20-HETE contributes to the acute fall in cerebral blood flow after subarachnoid hemorrhage in the rat

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20-HETE contributes to the acute fall in cerebral blood flow after subarachnoid hemorrhage in the rat. Am J Physiol Heart Circ Physiol 282: H1556–H1565, 2002. First published December 6, 2001; 10.1152/ajpheart.00924.2001.—This study examined the effects of blocking the formation of 20-hydroxyeicosatetraenoic acid (20-HETE) on the acute fall in cerebral blood flow after subarachnoid hemorrhage (SAH) in the rat. In vehicle-treated rats, regional cerebral blood flow (rCBF) measured with laser-Doppler flowmetry fell by 30% 10 min after the injection of 0.5 ml of arterial blood into the cisterna magna, and it remained at this level for 2 h. Pretreatment with inhibitors of the formation of 20-HETE, 17-octadecynoic acid (17-ODYA; 1.5 nmol intrathecally) and N-hydroxy-N-(4-buty1-2-methylphenyl)formamidine (HET0016; 10 mg/kg iv), reduced the initial fall in rCBF by 40%, and rCBF fully recovered 1 h after induction of SAH. The concentration of 20-HETE in the cerebrospinal fluid rose from 12 ± 2 to 199 ± 17 ng/ml after SAH in vehicle-treated rats. 20-HETE levels averaged only 15 ± 11 and 39 ± 13 ng/ml in rats pretreated with 17-ODYA or HET0016, respectively. HET0016 selectively inhibited the formation of 20-HETE in rat renal microsomes with an IC50 of <15 nM and human recombinant CYP4A11, CYP4F2, and CYP4F3 enzymes with an IC50 of 26, 125, and 100 nM, respectively. These results indicate that 20-HETE contributes to the acute fall in rCBF after SAH in rats.

CYP4A; stroke; 20-hydroxyeicosatetraenoic acid

SUBARACHNOID HEMORRHAGE (SAH) accounts for 5–10% of all strokes. The incidence is 2–20 events per 100,000 population per year with a case fatality rate of 20–60% (3, 21). SAH typically occurs after rupture of cerebral aneurysms or head trauma leading to subarachnoid bleeding and clot formation. After the initial bleed, cerebral blood flow initially falls because of an elevated intracranial pressure (ICP) that reduces cerebral perfusion pressure (CPP) combined with the release of vasoactive factors that increase cerebral vascular tone.

In humans, an early phase of cerebral vasospasm has been documented as early as 4 h after the onset of SAH (6, 40), and this is associated with neurological deficits and a high mortality (4, 7, 34).

Despite intensive investigation, the factors that produce the acute fall in cerebral blood flow after SAH are still uncertain (4, 22, 28, 36, 44). Endothelin (ET) levels increase after SAH (23, 35), and the acute fall in cerebral blood flow after SAH in rats is attenuated by inhibitors of the synthesis of ET or ET receptor blockers (9). Enhanced turnover of fatty acids and increased formation of vasoconstrictor metabolites of arachidonic acid (AA) have also been observed after SAH (10, 12). Previous reports that inhibitors of thromboxane synthesis ameliorate the fall in cerebral blood flow after SAH support a role for metabolites of AA in this response (24, 42).

Recent studies (15, 16) indicated that 20-hydroxyeicosatetraenoic acid (20-HETE) is the primary vasoconstrictor metabolite of AA produced in the cerebral circulation. 20-HETE is produced by enzymes of the CYP4A family that are expressed in vascular smooth muscle (VSM) cells in cerebral arteries. The members of the CYP4A family include CYP4A1, -2, -3, and -8 in rats, CYP4A11 in humans, and CYP4A10, -12 and -14 in mice. Enzymes of the CYP4F2 and -3 family have also been reported to produce 20-HETE in humans (5, 26, 32, 33). CYP4F2 is highly expressed in the kidney, whereas CYP4F3 is expressed in polymorphonuclear white blood cells that avidly produce 20-HETE (5, 33).

