Microvasospasms After Experimental Subarachnoid Hemorrhage Do Not Depend on Endothelin A Receptors

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Background and Purpose—Perturbations in cerebral microcirculation (eg, microvasospasms) and reduced neurovascular communication determine outcome after subarachnoid hemorrhage (SAH). ET-1 (endothelin-1) and its receptors have been implicated in the pathophysiology of large artery spasms after SAH; however, their role in the development of microvascular dysfunction is currently unknown. Here, we investigated whether inhibiting ET_A (endothelin A) receptors can reduce microvasospasms after experimentally induced SAH.

Methods—SAH was induced in male C57BL/6 mice by filament perforation of the middle cerebral artery. Three hours after SAH, a cranial window was prepared and the pial and parenchymal cerebral microcirculation was measured in vivo using two-photon microscopy before, during, and after administration of the ET_A receptor inhibitor clazosentan. In separate experiments, the effect of clazosentan treatment on neurological outcome was measured 3 days after SAH.

Results—Clazosentan treatment had no effect on the number or severity of SAH-induced cerebral microvasospasms nor did it affect neurological outcome.

Conclusions—Our results indicate that ET_A receptors, which mediate large artery spasms after SAH, do not seem to play a role in the development of microarterial spasms, suggesting that posthemorrhagic spasms are mediated by distinct mechanisms in large and small cerebral vessels. Given that cerebral microvessel dysfunction is a key factor for outcome after SAH, further research into the mechanisms that underlie posthemorrhagic microvasospasms is urgently needed.

Visual Overview—An online visual overview is available for this article. **(*Stroke*. 2018;49:693-699. DOI: 10.1161/STROKEAHA.117.020028.)**

Key Words: cerebrovascular circulation ■ endothelin-1 ■ mice ■ microcirculation ■ subarachnoid hemorrhage

Subarachnoid hemorrhage (SAH) is a devastating subtype of stroke most commonly caused by the rupture of a cerebral aneurysm. SAH predominantly occurs in relatively younger (ie, 40–60 years) patients and often leaves the survivor severely disabled. Strikingly, SAH incurs nearly the same socioeconomic cost as ischemic stroke, which affects 20× as many patients. Although early post-SAH mortality (ie, within the first 24 hours after the insult) has declined in recent decades because of improved emergency and surgical care, the 30-day mortality rate is still high.[1–4] Moreover, the cause of death of these closely monitored—and in many cases, apparently stable—patients is often unknown.

Poor outcome after SAH, including delayed mortality, is associated with delayed ischemic neurological deficits, which often cause secondary infarcts. For decades, delayed vasospasms of large arteries located primarily within the circle of Willis were thought to underlie the delayed ischemia and poor outcome after SAH. Moreover, a vast amount of research revealed that the vascular endothelin receptor subtype ET_A (endothelin A) is a primary contributor to posthemorrhagic large artery spasms.[5–11] However, although recent clinical trials showed that the ET_A receptor antagonist clazosentan can resolve large artery spasms, it did not improve patient outcome.[12,13] Therefore, these results raised doubts on whether large artery spasms indeed underlie the delayed ischemia after SAH.

In search for other mechanisms that underlie post-SAH ischemia and determine clinical outcome, researchers observed that despite normal cerebral perfusion pressure and the absence of large artery spasms, patients developed cerebral hypoperfusion within the first few days after SAH.[14,15] This paradoxical finding was also observed in animals after experimental SAH[16] and was associated with microvasospasms[17] that resulted in oligemia and lipid peroxidation,[16,18] ultimately

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leading to microvascular thrombosis[19] and complete loss of microvascular perfusion. Therefore, determining precisely why microvasospasms occur after SAH might provide a novel therapeutic strategy for improving tissue perfusion in these patients. However, the mechanisms that underlie the development of microvasospasms after SAH are largely unknown.

Here, we investigated whether inhibiting ET_A receptors—a strategy that can resolve large artery spasms after SAH—may also be effective in reducing cerebral microvasospasms after experimental SAH, providing a possible therapeutic strategy for preventing post-SAH cerebral microvasospasms. For this purpose, we chose a mouse model that reproduces the main features of early brain injury, early mortality, marked microcirculatory hypoperfusion, and microvasospasm.

