NEGATIVE RESULT

CO$_2$ has no therapeutic effect on early microvasospasm after experimental subarachnoid hemorrhage

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In addition to delayed vasospasm also early brain injury, which occurs during the first few days after subarachnoid hemorrhage (SAH) when large cerebral arteries are still fully functional, plays an important role for the outcome after SAH. In the current study, we investigated the hypothesis that carbon dioxide (CO$_2$), a strong cerebral vasodilator, has a therapeutic potential against early posthemorrhagic microvasospasm. C57BL/6 mice ($n = 36$) and Sprague-Dawley rats ($n = 23$) were subjected to sham surgery or SAH by filament perforation. The pial microcirculation in the mice was visualized 3 and 24 hours after SAH using intravital fluorescence microscopy. Partial pressure of CO$_2$ (PaCO$_2$) was modulated by hyper- or hypoventilation or by inhalation of 10% CO$_2$. In rats, CO$_2$-mediated changes in cerebral blood flow (CBF) were measured at the same time points using laser Doppler fluxmetry. Increased PaCO$_2$ caused vasodilatation in sham-operated animals. Following SAH, however, cerebral arterioles were nonreactive to CO$_2$. This lack of microvascular CO$_2$ reactivity was accompanied by a complete loss of CO$_2$-induced hyperemia. Our data show that CO$_2$ is not able to dilate spastic microvessels and to increase CBF early after SAH. Future therapeutic approaches will therefore need to address mechanisms beyond CO$_2$.

INTRODUCTION

Subarachnoid hemorrhage (SAH) underlies 6% to 10% of all stroke events yet accounts for 22% to 25% of cerebrovascular-related deaths.[1] Only ~10% of patients who experience SAH recover completely, and ~70% of initially comatose patients die within the first 6 months of the SAH.[2] In addition to the severity of the disease also the lack of causal therapeutic options is responsible for this less than optimal situation.

The pathophysiology of SAH can be divided into two distinct phases: (1) delayed brain injury associated with delayed macro-vasospasm, which occurs later than 5 days after SAH[3–5] and (2) early brain injury (EBI) that occurs within the first few days after SAH when large cerebral vessels are still fully functional. Nevertheless, EBI is associated with acute cerebral hypoperfusion.[6] Recently, several laboratories, including ours, showed that spasms of the cerebral microcirculation may be responsible for cerebral hypoperfusion/ischemia during EBI. Using histology, Sehba et al[7] described macro- and microvascular spasms after the induction of experimental SAH, and microvasospasms were recently observed in vivo early after SAH both in patients[8,9] and in experimental animals.[10,11] Accordingly, the appealing hypothesis was put forward that the application of vasodilators, ideally cerebral vasodilators, may be an appropriate way to dilate spastic posthemorrhagic microvessels thereby mitigating or even preventing EBI after SAH. Accordingly, the aim of the current study was to address this hypothesis by applying carbon dioxide (CO$_2$), a potent cerebral vasodilator, to mice and rats after experimental SAH and investigate whether this intervention was able to resolve posthemorrhagic microvasospasm and increase cerebral blood flow (CBF).

MATERIALS AND METHODS

Male 7 to 11 weeks old C57BL/6 mice (20 to 23 g, Charles River, Germany) and Sprague-Dawley rats (270 to 300 g, own breeding), were used for these experiments. All procedures were performed in accordance with and were approved by the respective regulatory bodies in Germany (Regierung von Oberbayern, Approval No. 6/04) and Poland (Nencki Institute of Experimental Biology Local Commission for the Care and Use of Laboratory Animals for Experimental Procedures, Approval No. 191/2002).

Induction of Subarachnoid Hemorrhage in Mice

The mice were anesthetized with 0.5 mg/kg BW medetomidine, 5 mg/kg BW midazolam, and 0.05 mg/kg BW fentanyl (intraperitoneally), intubated and ventilated under control of end-tidal partial pressure of CO$_2$ (PaCO$_2$) by microcapnometry. SAH was induced using the filament perforation model and monitored by the measurement of intracranial pressure in each animal as previously described.[12] Sham-operated mice were treated in the same way only that the filament was not advanced so far that hemorrhage was induced. Eleven mice died after SAH (9/47 operated animals; total mortality: 23%) and could therefore not be investigated further.