20-HETE is a potent constrictor of cerebral arteries (EC50 <10 nM) that activates protein kinase C (PKC) and depolarizes VSM cells by inhibiting the large-conductance, Ca2+-sensitive K+ channel (16, 18, 41). 20-HETE also increases Ca2+ influx via L-type Ca2+ channels in the cerebral vasculature (16) and plays a critical role in the mechanism underlying the autoregulation of cerebral blood flow in the rat (2, 15, 18). Vasocostrictor peptides like angiotensin II and ET stimulate the formation of 20-HETE in VSM, and 20-HETE.
HETE potentiates the vasoconstrictor response to these agents (11, 31). Nitric oxide (NO) inhibits the formation of 20-HETE in VSM, and this contributes to the vasodilator actions of NO in the cerebral circulation (2, 37). Given that the actions of 20-HETE share many properties previously associated with the acute fall in cerebral blood flow after SAH, the present study tested the hypothesis that 20-HETE production and release are enhanced after SAH and that 20-HETE contributes to the acute fall in blood flow in the rat after SAH.

METHODS

Experiments were performed on male Sprague-Dawley rats (9–12 wk old) purchased from Harlan Sprague Dawley (Indianapolis, IN). The Animal Care Program at the Medical College of Wisconsin is approved by the American Association for the Accreditation of Laboratory Animal Care. All protocols involving animals received approval by the Animal Care Committee of the Medical College of Wisconsin and conformed to the “Guiding Principles in the Care and Use of Animals” of the American Physiological Society and were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Surgical preparation. The rats were anesthetized with ketamine (Ketaject; 20 mg/kg im) and thiobutabarbital (Inactin; 50 mg/kg ip). A cannula was placed in the trachea, and the rats were artificially ventilated with a small-animal ventilator (SAR-830; CWE, Ardmore, PA) with a mixture of 30% O2 in N2. Cannulas were placed in the femoral vein for infusion of drugs and in the femoral artery for collection of arterial blood samples and measurement of mean arterial pressure (MAP). End-tidal PCO2 was maintained at 35 mmHg by adjusting the minute ventilation according to the reading of a CO2 analyzer (Capstar-100 ITTC, Woodland Hills, CA). Blood samples were collected at the beginning and end of the experiment and analyzed with a blood gas analyzer (ABL 300; Radiometer, Copenhagen, Denmark) to validate the end-tidal PCO2 readings. Anesthesia was maintained by administering additional amounts of thiobutabarbital (8 mg/kg iv) as needed. Body temperature was maintained at 37 ± 1°C with a thermoregulated heated pad, and the rats received an intravenous infusion of 0.9% NaCl solution containing 1% bovine serum albumin at a rate of 3 ml/h to replace fluid losses.

Induction of SAH and measurement of cerebral blood flow. SAH was induced by injection of 0.3 ml of unheparinized autologous freshly drawn arterial blood into the cisterna magna with a modification of a posterior craniocervical approach as described by Delgado et al. (13) and Solomon et al. (39). The head of the rat was placed in a stereotactic head holder, and the atlantooccipital membrane was exposed by separating the overlying neck muscles in the midline. With the use of a stereomicroscope a 30-gauge needle attached to a polyethylene (PE)-10 catheter was inserted into the cisterna magna by penetrating the atlantooccipital membrane. A small hole was drilled in the bone overlying the left parietal cortex. A PE-10 catheter pulled to a tip diameter of 100 μm was inserted under the dura and cemented in place with cyanoacrylate for monitoring ICP. A 3 × 5-mm area of the bone overlying the right parietal cortex was thinned using a hand-held drill until only a thin, translucent layer of cranial bone remained (thinned cranial window). A PF 102 laser-Doppler flow probe was positioned over the cranial window, and regional cerebral blood flow (rCBF) was monitored with a PF-3 laser-Doppler flowmeter (Perimed, Stockholm, Sweden). After control measurements of rCBF were obtained, freshly drawn unheparinized arterial blood was infused into the cisterna magna at a rate of 30 μl/min over a 10-min period. This created a massive SAH that was confirmed in all rats at autopsy. Clotted blood was found overlying the cerebello-medullary junction posteriorly and around the basal artery and the vessels of the circle of Willis on the ventral surface of the brain.