Materials and Methods

The authors declare that all supporting data are available within the article (and its online-only Data Supplement). All animal procedures, group size calculations, and statistical methods used were approved by the Government of Upper Bavaria (AZ#66-08). Sample size was calculated using SigmaPlot v13.0 (Jandel Scientific, Erkrath, Germany). The results of the study are reported in accordance with the ARRIVE guidelines. Given the relevant differences between genders with respect to stroke pathophysiology, and given that this study was intended to provide proof-of-principle rather than therapeutic intent, we used only male animals, thereby ensuring comparability of our results with previous studies performed using the same setup, model, and drug regimen.

Randomization and Blinding

After the induction of SAH, animals were randomly assigned to either the treatment or the control group by drawing lots. Where appropriate, the surgical preparation for SAH was performed in a blinded fashion. Neurological testing, data collection, and data analysis were performed by a researcher blinded with respect to the treatment group.

Anesthesia and Surgical Preparation

Male C57BL/6 mice (6–8 weeks, 24–26 g) were obtained from Charles River Laboratories (Kisslegg, Germany) and anesthetized in a chamber containing 4% isoflurane (Halocarbon Laboratories, Peachtree Corners, GA). Anesthesia was maintained by an intraperitoneal injection of a combination containing medetomidine (0.5 mg/kg bodyweight), midazolam (5 mg/kg), and fentanyl (0.05 mg/kg). The mice were intubated and mechanically ventilated with 30% O_2 and 70% air (MiniVent 845; Hugo-Sachs-Elektronik, March-Hugstetten, Germany) as described previously.[20] Ventilation was continuously monitored using microcapnometry (MicroCapnograph CI240; Columbus Instruments, Columbus, OH), and end-tidal CO_2 was held at 35 to 45 mm Hg by adjusting the respiration parameters. Systemic blood pressure and arterial blood gases were monitored via a femoral artery catheter. Body temperature was held at 37°C using a feedback-controlled heating pad. After induction of SAH and a 15-minute postinduction observation period, anesthesia was reversed by intraperitoneal injection of atipamezole (2.5 mg/kg), naloxone (1.2 mg/kg), and flumazenil (0.5 mg/kg), after which the animals were placed in an incubator at 33°C for 24 hours.

Measurement of Regional Cerebral Blood Flow

Regional cerebral blood flow (rCBF) was measured as described previously.[21] In short, a laser Doppler probe (Perimed, Järfälla, Sweden) was positioned perpendicular to the skull over the ipsilateral middle cerebral artery territory. Laser Doppler flux was recorded continuously from 10 minutes prior to 20 minutes after the induction of SAH. In in vivo microscopy experiments (3 hours after SAH), rCBF was measured after anesthesia induction and was recorded continuously through the observation period.

Measurement of Intracranial Pressure

Intracranial pressure (ICP) was measured using a microchip-based probe (Codman ICP monitor; Codman, Ascot, United Kingdom) placed in the epidural space over the contralateral hemisphere as described previously.[22] ICP was recorded 10 minutes before and for 20 minutes after the induction of SAH.

Blood Gas Analysis

Arterial blood samples were collected before SAH and at the end of the observation period and analyzed using a blood gas analyzer (Chiron Diagnostics Corporation, East Walpole, MA).

Induction of SAH

SAH was induced using the endovascular perforation technique, as described previously.[17,21] In brief, a 5-0 filament was inserted into the left external carotid artery and advanced toward the circle of Willis. Hemorrhage was confirmed by a rapid increase in ICP with a corresponding decrease in rCBF. The filament was then removed, the external carotid artery ligated. In sham surgery, the filament was inserted but not advanced far enough to produce a middle cerebral artery perforation.

Drug Application

The ET_A receptor–specific antagonist clazosentan was kindly provided by Actelion Pharmaceuticals (Basel, Switzerland). For the dose-finding and chronic (neuroscore) experiments, clazosentan was administered intravenously in the internal jugular vein via an ALZET osmotic mini-pump (Durect Corporation, Cupertino, CA) implanted subcutaneously. In the first set of experiments (dose finding; schematic drawing is given in Figure IA in the online-only Data Supplement), clazosentan was administered at 1, 3, 10, or 20 mg/kg bodyweight, and mean arterial blood pressure (MAP), rCBF, and ICP were monitored continuously for 180 minutes starting 15 minutes after successful pump implantation. Subsequent experiments evaluating post-SAH outcome for up to 72 hours (chronic group; schematic drawing is given in Figure IC in the online-only Data Supplement) were performed using 10 mg/kg clazosentan administered continuously via an osmotic mini-pump beginning 30 minutes before SAH induction. In in vivo microscopy experiments, clazosentan was continuously administered via a femoral vein catheter over a period of 30 minutes; cerebral microcirculation was monitored for 30 minutes before, during, and for 60 minutes after drug injection (a graphical summary is given in Figure IB in the online-only Data Supplement).