Intravital Microscopy

The mice were randomly assigned to one of the six treatment groups ($n = 6$ mice each) and reanesthetized 3 or 24 hours after SAH as described above. The pial microcirculation was visualized with the intravascular dye fluorescein isothiocyanate (FITC)-dextran (MW 150,000 Da) as previously described (Figures 1A and 1B).[10,13] The vessel diameter of three arterioles (30 to 50 μm diameter) surrounded by blood and therefore spastic (see Figure 1C and Friedrich et al[10] for details) were recorded every 15 minutes for 1 hour to establish the baseline values. The animals were then sequentially normoventilated, hyperventilated, normoventilated, and then finally hypoventilated (for 10 minutes each) to assess the responsiveness of

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the vessels to hypo- and hypercapnia. In three additional experimental groups (no SAH, SAH + 3 hours, and SAH + 24 hours; $n = 6$ mice each), the animals were ventilated with 10% CO_2 for 15 minutes, and vessel diameter was recorded as described above. The intravital microscopy images were analyzed offline by an investigator who was masked with respect to the treatment of the animals.[13]
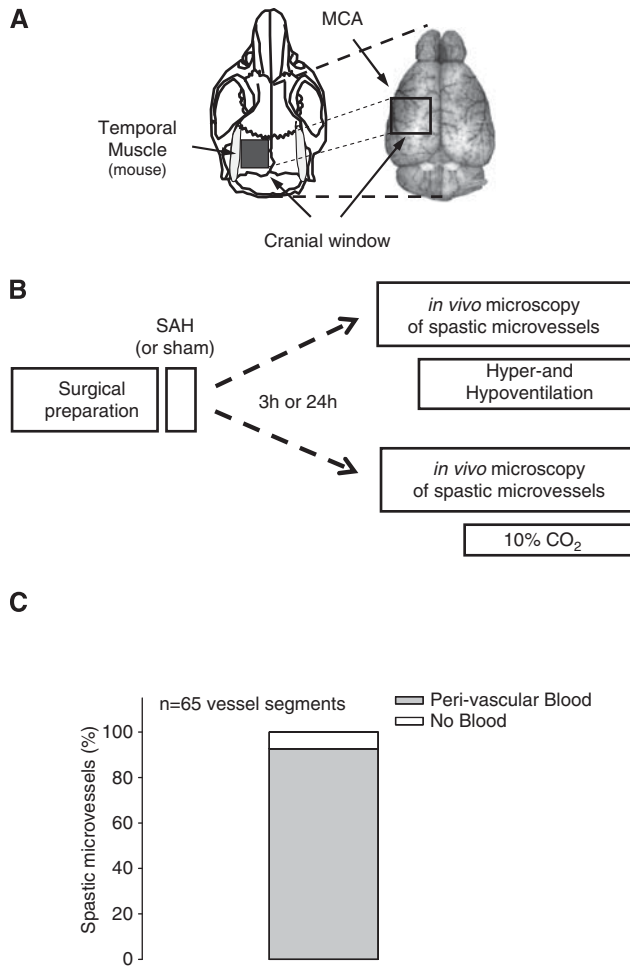

Figure 1. (**A**) Schematic drawing of the rodent skull (left) and a dorsal image of the brain (right) indicating the location of the cranial window used for intravital microscopy in mice. MCA, middle cerebral artery. (**B**) Schematic drawing of the experimental protocol used in this study. SAH, subarachnoid hemorrhage. (**C**) Percentage of spastic microvessels ensheathed by blood.

Induction of Subarachnoid Hemorrhage in Rats

Rats were anesthetized with chloral hydrate (3.6% solution intraperitoneally, 1 mL/100 g BW), intubated and ventilated with 30% O_2. Catheters were placed in the femoral or tail artery and in the femoral or tail vein. SAH was induced by the filament perforation technique and monitored by a sudden drop in regional CBF (rCBF) as previously described.[14]

CO_2 Reactivity in Rats

CO_2 reactivity in rats was evaluated 3 and 24 hours after SAH or sham surgery by ventilating the animals with 5% CO_2 for 5 minutes and measuring rCBF over the middle cerebral artery territory ipsilateral to the hemorrhage by laser Doppler fluxmetry. The results are expressed as a chemical index (CI_{CVR}) as previously described.[14] This CI is independent of changes in arterial blood pressure, as it is based on the change in cerebrovascular resistance (the ratio of arterial blood pressure to rCBF). Thus, CI_{CVR} is expressed as the relative change in cerebrovascular resistance per 1 mm Hg $PaCO_2$.