Pretreatment of rats with N-hydroxy-N-(4-butyl-2-methylphenyl)formamidine and 17-octadecenoyl acid. Experiments were performed in five separate groups of rats. After a 30-min equilibration period, vehicle-10% lecithin in saline (group 1) or N-hydroxy-N-(4-butyl-2-methylphenyl)formamidine (HET0016; 10 mg/kg iv; group 2) was given intravenously and rCBF was measured for 30 min. Other animals received 17-octadecenoyl acid (17-ODYA; 1.5 nmol; group 3) or vehicle-ethanol (1:1,000) in saline (group 4) in a volume of 50 μl that was injected directly in the cisterna magna with a micropipette. The average value of rCBF recorded over the last 5 min before induction of SAH served as the control value. SAH was induced, and rCBF was recorded over 2-min intervals at 10, 20, 30, 60, 90, and 120 min after induction of SAH. The rCBF data are expressed as the percent change in flow from control. Another group of rats (group 5) received an infusion of an equal volume of artificial cerebrospinal fluid (aCSF) instead of blood to control for changes in cerebral blood flow that might be related to the infusion and accompanying increase in ICP alone.

Videomicroscopy measurement of red blood cell flux and vessel diameters. Experiments were performed to determine whether the effects of HET0016 and SAH on cerebral blood flow are associated with changes in the supply rate of fluorescently labeled red blood cells (FRBC) in capillaries or changes in the diameter of small pial vessels on the surface of the cerebral cortex. These animals were anesthetized and surgically prepared as described in Surgical preparation. In addition, a closed cranial window was prepared over the right parietal cortex with techniques described previously (20). The rats were then transferred to the stage of an intravital microscope, paralyzed with 80 mg of gallamine, and artificially ventilated with a 30% O2-70% N2 mixture.

To facilitate the visualization of the microcirculation, red blood cells from a donor rat were labeled in vitro with fluorescein isothiocyanate (Sigma, St. Louis, MO) and injected intravenously to produce a ~4% labeled FRBC population. Small pial vessels on the surface of the brain were visualized with an epifluorescence videomicroscopy system and a ×40 objective. Effective magnification to the monitor was ×125. The exposure of the preparation to light was limited to brief periods during which the microcirculation was video recorded. Additionally, a heat filter and a 455-nm high-pass filter were used to block infrared and ultraviolet irradiation of the tissue.

Capillaries were brought into focus, and the supply rate of FRBC passing through individual intracortical capillaries was recorded and expressed as labeled cells per second during the control period. The rats received a bolus injection of HET0016 (10 mg/kg iv) or vehicle, and after a 30-min equilibration period the same capillaries were recorded. Next, SAH was induced, and the supply rate of FRBC in the capillaries was recorded at 10, 20, 30, and 60 min after induction of SAH.

Capillaries were identified by the presence of single-file FRBC flow (implying a vessel diameter <5 μm), arterioles by fast, diverging FRBC movement, and venules by slower,
converging FRBC movement. The diameter of larger arterioles and venules in the field was also measured throughout the experiment with a videomaging system (20).

**Measurement of 20-HETE levels in CSF.** At the end of many experiments, CSF (~100 μl) was collected from the cisterna magna with a 1-ml syringe and a 30-gauge needle. CSF was also collected from additional sham-operated rats that did not receive an injection of blood into the cisterna magna. 20-HETE concentration in CSF was measured with a fluorescent HPLC assay as described previously (27, 29). Briefly, 50 ng of an internal standard, 20-hydroxyeicosanoid acid (WIT-002) was added to the CSF samples (50 μl). The samples were extracted with 1 ml of ethyl acetate and dried under argon. The lipid fraction was labeled with 20 μl of acetone containing 36.4 mM 2-(2,5-naphthalimido)ethyltrifluoromethanesulfonate. N,N-diisopropyl ethylenamine (10 μl) was added to catalyze the reaction. The sample was reacted for 30 min at room temperature. Excess dye was removed with a Sep-Pak Vac column (no. WAT054955; Waters, Milford, MA), and the samples were dried under nitrogen, resuspended in 100 μl of methanol, and analyzed on a reverse-phase HPLC column (Waters) with a fluorescence detector. The amount of 20-HETE in the sample was determined by comparing the area of the 20-HETE peak to that of the internal standard.