Intravital Microscopy

In vivo two-photon microscopy was performed as described previously.[23,24] In brief, after induction of anesthesia and surgical preparation, a cranial window was prepared over the left middle cerebral artery territory (Figure IIA in the online-only Data Supplement). After an intravenous injection of 150 µL fluorescein isothiocyanate-dextran (MW 150 kDa; Sigma-Aldrich, Deisenhofen, Germany; Figure 1, for example image), the pial microvessels (ranging from 9.5 to ≈80 µm in diameter, 15–30 vessel segments per animal, with at least 4 measurements for each vessel category; n=8 mice per group) were visualized and subsequently categorized as either category 1 (9.5–25 µm diameter) or category 2 (>25 µm diameter). Three hours after SAH, two-photon microscopy was performed; after recording baseline values for 30 minutes, mice received an intravenous infusion of either clazosentan (10 mg/kg) or vehicle (phosphate-buffered saline; control) for 30 minutes, after which cerebral microcirculation was imaged for an additional 30 minutes. After an injection of fluorescein isothiocyanate-dextran, the cerebral microcirculation was scanned at 10-minute intervals and analyzed offline as described previously.[17,25] Several parameters were recorded to evaluate the severity, and degree

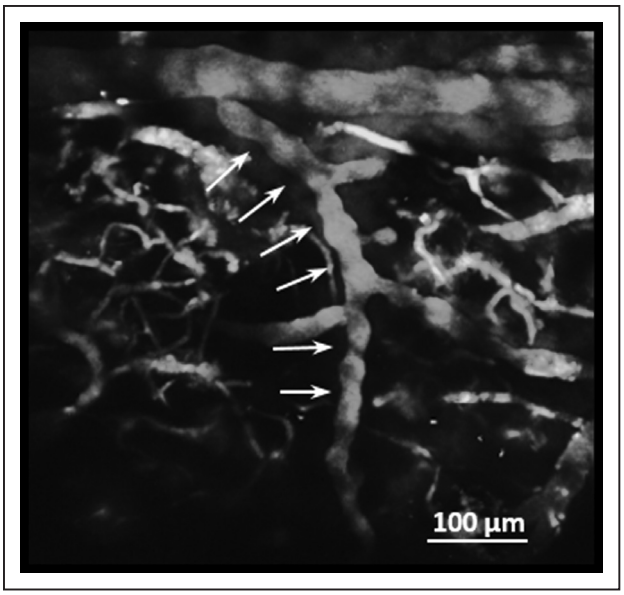

Figure 1. Intravital microscopy of cerebral microvesssels. Exemplar in vivo microscopy image taken 3 hours after subarachnoid hemorrhage; note the microvascular constriction and pearl string–like appearance (arrows).

of microvasospasm was recorded; specifically, vessel diameter was measured in a segment that was deemed to be nonspastic, and the severity of the spasm was assessed using a spasticity index shown in Figure IIB in the online-only Data Supplement (the constriction grade of the spastic vessel compared with a nonspastic vessel segment was calculated for each vessel at each time point and compared with the respective baseline value).

Neurological Function

Neurological outcome (n=9 mice per control group and n=8 per clazosentan group, 1 dropout that died within 20 minutes after SAH most probably because of rebleeding) was assessed by testing motor function (eg, the grasping reflex and the righting reflex), parameters reflecting general well-being (eg, bodyweight, fur grooming), and vigilance as described previously.[21] A composite score of 0 points was assigned to healthy animals, and the maximum deficit score was 33 points.

Statistical Analysis

All data are presented as median±SD. All data were assumed to be nonparametric. For comparisons between 2 groups, we used the Mann–Whitney rank-sum test. Multiple groups were analyzed using the Kruskal–Wallis ANOVA on ranks, and repeated measurements were analyzed using the Friedman test. Calculations were performed using a standard statistical software package (SigmaPlot 13.0; Jandel Scientific, Erkrath, Germany). Differences between groups were considered significant at $P<0.05$. Sample size calculations were performed using the following parameters: α error=0.05; β error=0.2; calculated SD 15% to 20% (depending on the parameter investigated); and biologically relevant difference 30%.