Statistical Analysis

Statistical analysis including calculation of sample size was performed using SigmaStat 3.0 (SPSS Science, Chicago, IL, USA). A one-way analysis of variance on ranks followed by the Dunn's or Tukey's test was used. Differences with a P value < 0.05 and a power of > 0.8 were considered to be statistically significant. Unless otherwise indicated, summary data are presented as the mean $\pm$ the standard error of the mean.

RESULTS

Physiologic Parameters

Body temperature (data not shown), blood pressure, and $PaCO_2$ were all constant throughout the baseline measurements in all groups (Tables 1 and 2). As expected, the mice that underwent SAH had lower blood pressure than sham-operated animals (Table 1). In both the sham and SAH-induced mice, blood pressure increased and decreased, and $PaCO_2$ decreased and increased, during hyperventilation and hypoventilation, respectively. During inhalation of 10% CO_2, both groups exhibited a drop in blood pressure and an increase in $PaCO_2$ to high levels (Table 1).

The rats that underwent SAH had the same blood pressure as sham-operated rats. During inhalation of 5% CO_2, blood pressure slightly increased in the sham-operated rats, but not in the SAH-induced rats (Table 2).

Relationship Between Microvasospasm and Perivascular Blood

As previously shown, 70% of pial microvessels become spastic after SAH.[10] To investigate whether perivascular blood was a possible cause for microvascular constriction, we determined the percentage of spastic microvessels surrounded by blood. Indeed, 93% of all spastic vessels were ensheathed by blood (Figure 1C) and no spasms were detected in vessels not being in contact with perivascular blood (data not shown). This strongly suggests that microvessels become only spastic when coming in direct contact

Table 1.	Physiologic parameters measured 3 hours after SAH or sham surgery in mice			
	Baseline	*10% CO_2*	*Hyperventilation*	*Hypoventilation*
Sham				
BP	92 ± 5 mm Hg	70 ± 8 mm Hg	92 ± 5 mm Hg	52 ± 5 mm Hg
$PaCO_2$	39 ± 3 mm Hg	274 ± 11 mm Hg	22 ± 2 mm Hg	49 ± 2 mm Hg
SAH				
BP	64 ± 3 mm Hg[a]	53 ± 3 mm Hg	72 ± 4 mm Hg	65 ± 5 mm Hg
$PaCO_2$	38 ± 2 mm Hg	278 ± 12 mm Hg	23 ± 3 mm Hg	47 ± 4 mm Hg
BP, blood pressure; SAH, subarachnoid hemorrhage. [a]$P < 0.05$ versus sham.				

with blood. Therefore only vessels covered with blood (and hence spastic) were used for the current study.

Responses to Changes in $PaCO_2$ by Variation of Ventilation

During baseline measurements, the diameter of the pial resistance vessels (i.e., arterioles) was constant. Following hypo- and

Table 2. Physiologic parameters measured 3 hours after SAH or sham surgery in rats

	Baseline	5% CO_2
Sham		
BP	105 ± 3 mm Hg	112 ± 3 mm Hg*
$PaCO_2$	37 ± 1 mm Hg	56 ± 1 mm Hg**
SAH		
BP	103 ± 8 mm Hg	110 ± 7 mm Hg
$PaCO_2$	38 ± 1 mm Hg	57 ± 2 mm Hg**

BP, blood pressure; SAH, subarachnoid hemorrhage. *$P < 0.05$, **$P < 0.005$.

hyperventilation, arterioles of sham-operated mice reacted as expected; specifically, vessels contracted during hyperventilation, returned to baseline upon normoventilation, and then dilated upon hypoventilation (Figure 2A, upper panels and Figure 2B, open circles). In the SAH-induced animals, the vessels that were affected by microvasospasm did not react to changes in ventilation either 3 or 24 hours after SAH (Figure 2A, lower panels and Figure 2B, light and dark gray circles). These findings indicate a complete loss of vessel reactivity in the cerebral microvessels that were affected by microvasospasm after SAH.