**HET0016 treatment protocol.** Additional experiments were performed to determine whether HET0016 could not only prevent, but also reverse, the fall in cerebral blood flow seen 30 min after induction of SAH. rCBF was measured with laser-Doppler flowmetry during the control period and for 30 min after induction of SAH. The rats then received an intravenous injection of HET0016 (10 mg/kg) or an equal volume of vehicle (10% lecithin in saline), and rCBF was measured for an additional 120 min.

**Characterization of HET0016 as a selective inhibitor of formation of 20-HETE.** These experiments examined the effects of various concentrations of HET0016 on the metabolism of AA in renal microsomes, which are a rich source of most of the cytochrome P-450 (CYP) isoforms known to metabolize AA to 20-HETE and epoxyeicosatrienoic acids (EETs). The renal cortex of rats was homogenized in a 10 mM potassium phosphate buffer (pH 7.7) containing (in mM) 250 sucrose, 1 EDTA, and 10 magnesium chloride. Microsomes were prepared by differential centrifugation as previously described (27). CYP4A and -4F enzyme activity was assayed by incubating renal cortical microsomes (0.5 mg protein) for 30 min at 37°C with [14C]-labeled AA (0.1 μCi/ml, 42 μM; Amersham, Arlington Heights, IL) in 1 ml of a 0.1 mM potassium phosphate buffer (pH 7.4) and 1 mM NADPH as previously described (27). The reactions were terminated by acidification to pH 3.5 with formic acid and extracted with ethyl acetate. Metabolites were separated by reverse-phase HPLC, and products were monitored with a radioactive flow detector as described above.

**Measurement of HET0016 levels in plasma and brain.** Rats received an intravenous injection of HET0016 (10 mg/kg) in 10% lecithin via the tail vein. After a 1-h equilibration period, the rats were anesthetized with pentobarbital (60 mg/kg ip) and a blood sample (1 ml) was collected from the jugular vein. The rats were then euthanized, and the brain was removed, weighed, and homogenized in 3 volumes of 0.9% NaCl solution with a Physcotron homogenizer (Nition-Ikikai, Chiba, Japan). The concentration of HET0016 in a 100-μl aliquot of plasma or a homogenate of the brain was determined by liquid chromatography/mass spectroscopy (LC/MS) with selective ion monitoring. The samples were mixed with 1 ml of acetonitrile and centrifuged, and 20 μl of the clear supernatant was injected into the LC/MS. The samples were separated on a 200 × 4.6-mm Capcellpak UG 120 ODS column (Shiseido, Tokyo, Japan) and eluted with acetonitrile:water (72/28 vol/vol). Peaks were monitored with a Sciex API 3000 mass spectrometer (Perkin Elmer Sciex) tuned to detect the precursor to product transitions specific for each analyte.

**Drugs and chemicals.** All chemicals were of HPLC grade. The fluorescent probe 2-(2,5-naphthalimido)ethyltrifluoromethanesulfonate was purchased from Molecular Probes (Eugene, OR). 17-ODYA was purchased from Sigma, and HET0016 and WIT-002 were synthesized and kindly provided by Taisho Pharmaceutical (Saitama, Japan).

**Statistics.** Mean ± SE values are presented. Significance of differences in mean values within and between groups was examined by a two-way ANOVA for repeated measures followed by the Duncan multiple-range test. A P value <0.05 was considered to be significant.

**RESULTS**

**Effects of pretreatment with HET0016 and 17-ODYA on rCBF after SAH.** The effects of two chemically and mechanistically different inhibitors of the formation of 20-HETE on the changes in rCBF after induction of SAH are presented in Fig. 1. There was no significant difference in the response of rats that received the vehicle for HET0016 or 17-ODYA. Thus the data from these two groups were combined and presented together in Fig. 1. Infusion of 0.3 ml of aCSF into the cisterna magna over 10 min increased ICP from 6 ± 4 to 35 ± 7 mmHg; however, rCBF was not significantly reduced at this time. ICP rapidly returned toward control over the next 10 min, and rCBF remained unaltered over the 2-h course of the experiment. In contrast, injection of 0.3 ml of autologous blood into the cisterna magna caused a rapid decline in rCBF in vehicle-treated rats. rCBF fell on average by 30% 10 min after the induction of SAH, and it remained at this level for the 2-h course of the experiment. ICP increased from 8 ± 2 to 50 ± 6 mmHg immediately after infusion of blood. ICP rapidly returned toward control and averaged 19 ± 3 mmHg 10 min after injection of blood. ICP then remained between 10 and 14 mmHg for the duration of the experiment.

