Hemoglobin, NO, and 20-HETE interactions in mediating cerebral vasoconstriction following SAH

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Takeuchi, Kazuhiko, Noriyuki Miyata, Marija Renic, David R. Harder, and Richard J. Roman. Hemoglobin, NO, and 20-HETE interactions in mediating cerebral vasoconstriction following SAH. Am J Physiol Regul Integr Comp Physiol 290: R84–R89, 2006. First published September 15, 2005; doi:10.1152/ajpregu.00445.2005.—Recent studies have indicated that 20-hydroxyecosatetraenoic acid (20-HETE) contributes to the fall in cerebral blood flow (CBF) after subarachnoid hemorrhage (SAH), but the factors that stimulate the production of 20-HETE are unknown. This study examines the role of vasoactive factors released by clotting blood vs. the scavenging of nitric oxide (NO) by hemoglobin (Hb) in the fall in CBF after SAH. Intracisternal (icv) injection of blood produced a greater and more prolonged (120 vs. 30 min) decrease in CBF than that produced by a 4% solution of Hb. Pretreating rats with Nω-nitro-L-arginine methyl ester (L-NAME; 10 mg/kg iv) to block the synthesis of NO had no effect on the fall in CBF produced by an icv injection of blood. L-NAME enhanced rather than attenuated the fall in CBF produced by an icv injection of Hb. Blockade of the synthesis of 20-HETE with TS-011 (0.1 mg/kg iv) prevented the sustained fall in CBF produced by an icv injection of blood and the transient vasoconstrictor response to Hb. Hb (0.1%) reduced the diameter of the basilar artery (BA) of rats in vitro by 10 ± 2%. This response was reversed by TS-011 (100 mM). Pretreatment of vessels with l-NAME (300 μM) reduced the diameter of BA and blocked the subsequent vasoconstrictor response to the addition of Hb to the bath. TS-011 returned the diameter of vessels exposed to l-NAME and Hb to that of control. These results suggest that the fall in CBF after SAH is largely due to the release of vasoactive factors by clotting blood rather than the scavenging of NO by Hb and that 20-HETE contributes the vasoconstrictor response of cerebral vessels to both Hb and blood.

Cerebral vasospasm is a critical complication of subarachnoid hemorrhage (SAH). Vasospasm occurs in 70% of patients with aneurysmal SAH and leads to ischemic deficits in 36% of patients (5). Despite extensive investigation, the factors that trigger the decline in cerebral blood flow (CBF) after SAH remain to be determined.

The fall in CBF after SAH correlates with the amount of hemoglobin (Hb) released into cerebrospinal fluid (CSF), and vasospasm can be triggered by an injection of Hb alone into the CSF (31, 51). Hb induces hemeoxygenase-1 (24) that increases iron levels, which generate superoxide radicals (30). Superoxide radicals at low concentration have been reported to contract vessels in several vascular beds in part by decreasing the levels of nitric oxide (NO) (13, 42), and there is a recent report that supports a similar mechanism in cerebral arteries (55). Free radicals also increase the production of lipid peroxides and isoprostanes (20, 44) that are potent constrictors of cerebral arteries (19).

Previous studies have focused on the role of various vasoconstrictor pathways in the development of cerebral vasospasm. The levels of endothelin (46), thromboxane (36), ATP (31), isoprostanes (44), glutamate (4), platelet-activating factor (PAF) (17) and serotonin (5-HT) (6, 43) in CSF all increase after SAH, and the response of cerebral arteries to most of these constrictors is enhanced. Cerebral vasospasm after SAH has been reported to be attenuated by inhibitors of endothelin synthesis or receptors (10, 12), by 5-HT receptor antagonists (6), and by inhibitors of the downstream effectors of these vasoconstrictors, including Ras, Rho, mitogen-activated protein kinase (MAPK), and protein kinase C (PKC) (25, 26, 33, 52). There is also enhanced release of fatty acids (37) after SAH that increases the formation of vasoactive metabolites of arachidonic acid (AA) (7, 8). More recent studies have focused on the role of 20-hydroxyecosatetraenoic acid (20-HETE) in the development of cerebral vasospasm. 20-HETE is a potent vasoconstrictor that is produced by enzymes of the cytochrome P450 (CYP) 4A and 4F families in cerebral arteries and polymorphonuclear leukocytes (40). 20-HETE shares many of the properties associated with the pathogenesis of cerebral vasospasm. It activates PKC, Ras, tyrosine kinase, and MAPK signal transduction cascades (41), and it promotes calcium entry by depolarizing (29) cerebral arteries secondary to blockade of the large conductance Ca2++-activated K+ (KCa) channel (16, 27, 57). 20-HETE also increases Ca2+ influx by activating l-type Ca2+ channels in the cerebral vasculature (11).