Inhalation with 10% CO_2

To exclude the possibility that the observed lack of CO_2 reactivity after SAH was not simply caused by a reduced sensitivity of posthemorrhagic vessels to CO_2 (i.e., dependent on the dose of the applied CO_2), $PaCO_2$ was increased to supraphysiologic levels by ventilation with 10% CO_2. In the sham-operated mice, inhalation of 10% CO_2 triggered an immediate and significant dilatation of the pial resistance vessels to 162% of baseline ($P = 0.002$ versus baseline; Figure 3A, upper panels and Figure 3B,
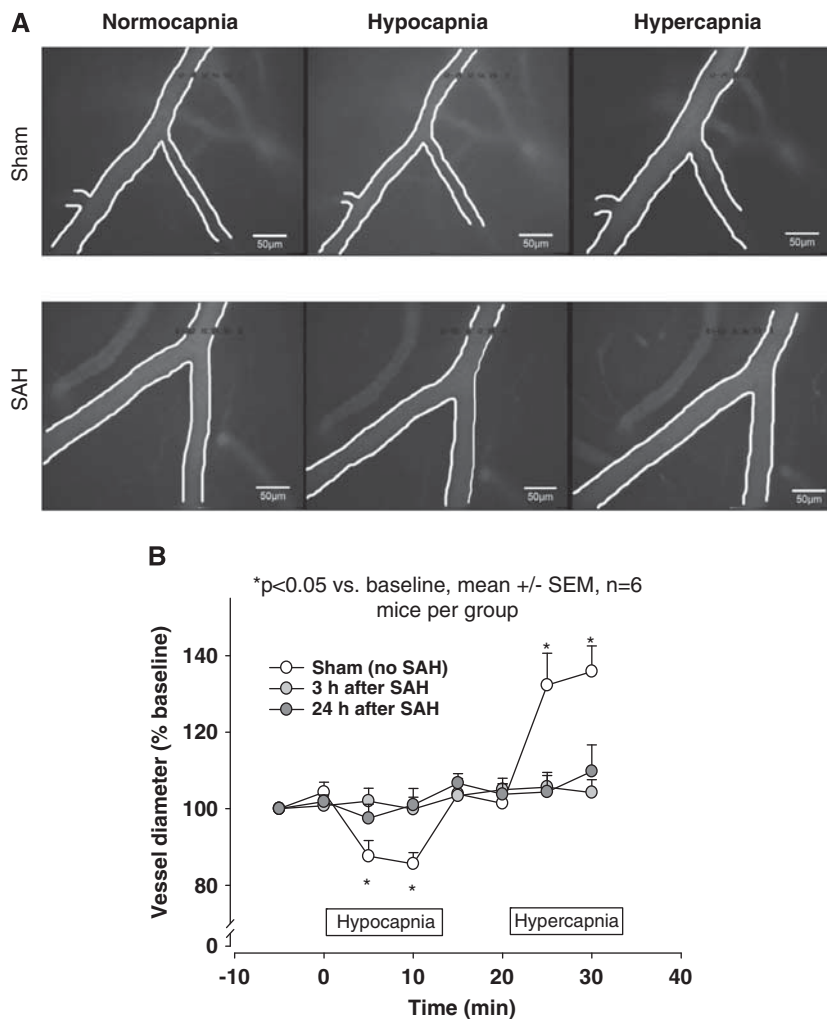

Figure 2. (**A**) *In vivo* microscopy images of mouse pial arterioles and their response to various ventilation frequencies 3 hours after sham surgery (upper panels) or SAH (lower panels). For each animal, the left, middle, and right panels show the same vessel. (**B**) Quantification of these data show that sham-operated mice exhibited a significant decrease and increase in vessel diameter during hypocapnia and hypercapnia, respectively (open circles). In contrast, vessels in mice that underwent SAH showed no change in vessel diameter in response to various ventilation frequencies (gray circles). SAH, subarachnoid hemorrhage; SEM, standard error of the mean. *$P < 0.05$ versus baseline; $n = 6$ mice per group.

open circles). Again, the spastic microvessels in the SAH-induced mice showed no reaction to inhaled CO$_2$ either 3 or 24 hours after SAH, indicating that spastic microvessels were indeed nonresponsive to CO$_2$ (Figure 3A, lower panels and Figure 3B, gray circles).