**Pretreatment of rats with the irreversible inhibitor of the formation of 20-HETE and EETs, 17-ODYA, or the novel selective inhibitor of the formation of 20-HETE, HET0016, had no significant effect on baseline...
rCBF. Both CYP inhibitors, however, attenuated the initial fall in rCBF recorded at 10 min after induction of SAH by ~40%, and rCBF completely recovered within 60 min after induction of SAH in rats treated with 17-ODYA and within 90 min in rats treated with HET0016. HET0016 and 17-ODYA did not alter the changes in ICP produced by SAH. ICP still rose to a peak value of 50 mmHg at the end of the injection of blood and returned to values near 15 mmHg within 10 min after infusion of blood into the CSF.

A comparison of the MAP and end-tidal PCO2 data in the various groups of rats is presented in Table 1. There were no significant differences in end-tidal PCO2 at any time during the experiment in rats that received vehicle, HET0016, 17-ODYA, or aCSF. MAP tended to decline during the experiments in all groups, but there was no significant difference in blood pressure between rats treated with vehicle, 17-ODYA, HET0016, or aCSF at any time during the experiment.

Videomicroscopy studies. The effects of SAH and HET0016 on the FRBC supply rate in intracortical capillaries and the diameter of small arterioles and venules on the surface of the brain are presented in Fig. 2. The supply rate of FRBCs in cerebral capillaries decreased significantly by 30% 10 min after injection of blood into the CSF of vehicle-treated rats and remained at this level over the course of the experiment. In HET0016-pretreated rats, the FRBC supply rate also initially fell by 30% but returned to control within 30 min after induction of SAH.

The decrease in FRBC supply rate after SAH in vehicle-treated rats was not associated with changes in the diameter of small pial arteries and veins on the surface of the brain. Similarly, no significant change could be detected in the diameter of small arteries and veins after SAH in the rats pretreated with HET0016, either during the initial 20-min period when FRBC supply rate was reduced in these animals or in the later time periods after the FRBC supply rate returned to control level.

Measurement of 20-HETE levels in CSF. The concentration of 20-HETE in the CSF of sham-operated control rats and 2 h after SAH in rats given vehicle, 17-ODYA, and HET0016 is presented in Fig. 3. Rats that received vehicle had a 17-fold increase in the concentration of 20-HETE in CSF 2 h after SAH compared to control rats and 2 h after SAH in rats given vehicle, 17-ODYA, and HET0016.

Table 1. Mean arterial blood pressure and end-tidal PCO2 in rats treated with vehicle, 17-ODYA, and HET0016