Results

First, we continuously infused 1, 3, 10, or 20 mg/kg clazosentan into the internal jugular vein of healthy C57BL/6 mice for 3 hours while measuring MAP (Figure 2A) and rCBF (Figure 2B). Although clazosentan had no effect on either MAP or rCBF at any dose, 20 mg/kg caused a slight decrease in MAP toward the end of the observation period; therefore, we used 10 mg/kg clazosentan for the subsequent experiments.

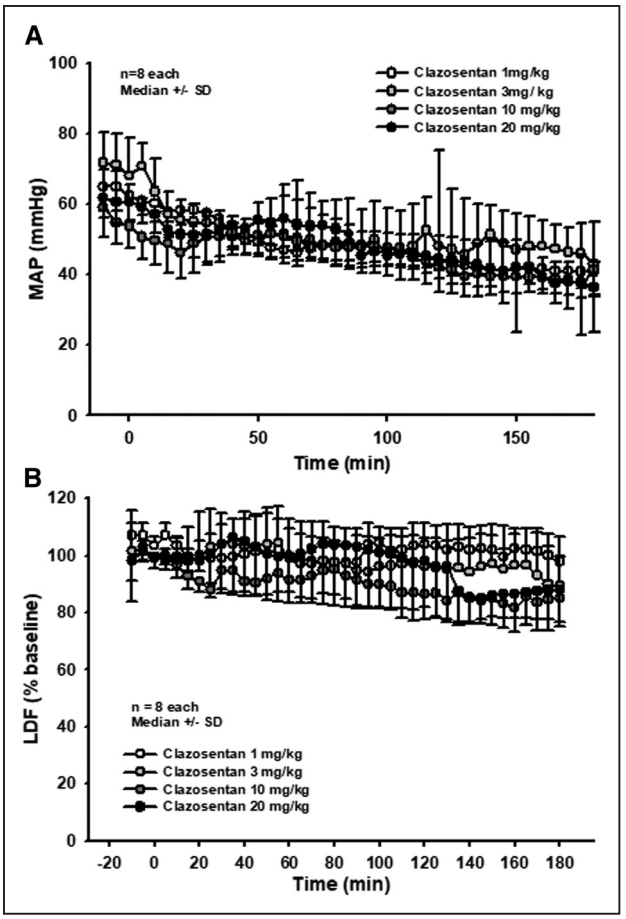

Figure 2. Dose-finding experiment. Clazosentan at the indicated dosages or saline (control) was administered by intravenous infusion as shown in Figure 1, and mean arterial blood pressure (MAP; **A**) and cerebral blood flow (**B**) were monitored for 3 hours.

SAH was induced using the middle cerebral artery filament perforation model; it caused both a rapid increase in ICP (Figure 3A) and a rapid decrease in rCBF (Figure 3B); ICP remained significantly elevated until the end of the observation period, CBF normalized within 10 minutes. Sham-operated mice did not show any increase in ICP or decrease in cerebral blood flow (data not shown). There was no significant difference in either ICP or rCBF between the control and clazosentan groups; physiological parameters directly after SAH induction were within normal limits in both groups (Table II in the online-only Data Supplement). Animals were allowed to wake up afterward; 2 hours after SAH, mice were reanesthetized and prepared for intravital microscopy. After baseline recording, they were randomized to the clazosentan or control group. MAP was not affected by clazosentan treatment and remained within the physiological range throughout the observation period (Figure 3C). Blood gases measured at the end of the observation period did not differ between the 2 groups (Table III in the online-only Data Supplement). As illustrated in Figure 1, 3 hours after SAH, we observed a global narrowing of the arterioles and vessels, with a pearl string–like appearance, reflecting the presence of microvasospasms, consistent with previous reports.[17,26] Sham-operated mice did not exhibit any alterations of the cerebral