Recent studies have revealed that the concentration of 20-HETE increases in the CSF of rats (6, 22), dogs (14), and humans (15, 38) after SAH and that inhibitors of the synthesis of 20-HETE or its vasoconstrictor actions prevent the acute fall in CBF after SAH in rats (6, 22, 23, 33) and reverse delayed vasospasm in both dogs (14) and rats (49). However, the factors released by clotting blood that stimulate the release of 20-HETE in CSF after SAH, and the mechanisms by which 20-HETE interact with the other constricting factors to contribute to the fall in CBF after SAH are unknown. Thus the present study examined the relative importance of the release...
of vasoactive factors by clotting blood vs. scavenging of NO by Hb in triggering the fall in CBF after SAH. We also examined the effects of blocking the synthesis of NO with N^\text{6}-\text{nito}-l-arginine methyl ester (l-NAME) and 20-HETE with TS-011 on the fall in CBF produced by injections of blood and Hb into the cisterna magna of rats in vivo and on the vasoconstrictor response of rat basilar arteries (BAs) to Hb in vitro.

**MATERIALS AND METHODS**

**General.** Experiments were performed on male Sprague-Dawley rats purchased from Taconic Farms (Germantown, NY) that weighed 250–350 g. The rats were housed in an American Association for Accreditation of Laboratory Animal Care-approved Animal Care Facility at the Medical College of Wisconsin, and they had free access to food and water. All protocols were approved by the Animal Care and Use Committee of the Medical College of Wisconsin.

**Induction of SAH and measurement of cerebral blood flow.** Rats were surgically prepared for induction of SAH and measurement of CBF using laser Doppler flowmetry (Perimed, Stockholm, Sweden), as previously described (6, 22). Briefly, the rats were anesthetized with isoflurane (2.0%), and the left femoral artery and vein were cannulated for measurement of arterial pressure and for the infusion of drugs. Body temperature was maintained at 37°C with a heating pad. The rats were positioned in a stereotaxic device, and a 3 mm area next to the left parietal bone overlying the irrigation area of the middle cerebral artery (MCA) was thinned with hand-held drill until the pial vessels were visible. A laser-Doppler probe was placed above the cranial window for measurement of regional CBF. The atlanto-occipital membrane was exposed, and a 30-gauge needle attached to a PE-10 catheter was inserted into the cisterna magna for withdrawal of CSF and for injection of blood, saline, or 0.3 ml of autologous, unheparinized arterial blood or a 4% solution of bovine Hb (Sigma, St. Louis, MO) in saline containing 40 mM OxyHb. The concentration of OxyHb in the 4% solution of Hb was calculated by measuring the absorbance of the solution at 577 and 630 nm using the following equation: OxyHb (mM) = (66.5A577 nm - 80.6A630 nm)/4.

**CBF was continuously monitored using digital recording software (WinDaq, Dataq Instruments, Akron, OH).** After surgery and a 30-min equilibration period, the average value of CBF recorded over a 5-min period was taken as a control value. Experimental values of CBF were taken as the mean value recorded over 2-min periods, 30, 60, 90, and 120 min after an intracisternal (icv) injection of blood, Hb or saline. CBF is expressed as a percentage of the control value measured in the same animal.

**Protocol 1: comparison of the effects of icv injections of blood and Hb on CBF.** After surgery and a 30-min equilibration period, CBF was recorded for a 5-min control period. The rats in group 1 (blood, n = 9) received an infusion of 0.3 ml of autologous, unheparinized arterial blood into the cisterna magna over a 10-min period. CBF was continuously monitored for 2 h. Group 2 (Hb, n = 6) received an infusion of 0.3 ml of 4% solution of Bovine Hb in the cisterna magna over a 10-min period; the change in the diameter of the vessel was determined. Additional experiments were performed to better define the relative importance of NO vs. 20-HETE in mediating the vasoconstrictor response to Hb. The vessels were pretreated with l-NAME (300 µM) to block the formation of NO, and the change in the diameter was determined. Then, Hb (0.1%) was added to the bath, and the change in vascular diameter was redetermined. Finally, TS-011 (100 nM), an inhibitor of the synthesis of 20-HETE, was added to the bath to assess the contribution of 20-HETE to the vasoconstrictor response to combined addition of l-NAME and Hb to the bath.