<table>
<thead>
<tr>
<th>End-tidal PCO2</th>
<th>Control (15)</th>
<th>10 min</th>
<th>20 min</th>
<th>30 min</th>
<th>60 min</th>
<th>90 min</th>
<th>120 min</th>
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<tbody>
<tr>
<td>Vehicle (15)</td>
<td>35 ± 1</td>
<td>34 ± 1</td>
<td>34 ± 1</td>
<td>34 ± 1</td>
<td>34 ± 1</td>
<td>34 ± 1</td>
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<tr>
<td>17-ODYA (7)</td>
<td>37 ± 1</td>
<td>38 ± 1</td>
<td>38 ± 1</td>
<td>37 ± 1</td>
<td>37 ± 1</td>
<td>37 ± 1</td>
<td>37 ± 1</td>
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<tr>
<td>HET0016 (9)</td>
<td>36 ± 1</td>
<td>37 ± 1</td>
<td>37 ± 1</td>
<td>36 ± 1</td>
<td>36 ± 1</td>
<td>36 ± 1</td>
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<tr>
<td>Control (aCSF) (9)</td>
<td>36 ± 1</td>
<td>37 ± 1</td>
<td>37 ± 1</td>
<td>36 ± 1</td>
<td>36 ± 1</td>
<td>36 ± 1</td>
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<tr>
<td>MAP</td>
<td>117 ± 4</td>
<td>118 ± 4</td>
<td>115 ± 4</td>
<td>111 ± 4</td>
<td>109 ± 4</td>
<td>106 ± 4</td>
<td>103 ± 3</td>
</tr>
<tr>
<td>Vehicle (15)</td>
<td>106 ± 4</td>
<td>107 ± 5</td>
<td>105 ± 5</td>
<td>102 ± 4</td>
<td>98 ± 3</td>
<td>98 ± 3</td>
<td>97 ± 3</td>
</tr>
<tr>
<td>17-ODYA (7)</td>
<td>122 ± 5</td>
<td>121 ± 5</td>
<td>118 ± 5</td>
<td>115 ± 4</td>
<td>111 ± 4</td>
<td>106 ± 3</td>
<td>106 ± 3</td>
</tr>
<tr>
<td>HET0016 (9)</td>
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<td>105 ± 6</td>
<td>115 ± 5</td>
<td>116 ± 6</td>
<td>114 ± 5</td>
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Values (in mmHg) are means ± SE. Nos. in parenthesis indicate no. of animals studied. MAP, mean arterial pressure; 17-ODYA, 17-octadecynoic acid; HET0016, N-hydroxy-N'-(4-butyl-2-methylphenyl)-formamide; aCSF, artificial cerebrospinal fluid. There were no significant differences in the values between the groups.
Effects of HET0016 on synthesis of 20-HETE by human CYP isoforms. We also examined the ability of HET0016 to inhibit the production of 20-HETE by the 3 CYP isoforms, CYP4A11, CYP4F2, and CYP4F3, known to produce 20-HETE in humans (26, 32). We found that all three recombinant enzymes only produced a single peak when incubated with AA (Fig. 6). This product coeluted with a 20-HETE standard, and we confirmed using LC/MS that the product formed had the expected mass of 319 and had a fragmentation pattern identical to that of authentic 20-HETE. CYP4F3 had the greatest activity, followed by CYP4F2 and CYP4A11. HET0016 inhibited the formation of 20-HETE by all three isoforms in a concentration-dependent manner (Fig. 6). The IC50 was 42 nM for CYP4A11, and it averaged 100 and 125 nM for the CYP4F3 and CYP4F2 isoforms, respectively.

Measurement of HET0016 levels in plasma and brain of rats. Levels of HET0016 averaged 570 ± 90 ng/ml (n = 3; 2.8 μM) in the plasma and 1,488 ± 104 ng/g (n = 3; 7.2 μM) in the brain 1 h after intravenous injection of a 10 mg/kg dose of HET0016.

DISCUSSION

The present study examined the role of 20-HETE in the acute fall in rCBF after induction of SAH in rats. The results indicate that the concentration of 20-HETE in CSF increased 17-fold, from 12 ± 4 to 199 ± 24 ng/ml, 2 h after injection of blood into the cisterna magna of rats. This was associated with a fall in rCBF to 71 ± 3% of preinjection values 10 min after induction of SAH, which remained at this level for the entire 2-h course of the experiment in rats treated with vehicle (69 ± 3% at 120 min). The fall in perfusion in the cerebral cortex after SAH was confirmed by measuring a similar fall in the supply rate of FRBCs in capillaries on the cortical surface with videomicroscopy. The time course and magnitude of the changes in cerebral blood flow in the present study are similar to those reported by Jackowski et al. (22), who found that cerebral blood flow measured with hydrogen clearance fell to 57 ± 11% of control for up to 3 h after injection of blood into the cisterna magna of rats. In their study, rCBF remained reduced at 85 ± 15% of control at 24 h and returned to control 48 h after induction of SAH.