To determine whether the changes that were observed at the microcirculation level also had an effect on CBF, and to evaluate

whether the same effect also occurs in another species, similar experiments—with the addition of rCBF measurements using laser Doppler flowmetry—were performed in rats as showed by an original recording 3 hours after sham surgery or SAH (Figure 4A). Inhalation of 5% CO$_2$ triggered a large increase in rCBF and a
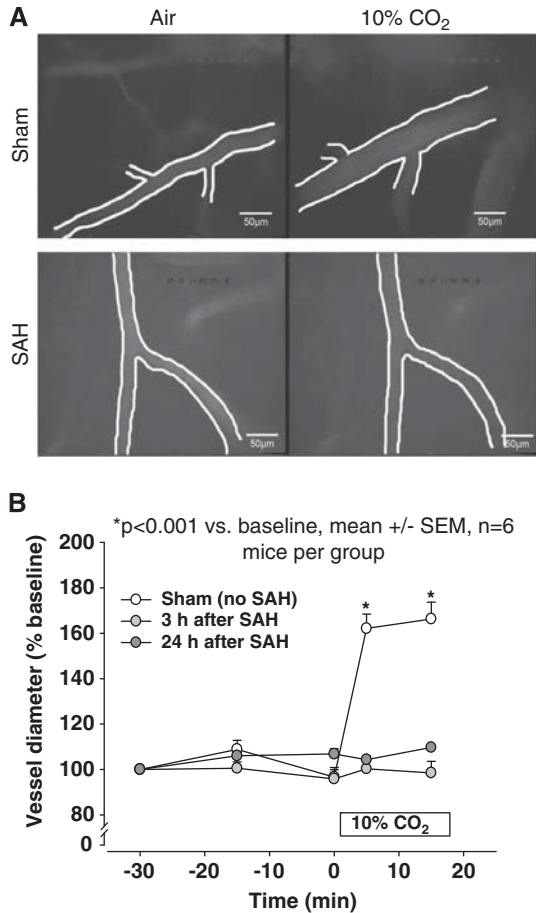
Figure 3. (**A**) Representative *in vivo* microscopy images of mouse pial arterioles and their response to inhalation of 10% CO$_2$ 3 hours after sham surgery (upper panels) or SAH (lower panels). For each animal, the left and right panels show the same vessel. (**B**) Quantification of experiments shown above. Vessel diameter in the sham-operated animals increased immediately upon ventilation with 10% CO$_2$. In contrast, the diameter of cerebral arterioles of mice subjected to SAH showed no change in response to elevated PaCO$_2$. SAH, subarachnoid hemorrhage; SEM, standard error of the mean. *$P < 0.001$ versus baseline; $n = 6$ mice per group.
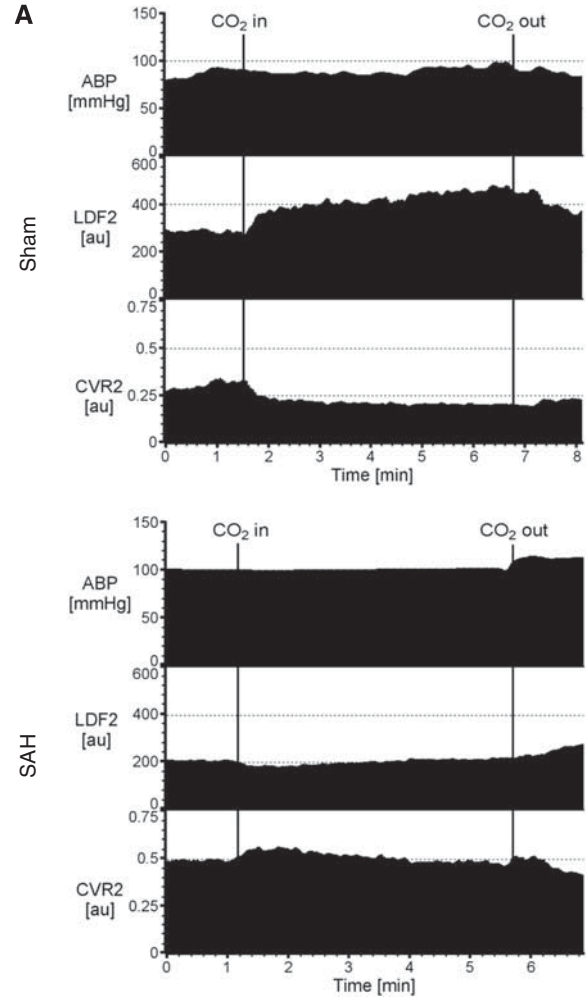