**Statistical analysis.** Mean values ± SE are presented. The significance of differences in mean values between and within groups was evaluated using an ANOVA for repeated measures followed by Holm-Sidak test. A P value of <0.05 was considered to be significant.

**RESULTS**

**Protocol 1: comparison of the effects of icv injections of blood and Hb on CBF.** The results of these experiments are presented in Fig. 1. An icv injection of saline had no effect on CBF. CBF fell to 51.1 ± 9.6% of control after an icv injection of saline, while a 30-min equilibration period, the change in CBF was recorded. The rats then received an infusion of 0.3 ml of autologous, unheparinized arterial blood or a 4% solution of bovine Hb in the cisterna magna, and CBF was continuously measured for an additional 2 h.

**Protocol 2: role of NO in mediating the fall in CBF induced by an icv injection of Hb or blood.** The rats were surgically prepared for induction of SAH and the baseline level of CBF was determined. The rats then received an intravenous injection of l-NAME (10 mg/kg) to block the synthesis of NO, and CBF was observed for 30 min. After determining the effect of l-NAME on CBF, the rats received an infusion of 0.3 ml of autologous, unheparinized arterial blood or a 4% solution of bovine Hb into the cisterna magna over a 10-min period. CBF was continuously monitored for an additional 2 h.

**Protocol 3: effects of TS-011, an inhibitor of the synthesis of 20-HETE, on the fall in CBF induced by an icv injection of Hb or blood.** After surgery and measurement of baseline CBF, the rats received an intravenous injection of TS-011 (0.1 mg/kg) (31) and after
of blood and it remained 30% below control for the entire 2-h course of the experiment. CBF fell to 63.5 ± 7.2% of control immediately after an icv administration of Hb. However, the response was transient and returned to levels not significantly different from control within 1 h.

Protocol 2: effects of blockade of NO on the fall in CBF after an icv injection of blood or Hb. Pretreatment of the rats with L-NAME reduced baseline CBF by 15% (Fig. 2). However, CBF still fell by an additional 22% after an icv administration of blood. The fall in CBF in rats pretreated with L-NAME was of the same magnitude and duration as the response seen in control rats. CBF fell by 28% immediately following an icv injection of Hb in rats pretreated with L-NAME. The magnitude of the fall in CBF after administration of Hb tended to be slightly, but not significantly, greater in rats treated with L-NAME than that seen in the control rats, and the duration of the response was more prolonged (120 vs. 30 min).

Protocol 3: effects of TS-011 on the fall in CBF following an icv injection of blood or Hb. The effects of TS-011, a selective inhibitor of the synthesis of 20-HETE (31), on the fall in CBF induced by an icv injection of blood or Hb are presented in Fig. 3. Blockade of the synthesis of 20-HETE had no significant effect on baseline CBF. TS-011 had no effect on the immediate fall in CBF seen following icv injection of blood or Hb solution, which is associated with the transient rise in intracerebral pressure. However, TS-011 completely blocked the sustained fall in CBF seen after an icv administration of blood. It also prevented the transient fall in CBF seen at 30 min after icv administration of Hb.

Protocol 4: role of 20-HETE and NO in mediating the vasoconstrictor response of the BA to Hb in vitro. The results of these experiments are presented in Fig. 4. The control diameter of the BA averaged 194.2 ± 0.2 μm. Addition of Hb (0.1%) to the bath reduced the diameter of the BA by 20.0 ±
solution of Hb alone. Moreover, pretreatment of the rats with a dose of L-NAME (10 mg/kg iv) that completely blocks the synthesis of NO in brain homogenates (9, 21) lowers baseline CBF but did not block the fall in CBF produced by an icv administration of Hb. Rather, the duration of the vasoconstrictor response to Hb was prolonged in L-NAME-treated rats relative to that seen in control animals. These results indicated that scavenging of NO plays little, if any, role in transient fall in CBF produced by an icv administration of Hb. The results further suggest that upregulation of production of NO in the brain appears to oppose the sustained vasoconstrictor response to Hb, and this likely explains the transient nature of the fall in CBF following icv administration of Hb.

In contrast, blockade of the synthesis of NO with L-NAME had no effect on either the magnitude or duration of the fall in CBF produced by an icv injection of blood. This finding suggests that factors other than scavenging of NO contribute to the fall in CBF following the icv injection of blood and SAH. It is likely that the release of vasoconstrictor mediators by clotting blood plays a major role in triggering the fall in CBF after SAH. This conclusion is further supported by previous findings that the levels of endothelin (46), thromboxane (36), ATP (31), isoprostanes (44), glutamate (4), PAF (18), and 5-HT (6, 43) in CSF all increase after SAH. In addition, this view is consistent with the evidence that cerebral vasospasm after SAH is attenuated by inhibitors of endothelin synthesis or receptors (10, 12), 5-HT receptor antagonists (6), and inhibitors of downstream second messengers of cerebral vasoconstriction, including Ras, Rho, MAPK, and PKC (25, 26, 33, 52).