The sustained fall in rCBF after SAH observed in the present study is not due to a rise in ICP and reduced CPP. Indeed, we found that, although CSF pressure increased to 50 mmHg immediately after completion of the injection of the blood, it rapidly decreased to values only 5 mmHg above control 10 min after induction of SAH; however, cerebral blood flow remained 30% below baseline over the 2-h course of the experiment. Moreover, injection of an equal volume of aCSF produced a similar transient rise in ICP but had no significant effect on rCBF. This finding is consistent with previous reports that cerebral blood flow is well autoregulated and can be maintained at or near normal levels after elevations in ICP or reductions of CPP of up to 40 mmHg (14). Therefore, the changes in rCBF after
Induction of SAH cannot be explained on the basis of concurrent changes in ICP and CPP alone. Thus the sustained reduction of rCBF is likely due to the presence of blood or blood products in the CSF that acutely alter the cerebrovascular tone in the rat. In this regard, Delgado et al. (13) presented angiographic evidence of a 15–40% narrowing of the large cerebral arteries near the base of the brain in the time frame of 10 min to 48 h after induction of SAH in the rat. Others using videomicroscopy have repeatedly demonstrated that the diameter of the basilar artery of the rat is also decreased after SAH (37, 38). The present videomicroscopy results also indicate that the fall in rCBF flow in the cerebral cortex of the rat after SAH is not associated with narrowing of small vessels on the surface of the cerebral cortex in which the flow was measured. Together, these results indicate that an increase in cerebral vascular resistance accounts for the acute fall in rCBF after SAH in the rat. Moreover, this rise in vascular resistance most likely occurs in the upstream feed vessels near the base of the brain, because the diameter of the small vessels on the surface of the brain was unaltered. On the other hand, this observation does not mean that SAH and HET0016 have no effect on the vascular tone of the pial arterioles. Indeed, constriction of larger upstream cerebral arteries should elicit an autoregulatory vasodilation of pial arterioles to maintain flow. Thus the finding that SAH and HET0016 had no effect on the diameter of these vessels implies that SAH and 20-HETE may increase and blockade of 20-HETE production with HET0016 reduces vascular tone in pial arterioles.

Previous studies indicated that the turnover of fatty acids and the formation of thromboxane and other
vasoconstrictor metabolites of AA are elevated in CSF after SAH (12, 17). Activated platelets in clotting blood are the likely source of thromboxane and 12-HETE in CSF after SAH. 20-HETE is one of the major metabolites of AA produced by the cerebral vasculature (1, 15, 16, 25) and by polymorphonuclear leukocytes (PMNs) (5, 33). Thus the levels of 20-HETE in CSF were measured after SAH in the present study. We found that the levels of 20-HETE were 17-fold higher in CSF 2 h after induction of SAH in vehicle-treated rats compared with the levels measured in sham-operated rats. Pretreating the rats with two structurally and mechanistically different inhibitors of the synthesis of 20-HETE markedly reduced the levels of 20-HETE in the CSF after SAH. Because thrombin and the clotting cascade are known to stimulate the release of AA in PMNs, it is likely that these cells are one of the sources of elevated levels of 20-HETE in CSF after SAH. Moreover, because ET, vasopressin, angiotensin II (8, 11, 31, 43), and other vasoconstrictors are known to stimulate the formation of 20-HETE in VSM, it is possible that these substances that are released by clotting blood also stimulate the release of AA and formation of 20-HETE within the cerebral arteries.

To examine the contribution of elevated levels of 20-HETE to the acute fall in rCBF after SAH, we used 17-ODYA, which is an irreversible inhibitor of the formation of 20-HETE and EETs (45). 17-ODYA was administered intrathecally via injection into the cisterna magna because it is highly bound to plasma proteins and does not cross the blood-brain barrier when given intravenously. We found that pretreatment of rats with 17-ODYA had no significant effect on baseline rCBF but restored rCBF to control within 60 min after induction of SAH. In contrast, rCBF showed no tendency to recover in rats treated with vehicle. Assuming that the volume of CSF is 300 μl, the dose of 17-ODYA used in the present study should have produced a concentration of 5 μM in CSF, which is high enough to completely block the formation of 20-HETE and EETs (45). Indeed, we found that 17-ODYA completely blocked the increase in 20-HETE levels in CSF after SAH. However, as mentioned earlier, 17-ODYA is equally effective in blocking the formation of EETs and 20-HETE (45). Therefore, one could not exclude a role for EETs in mediating the reduction of rCBF based on these experiments using 17-ODYA.