Figure 4. (**A**) Representative original recording of arterial blood pressure (ABP), regional cerebral blood flow (LDF), and cerebrovascular resistance (CVR) 3 hours after sham operation (upper recordings) or subarachnoid hemorrhage (SAH; lower recordings) in rats. Upon carbon dioxide (CO$_2$) inhalation regional cerebral blood increased and CVR decreased in sham-operated animals, while no significant changes were observed after SAH. (**B**) Chemical index (CI) of rats subjected to SAH or sham surgery. CI is indicated as a relative change of CVR per 1 mm Hg partial pressure of CO$_2$ (PaCO$_2$) during inhalation of CO$_2$. Sham-operated rats showed a normal reactivity to 5% inhaled CO$_2$ (open bar; CI = 1.9 ± 0.1%/1 mm Hg PaCO$_2$). Three and twenty-four hours after SAH the cerebral cortex of rats subjected to SAH showed a complete lack of CO$_2$ reactivity as showed by CI values close to zero. *$P < 0.01$ versus sham-operated rats; $n = 5$ to 10 rats per group.
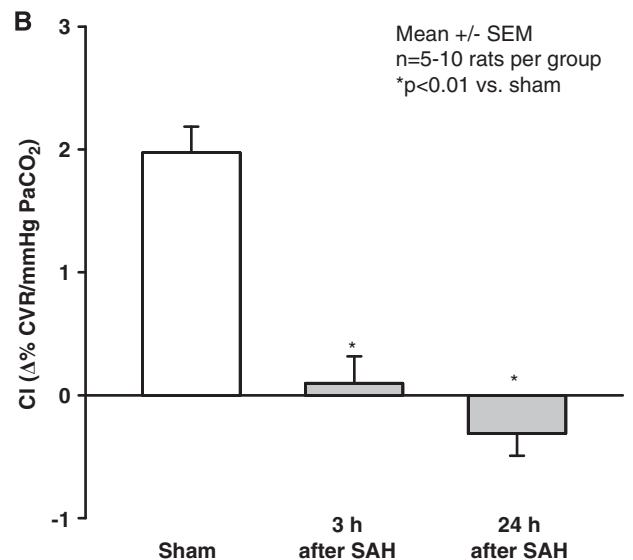
decrease in cerebrovascular resistance in sham-operated rats (CI = 1.9 ± 0.1%/mm Hg PaCO$_2$). However, 3 and 24 hours after SAH, CO$_2$ reactivity was completely lost, as CI was 0.1 ± 0.2 and − 0.3 ± 0.2%/mm Hg PaCO$_2$, respectively (Figure 4B).