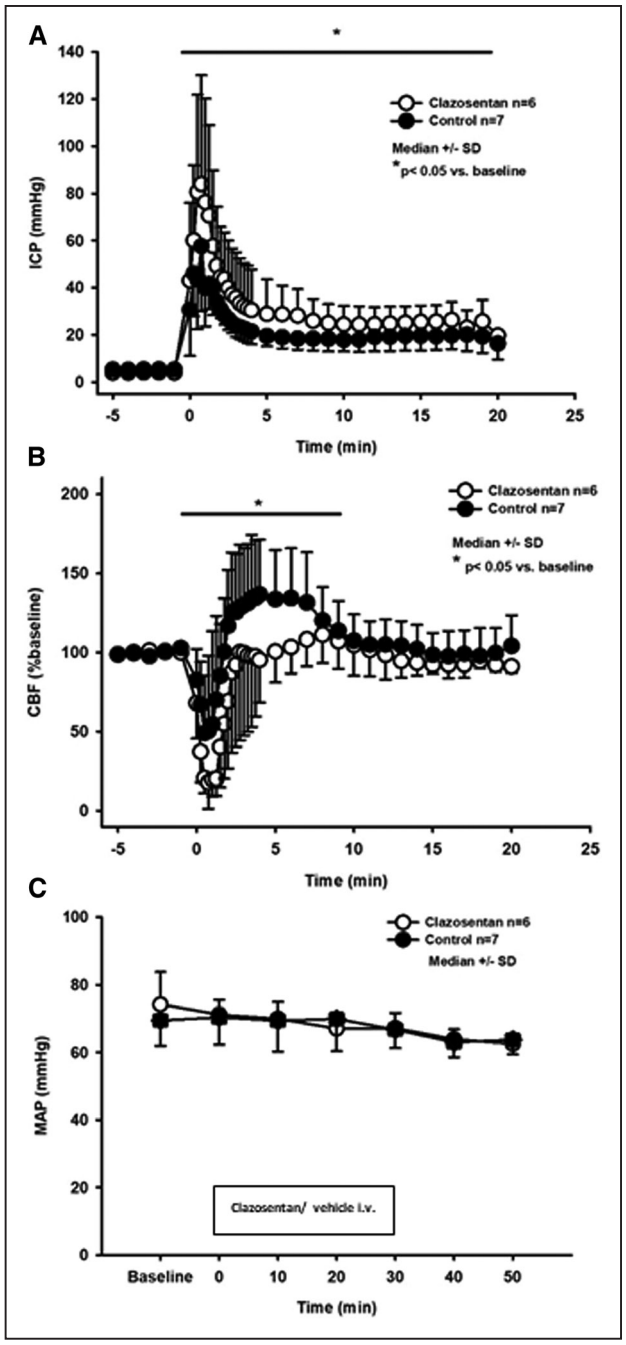

Figure 3. Induction of subarachnoid hemorrhage (SAH). SAH was successfully induced in all animals, reflected by a significant, rapid increase in intracranial pressure (ICP) peak (**A**) and decrease in regional cerebral blood flow (rCBF; **B**) at the time of middle cerebral artery perforation. Three hours later, mice were reanesthetized and prepared for in vivo microscopy (IVM); randomization to the treatment or clazosentan group was performed after surgical preparation and recording of baseline. **C**, Mean arterial blood pressure (MAP) was measured during IVM and was not affected by clazosentan infusion (baseline refers to 3 hours after SAH).