For this reason, we repeated these experiments with HET0016, a new, highly selective competitive inhibitor of the formation of 20-HETE (30). We confirmed that HET0016 is a potent inhibitor of the formation of 20-HETE in microsomes prepared from the kidney of rats. The IC50 for inhibition of 20-HETE formation by HET0016 in rat renal microsomes is ~15 nM, which is 100 times lower than that reported for any previously described inhibitor of this pathway, including N-meth-
HET0016 was found to be an extremely selective inhibitor of the \( \text{H}9275 \)-hydroxylation of AA (30). Unlike these other inhibitors, HET0016 had no significant effect on the formation of EETs and dihydroxyeicosatrienoic acids (diHETEs) in renal microsomes even when used at a concentration of 1,000 nM. Furthermore, we demonstrated that HET0016 is also an effective inhibitor of all of CYP isoforms, CYP4A11, CYP4F2, and CYP4F3, known to produce 20-HETE in humans.

Like 17-ODYA, pretreatment of the rats with HET0016 attenuated the initial fall in rCBF by \( \approx 50\% \). It also markedly increased rCBF throughout the 2-h course of the experiment relative to the values observed in the vehicle-treated rats. Using LC/MS, we confirmed that the plasma levels of HET0016, after a 10 mg/kg iv administration, were much higher than the IC\(_{50}\) needed for inhibition of 20-HETE synthesis. HET0016 levels were also 2.8 times higher in brain tissue than in plasma, indicating that HET0016 avidly crosses the blood-brain barrier. Moreover, we demonstrated that pretreatment of the rats with HET0016 reduced the increase in 20-HETE levels in CSF after SAH. Overall, the results of the present study indicate that 20-HETE levels are acutely elevated after SAH and that two chemically distinct inhibitors of the formation of 20-HETE attenuate the acute fall in rCBF after SAH.

Although it is interesting that pretreatment of rats with HET0016 can mitigate the acute fall in rCBF after SAH, it was important to determine whether this compound might have therapeutic potential and reverse the fall in rCBF after SAH. As can be seen in Fig. 4, intravenous administration of HET0016 returned rCBF to control in animals with preexisting SAH. These results indicate that an inhibitor of the formation of 20-HETE can not only prevent but actually reverse the acute fall in rCBF after SAH.
The cell types involved in elevating 20-HETE levels after SAH remain to be determined. Recent studies indicating that activated PMNs and cerebral arteries avidly produce 20-HETE (2, 15, 16, 18, 25) suggest that they could be the source of 20-HETE after SAH. 20-HETE is a potent vasoconstrictor that inhibits K⁺ channels and depolarizes cerebral arterial VSM by activating PKC (18) and also potentiates the vasoconstrictor response to a wide variety of constrictor agents including ET (31). On the other hand, NO blocks the formation of 20-HETE in cerebral VSM cells, and inhibition of the formation of 20-HETE contributes to the activation of K⁺ channels and the vasodilator response to NO in the cerebral circulation (2, 19, 41). We reported previously (2, 41) that preventing the fall of 20-HETE completely blocks the activation of K⁺ channels in VSM cells and reduces the response to NO in middle cerebral arteries of the rat by >50%. In isolated vessel studies at a concentration of 1 μM, 20-HETE reduced rat renal (1) and cerebral (15) arterial diameter by 19 ± 3% and 25 ± 2%, respectively, and cat middle cerebral arterial diameter by 29 ± 8% (25). Thus the high concentration of 20-HETE measured in CSF after SAH (0.6 μM) could account for the 30–40% fall in rCBF observed in the present study. An elevation in 20-HETE production provides a unifying paradigm to explain how the initiation of a clotting reaction in CSF could lead to the formation of a vasoconstrictor substance (20-HETE) by PMNs or cerebral VSM cells that mediates vasoconstriction and potentiates the vascular responses to vasoconstrictors, including ET. The present study also suggests that 20-HETE plays an important role in the pathogenesis of acute cerebral hypoperfusion after hemorrhagic stroke and that inhibitors of this pathway may have potential as therapeutic agents to treat this serious, life-threatening condition.

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