DISCUSSION

The aim of this study was to investigate whether early posthemorrhagic microvasospasm after SAH, which is associated with microthrombosis, cerebral ischemia, and the development of early-onset brain injury could be reduced or resolved by increasing the plasma concentration of CO$_2$, a potent cerebral vasodilator. Our results show that cerebral arterioles that are covered with blood and therefore affected by posthemorrhagic microvasospasm undergo a complete loss of CO$_2$ reactivity both 3 and 24 hours after SAH, and this loss of reactivity results in the inability of the brain to increase its blood flow in response to elevated PaCO$_2$ levels. This lack of microvascular CO$_2$ reactivity suggests that the cerebral autoregulation is severely compromised after SAH and this may render the brain vulnerable to decreases in blood pressure and/or increases in brain metabolism, thereby causing additional brain damage. In addition, our results show that increasing PaCO$_2$ is not a viable therapeutic option against posthemorrhagic microvasospasm after SAH and that hyperventilation clinically used to reduce increased intracranial pressure by constricting cerebral vessels may not be appropriate after SAH.

In the present study, we induced SAH using the intraluminal perforation model, a well-characterized model that does not require opening of the skull and mimics quite nicely the acute processes that are triggered by the rupture of an intracranial aneurysm (e.g., elevated intracranial pressure, cerebral ischemia, and hippocampal damage) as well as the long-term squeals of SAH in patients (e.g., mortality, cerebral edema, and hydrocephalus). The induction of SAH was confirmed in each animal by a typical posthemorrhagic increase in intracranial pressure in mice and a sharp bilateral decrease of rCBF in the territory of the middle cerebral artery in rats. Importantly, the physiologic parameters were monitored closely and kept constant during all procedures including intravital microscopy.[12] Thus, we believe that our setup—though technically demanding—minimized the likelihood of potential experimental artifacts.

Data regarding cerebral vessel reactivity were collected in vivo and in real time using epi-fluorescence microscopy. Although this technique has a limited penetration depth and therefore does not allow the investigation of deep parenchymal vessels, it is sufficient for the current study that focused on spastic arterioles in the subarachnoid space.

CO$_2$ is one of the most important local regulators of cerebral vessel diameter and thus CBF. Accordingly, several clinical and experimental trials investigated whether CO$_2$ could be used to treat delayed cerebral vasospasm after SAH.[3,15–25] In patients suffering from delayed vasospasm CO$_2$ reactivity was decreased and was found to be an accurate predictor of delayed cerebral ischemia.[3,16] However, so far, CO$_2$ reactivity and the potential therapeutic benefits of CO$_2$ on the recently described early, that is EBI associated, posthemorrhagic microvasospasm has not been investigated.

Our current and previous results suggest that the cerebral microvessels that are located in the subarachnoid space and which come into contact with sufficient amounts of blood become spastic and nonresponsive to CO$_2$.[10] Although, the mechanisms by which SAH causes microvasospasm are not yet fully understood, the cooccurrence of microvasospasm together with an attenuated CO$_2$ response in the very same microvessels strongly suggests that a common mechanism may underlie both phenomena. Experiments by Iadecola and colleagues revealed that changes in CBF because of hypercapnia in the physiologic range are mediated primarily by nitric oxide (NO),[26,27] and these findings are supported by the fact that CO$_2$ elevates the classic NO-induced messenger molecule cyclic guanosine monophosphate in the smooth-muscle cells of cerebral vessels.[28] Because hemoglobin is a potent NO scavenger,[29] and because NO is required for both CO$_2$-mediated vasodilatation and to maintain physiologic arteriolar diameter, we suggest that hemoglobin in the blood surrounding cerebral microvessels after SAH scavenges vascular (and/or perivascular) NO, thereby causing microvasospasm and a complete lack of CO$_2$ reactivity in these vessels. This hypothesis is supported, among others, by a recent nonhuman primate study that showed that replenishing vascular (or perivascular) NO by administering intravenously sodium nitrite—a strategy that is often used to deliver NO to endothelial cells—reversed SAH-induced macrovasospasm.[30] A possible argument against this interpretation of our findings may be provided by a recent study performed on brain slices obtained from rats subjected to SAH, showing that on electrical stimulation of the neighboring parenchyma not perfused and hence not pressurized vessels show inverse neurovascular coupling, i.e., rather constriction than dilatation.[31] Which interpretation represents the situation after SAH in vivo will have to be showed by ongoing studies.

Here, we report that the cerebral microvessels that are affected by early posthemorrhagic microvasospasms lose their ability to dilate in the face of increased levels of CO$_2$. These findings suggest that post-SAH microvasospasm does not impair cerebral perfusion only by vasoconstriction and the formation of microthrombi; the impaired perfusion may also be because of either a lack of responsiveness to increased metabolic demand and/or reduced cerebral perfusion pressure. Our data also suggest that CO$_2$ has no therapeutic effect against early microvasospasm and, hence, against EBI. Mechanistically, our results point to the possibility that the severe SAH-induced microvascular dysfunction is caused by a lack of vascular NO. Therefore, our negative results concerning CO$_2$ may suggest that increasing vascular NO could be a promising strategy for treating patients who have EBI after SAH.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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