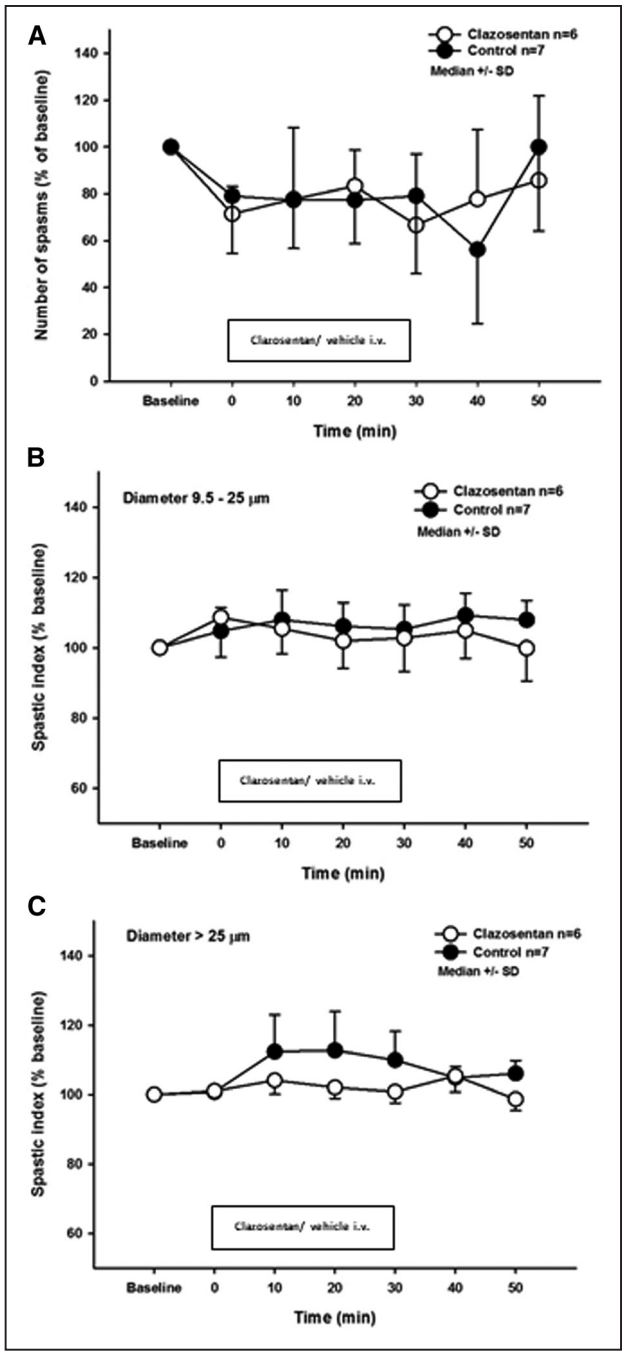

Figure 4. Clazosentan does not improve cerebral microcirculation after subarachnoid hemorrhage (SAH). Time course of the total number of vasospasms (**A**) and the spasticity index in small-diameter (**B**) and larger-diameter vessels (**C**) in mice treated with clazosentan or saline (control), expressed relative to baseline recorded before randomization to treatment at *t*=3 hours after SAH.

microcirculation such as microarterial constriction or microvasospasm. Clazosentan treatment had no significant effect on the total number of microvasospasms (Figure 4A); similarly, clazosentan had no significant effect on the severity of microvasospasms in small or large microvessels (Figure 4B and 4C, respectively). Moreover, clazosentan had no significant effect on the diameter of nonspastic segments of vessels (Figure 5). Last, we examined whether pretreating mice with clazosentan can improve neurological outcome after SAH (Figure IC in the online-only Data Supplement). Consistent with our results on microvasospasms, we found that clazosentan did not improve neurological outcome assessed using a multivariate neurological score (Figure 6).

The numeric data for all groups/experiments are provided in Tables IV through XIV in the online-only Data Supplement.

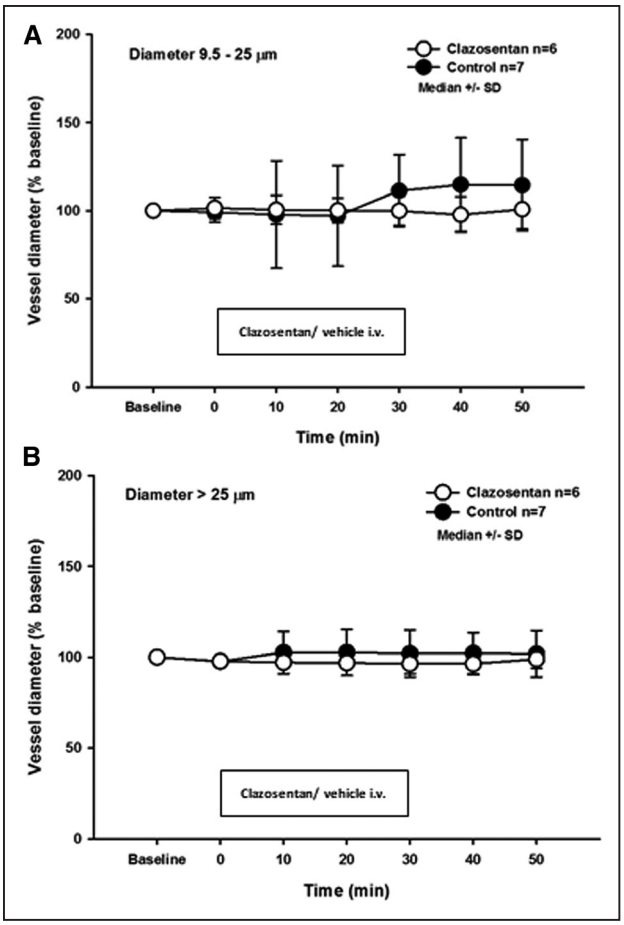

Figure 5. Clazosentan does not affect the diameter of spastic microvessels. Vessel diameter was measured in the nonspastic segments of small-diameter (**A**) and larger-diameter (**B**) vessels in mice treated with clazosentan or saline (control), expressed relative to baseline.