The present study also explores the role of 20-HETE in mediating the vasoconstrictor response to icv administration of Hb and blood, since previous studies have indicated that the vasoconstrictor response to 20-HETE in cerebral arteries mimics the changes in vascular tone and reactivity associated with cerebral vasospasm (53). The present finding that blockade of the synthesis of 20-HETE with TS-011 prevents the sustained fall in CBF following an icv administration of blood is consistent with the results of previous studies using less selective inhibitors of the synthesis of 20-HETE (6, 22). 20-HETE is a potent constrictor of cerebral arteries that reduces the open state probability of KCa channels through activation of PKC (27). It also increases the sensitivity of the contractile apparatus to Ca2+ by activating Rho kinase (40). The formation of 20-HETE in vascular smooth muscle (VSM) is stimulated by ANG II (2, 34), endothelin (34), 5-HT (6), and other vasoconstrictors, and blockade of the synthesis of 20-HETE attenuates the vasoconstrictor response to ANG II (2, 34), endothelin (35), vasopressin (50), ATP (56), and 5-HT (6). Thus it seems likely that endothelin, 5-HT, ATP, and other vasoconstrictors that are released in large quantities by clotting blood stimulate the production of 20-HETE in cerebral arteries. 20-HETE then acts to potentiate the vasoconstrictor actions of these mediators by depolarizing cerebral VSM cells secondary to blocking the KCa channels (27, 29). This hypothesis that converges on 20-HETE in the common final pathway leading to cerebral vasospasm after SAH helps explain how seemingly unrelated inhibitors like endothelin and 5-HT receptor antagonists (6, 10), inhibitors of the synthesis and action of 20-HETE (6, 22, 23, 32) and blockers of PKC and Rho kinase (26, 33), which
are downstream effectors of 20-HETE (41), all attenuate the fall in CBF following SAH.

One of the new findings of the present study is that inhibition of the formation of 20-HETE with TS-011 attenuated the transient fall in CBF produced by an icv administration of Hb and the vasoconstrictor response of isolated perfused BA to Hb in vitro. These studies further suggest that the vasoconstrictor response of the cerebral arteries to Hb in vitro, which appeared to be largely mediated by scavenging of NO, as it was completely blocked by L-NAME, is dependent on the formation of 20-HETE. This finding does not fit with the generally accepted view that the vasodilator response to NO is secondary to activation of soluble guanylate cyclase and elevations in cGMP (28). However, we and others have reported that NO directly binds to heme in CYP4A enzymes and inhibits the formation of 20-HETE (47). We have also shown that NO induced activation of the K_{Ca} channels in rat renal interlobular arteries and in the middle cerebral artery of the rat is cGMP independent and is associated with inhibition of the endogenous formation of 20-HETE in VSM cells (47, 48). Further studies have shown that inhibitors of the synthesis of 20-HETE reduce the vasodilator response to NO in renal and cerebral arteries by 50–75% (1, 3) and the pressor response of rats to blockade of NO synthesis with L-NAME in vivo (17). Thus the present data suggest that the vasodilator action in the cerebral vessels to scavenging of NO by Hb (at least in vitro) is due, in part, to an increase in 20-HETE production in cerebral arteries, which blocks K_{Ca} channels and depolarizes VSM cells. Moreover, these findings suggest that the success of recent therapeutic strategies using NO donors and NO synthase gene therapy for the treatment of cerebral vasospasm (38, 45) will likely depend on the levels of expression of CYP4A enzymes in cerebral arteries and elevated levels of 20-HETE.

**Perspectives**

The results of the present study indicate that the fall in CBF after SAH is largely due to the release of vasoactive factors by clotting blood rather than the scavenging of NO by free Hb and that 20-HETE plays a role in the vasoconstrictor response of cerebral vessels to both Hb and blood. These results further suggest that inhibitors of 20-HETE may be useful for the treatment of vasospasm after SAH, as 20-HETE plays a role in both the fall in NO levels and the release of vasoactive mediators (endothelin, ATP, 5-HT) that trigger cerebral vasospasm when Hb or blood are introduced into CSF.

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