HET0016 and isamoltane had no effect on the peak response in [Ca2+]i after administration of 5-HT in VSM isolated from the MCA; however, both treatments significantly attenuated the steady state increase in [Ca2+]i.

**Discussion**

The present study examined the interactions between 20-HETE and 5-HT in mediating the acute fall in rCBF after SAH in rats. The results indicate that the levels of 20-HETE and 5-HT in CSF were elevated 2 hours after induction of SAH and that this was associated with a sustained 30% fall in...
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rCBF. At this time, ICP was elevated only minimally by 10 mm Hg, and CPP averaged 80 mm Hg, well above the threshold for autoregulation of rCBF. This indicates that the sustained fall in rCBF after SAH is not due to a fall in CPP but is related to some impairment in autoregulation of rCBF that produces an inappropriate elevation in cerebral vascular resistance. We found that blockade of 5-HT<sub>1B</sub> receptors with isamoltane prevented the rise in 20-HETE levels in CSF and the sustained fall in rCBF after SAH without altering the effects of SAH on ICP or CPP. We also found that injection of 5-HT into CSF mimicked the effects of SAH by increasing 20-HETE levels in CSF and reducing rCBF in the absence of sustained changes in ICP or CPP. Blockade of the synthesis of 20-HETE with HET0016 prevented the increase in 20-HETE levels in CSF and the fall in rCBF produced by injection of 5-HT into CSF. Overall, these findings confirm previous observations that 5-HT levels are elevated in CSF after SAH. They also indicate that 5-HT stimulates the formation and/or release of 20-HETE in CSF after SAH through activation of 5-HT<sub>1B</sub> receptors and suggest that 20-HETE contributes to the acute fall in rCBF in rats not only after SAH, as we reported previously, but also in response to administration of 5-HT in CSF. The source of 5-HT and 20-HETE in CSF after SAH is uncertain. Platelets avidly store and release 5-HT when activated during clotting reaction. Thus, they could be the source of 5-HT in CSF after SAH. 20-HETE is known to be produced by activated polymorphonuclear white blood cells and/or cerebral VSM. These may be the sources of the elevated levels of 20-HETE in CSF after SAH and after administration of 5-HT into the CSF.

Although it has long been known that 5-HT is released into CSF after SAH and that 5-HT constricts cerebral arteries, the results of previous studies have largely excluded a role for 5-HT in mediating the acute or delayed vasospasm associated with SAH. However, most of these previous studies used either the 5-HT<sub>2</sub> receptor antagonist BW501C67, the nonselective 5-HT<sub>1</sub> receptor antagonist methysergide, or the inhibitors of vesicular monoamine transport reserpine and kanamycin to alter the actions of 5-HT after SAH. More recent studies have indicated that 5-HT<sub>1B</sub> receptors are expressed on cerebral VSM cells and that these receptors largely mediate the vasoconstrictor response to 5-HT in the cerebral circulation. This view is further supported by the present finding that a selective inhibitor of 5-HT<sub>1B</sub> receptors, isamoltane, inhibited the vasoconstrictive response to 5-HT in rat MCA studied in vitro and the 5-HT-induced rise in Ca<sup>2+</sup>, in VSM cells isolated from rat MCA. Moreover, our finding that isamoltane also blocked the fall in rCBF and rise in 20-HETE levels after SAH provides the direct evidence implicating 5-HT<sub>1B</sub> receptors in triggering the rise in 20-HETE levels that, together with 5-HT, appears to mediate the acute fall in rCBF after SAH in rats.

The mechanism by which 20-HETE contributes to the fall in rCBF after SAH and intrathecal administration of 5-HT was also explored in the present study. Administration of HET0016, a highly selective inhibitor of the synthesis of 20-HETE, reduced the vasoconstrictor response of rat MCA to 5-HT in vitro by 60%. The degree of inhibition of the vasoconstrictor response to 5-HT in these arteries was similar to that seen after blockade of 5-HT<sub>1B</sub> receptors. Exogenous administration of the stable 20-HETE agonist WIT003 partially restored the response to 5-HT in MCA in which the endogenous synthesis of 20-HETE was blocked with HET0016. HET0016 also blunted the rise in Ca<sup>2+</sup> concentration in VSM cells isolated from rat MCA. These findings are consistent with previous findings indicating that 20-HETE serves as an endogenous inhibitor of the large-conductance, calcium-activated K<sup>+</sup> channel in VSM and potentiates the myogenic response of renal and cerebral arteries as well as the vasoconstrictor responses to endothelin and angiotensin II in renal arteries both in vitro and in vivo by preventing repolarization of VSM cells, thereby enhancing Ca<sup>2+</sup> influx.

Blockade of 5-HT<sub>1B</sub> receptors and the synthesis of 20-HETE reduced the vasoconstrictor response to 5-HT in isolated cerebral vessels by 60% and prevented the sustained increase in intracellular Ca<sup>2+</sup> concentration in cerebral VSM cells. These findings indicate that there are 2 mechanisms mediating the vasoconstrictor response to 5-HT in cerebral vessels: 1 that is mediated by activation of 5-HT<sub>1B</sub> receptors and is dependent on the synthesis of 20-HETE and 1 that is probably mediated by activation of some other 5-HT receptors. Our in vivo data are also consistent with the
hypothesis that the vasoconstrictor response to 5-HT in the cerebral circulation is mediated by both 5-HT₁a– and 20-HETE– dependent and –independent pathways. In this regard, while isomitoline prevented the sustained fall in rCBF, it only attenuated the initial fall in rCBF after SAH. Similarly, blockade of the synthesis of 20-HETE had little effect on the initial fall in rCBF after induction of SAH.

Previous studies have indicated that cerebral arteries become sensitized to the vasoconstrictor effect of 5-HT in the acute and chronic phases of SAH. Since 5-HT in VSM cells of cerebral arteries is known to activate phospholipase A₂ and the release of arachidonic acid21,22 and in the present study we have shown that 5-HT also increases 20-HETE levels in CSF, it is possible that elevated concentration of 20-HETE in cerebral arteries after SAH contributes to the increased response of vessels to 5-HT after SAH. This mechanism is also consistent with the increase sensitivity of cerebral arteries to 5-HT seen in MCA when transmural pressure is increased, an effect that is known to be 20-HETE dependent.28

We previously reported that nitric oxide inhibits the synthesis of 20-HETE in VSM of MCAs through cGMP– dependent pathways.29 Since in SAH hemoglobin binds nitric oxide and prevents diffusion of nitric oxide to VSM, there is a possibility that a low concentration of nitric oxide in VSM after SAH also acts in conjunction with elevated 5-HT levels to stimulate the production of 20-HETE in cerebral arteries.

In conclusion, the present results indicate that 5-HT is released into CSF after SAH and stimulates the synthesis and/or release of 20-HETE by activating 5-HT₁a receptors. 20-HETE potentiates the vasoconstrictor response to 5-HT in cerebral arteries and contributes to the acute fall in rCBF after SAH in rats. New diagnostic techniques will be important to determine whether similar acute changes in brain perfusion occur in patients after SAH. If true, then our results in rats suggest a potential for inhibitors of 20-HETE synthesis and/or release of 20-HETE by activating 5-HT₁ receptors in human cerebral arteries to 5-HT seen in MCA when transmural pressure is increased, an effect that is known to be 20-HETE dependent.28

References


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