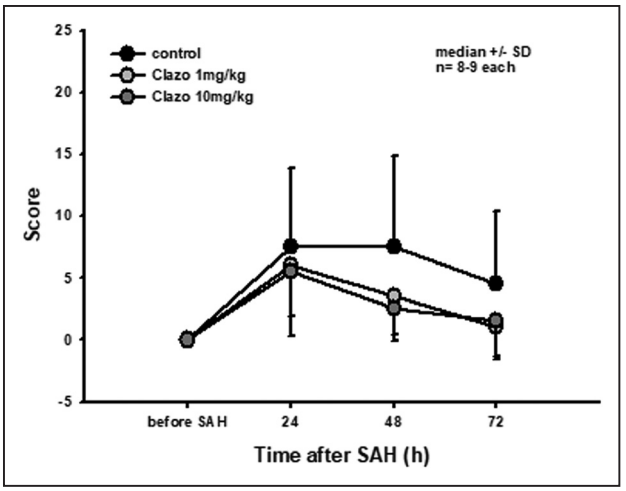

Figure 6. Clazosentan does not improve neurological outcome after experimental subarachnoid hemorrhage (SAH). Neurological outcome was assessed using a multivariate neurological score 1 day before SAH and for 3 days after SAH. Where indicated, the mice were treated with saline (control), 1 mg/kg clazosentan, or 10 mg/kg clazosentan.

Discussion

The CONSCIOUS-1 trial (Clazosentan to Overcome Neurological Ischemia and Infarction Occurring After Subarachnoid Hemorrhage), a large phase 2 clinical trial designed to investigate the role of ET_A receptors in post-SAH ischemia and outcome, reported that ET_A receptor inhibitor clazosentan significantly reduced SAH-induced angiographic vasospasms.[12] Thus, researchers hoped that delayed vasospasm—which was thought to be the key factor in delayed cerebral ischemia and neurological outcome—would finally be manageable. However, subsequent trials found that inhibiting ET_A receptors had no beneficial effect on ischemic infarcts or functional outcome.[13,27,28] More recent studies therefore primarily focused on the early posthemorrhagic phase (ie, within 72 hours of the SAH), which is characterized by a reduction in cerebral blood flow with no change in cerebral perfusion pressure[14,15] followed by microcirculatory dysfunction. A variety of mechanisms collectively referred to as early brain injury have been proposed to contribute to this phenomenon[16,18] and are now considered to be the pathophysiological correlate of delayed ischemic changes after SAH. By directly visualizing the cerebral microcirculation soon after SAH, microarteriolar constriction (ie, microvasospasm) can be observed under both experimental[17,29,30] and clinical[26,31] conditions. To date, the mechanisms that underlie microvasospasm remain poorly understood. Our group[29] and others[32,33] have found evidence suggesting that global or local depletion of nitric oxide (NO), a potent vasodilator, plays a causal role in the formation of posthemorrhagic microcirculatory dysfunction; specifically, increasing NO levels causes a rapid reduction in microarteriolar constriction and significantly improves both mortality and outcome,[29] demonstrating that reducing microvasospasms can indeed improve both structural and functional outcomes. As NO depletion is also a major pathophysiological feature in delayed-onset vasospasm,[34,35] it seemed reasonable to assume that spasms of large and microvessels after SAH may have some pathophysiological similarities. ET-1 (endothelin-1) is a potent, long-lasting endogenous vasoconstrictor that has been implicated in the delayed arterial vasospasm in the circle of Willis.[36,37] Moreover, ET-1 levels are elevated both in patients[9] and in animal models after experimental SAH.[38] Although most clinical studies measured increased ET-1 in the cerebrospinal fluid or plasma at relatively later time points (eg, >48 hours after hemorrhage), there is experimental evidence showing that ET-1 levels are significantly elevated as early as 30 minutes after the induction of SAH.[8] The vasoconstrictive effect of ET-1 after SAH seems to be mediated primarily by the ET_A receptor subtype.[39,40] Using a peptide-based ET_A receptor antagonist, Clozel and Watanabe[6] found that cerebral blood flow was significantly increased in a rat model of SAH; however, the antagonist was effective only when administered intracerebroventricularly. This practical limitation was overcome with the introduction of the selective nonpeptide ET_A receptor inhibitor R0 61–1790, which was later named clazosentan; intravenous injection of clazosentan before the induction of SAH significantly improved post-SAH cerebral blood flow in both rats[41] and mice.[42] Moreover, clazosentan improved the initial widespread hypoperfusion and delayed the onset of vasospasm; however, clazosentan did not reduce ischemia or improve neurological outcome after SAH in

clinical studies. However, as these trials were specifically designed to assess angiographic vasospasm at later time points after SAH and did not include measures to investigate early brain injury, it remained unclear whether a possible effect of ET_A receptor inhibition on early microcirculatory dysfunction may have been missed.

This is the first study to directly examine the putative role of ET_A receptors in early post-SAH changes in the cerebral microcirculation. We found that pharmacological inhibition of ET_A receptors had no effect on either the number or severity of microvasospasms after SAH, nor did it affect the global constriction of arterial microvessels. Furthermore, we found no significant improvement in neurological outcome after SAH. These results suggest that ET_A receptors do not likely play a major role in the development of microvasospasms after experimentally induced SAH. Given that inhibiting ET_A receptors reduces spasms in large cerebral arteries after SAH, but has no apparent effect on microvessels, these results also suggest that large-vessel spasms and microvasospasms are mechanistically distinct phenomena; thus, findings on large cerebral vessels may not necessarily apply to cerebral microvessels. This may be particularly relevant when attempting to relate in vitro results obtained from isolated vessels to in vivo results.

Microvascular constriction can occur as early as 3 hours after SAH, and it can last up to 72 hours or longer.[17] In a previous study, we demonstrated that reduction of microarteriolar constriction and microvasospasm led to a significant reduction of posthemorrhagic brain damage and improved neurological outcome thereby stressing the importance of early microcirculatory phenomena for structural and functional outcome after SAH.[29] We found that inhibiting ET_A receptors at the time of peak microvasospasm did not reduce the number of microvasospasms, nor did it affect global microcirculatory constriction. In addition, clazosentan did not improve neurological function measured for 3 days after SAH, stressing the importance of early changes in the microcirculation for neurological outcome. Our results are consistent with results obtained by others who found no significant effect of inhibiting ET_A receptors on microthrombosis,[42–44] cell death,[42,43] or endothelial nitric oxide synthase uncoupling.[42] Therefore, we conclude that ET_A receptors do not play a major role in the pathophysiology of early microvasospasms and—subsequently—early brain injury after SAH. Our findings may also explain the apparent failure of inhibiting ET_A receptors inhibition after SAH in a clinical trial: because post-SAH ischemia is caused by a perturbation in the microcirculation and because inhibiting ET_A receptors affects large but not small arterioles, this approach is not likely to improve ischemia or neurological outcome after SAH.

In our study, we used clazosentan to inhibit ET_A receptors because its pharmacokinetics and its ability to selectively and potently inhibit ET_A receptors have been studied in detail, including in rodents.[45–47] Nevertheless, we cannot exclude the possibility that clazosentan-specific side effects may have counteracted a possible beneficial effect of inhibiting ET_A receptors. However, this is unlikely, given that clazosentan did not affect the animals' basic physiological parameters and we did not detect adverse events during dose finding. Furthermore, clazosentan was applied by continuous intravenous infusion, thereby minimizing any possible effects of pharmacokinetics.

In summary, we present data suggesting that ET_A receptors do not play a major role in the development of microvasospasm or microarteriolar constriction after SAH, suggesting that macrovasospasms and microvasospasms are mediated by distinct mechanisms which have far-reaching consequences for future treatment strategies and therapeutical approaches. Given that maintaining the function of the cerebral microcirculation is a prerequisite for improving outcome after SAH, additional research is needed to determine the mechanisms that underlie SAH-induced microvascular dysfunction.

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Disclosures

None.

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