Effects of subarachnoid hemorrhage on vascular responses to calcitonin gene–related peptide and its related second messengers

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Calcitonin gene–related peptide (CGRP) is a potent vasodilator and a primary signaling molecule in neurovascular communication. In the present study, the authors examined cerebrovascular responses to CGRP and its related second messenger systems during cerebral vasospasm induced by subarachnoid hemorrhage (SAH). Tension measurements were performed in vitro on ring strips of basilar arteries obtained from rabbits subjected to artificial SAH and from control (non-SAH) animals. In vessels from SAH animals, which were preconstricted with serotonin, the vasorelaxant response to CGRP was attenuated. Because it has been suggested that vasodilation elicited by CGRP is mediated by cyclic 3′,5′-adenosine monophosphate (cAMP) and/or cyclic 3′,5′-guanosine monophosphate (cGMP), the vascular effects of directly activating these second messenger systems were also examined. The relaxant effect of forskolin, which activates adenylate cyclase directly, was slightly enhanced after SAH. In contrast, the relaxant effect of nitroglycerin (GTN), which activates soluble guanylate cyclase directly, was unchanged after SAH.

The attenuation of CGRP-induced vasorelaxation could be the result of a modification in its ability to stimulate the production of second messengers. Experiments testing the capacity of CGRP to elevate cAMP levels showed no significant differences between vessels from non-SAH and SAH animals. Similarly, the resting levels of cAMP and the forskolin-induced elevations of cAMP did not differ between non-SAH and SAH animals. In contrast, cGMP levels were lower in resting and CGRP-treated vessels from SAH animals than in those from non-SAH animals. No significant differences in the levels of cGMP were observed between non-SAH and SAH vessels treated with GTN.

This study indicates that CGRP-induced vasodilation is attenuated during vasospasm in a rabbit model of SAH. The findings also demonstrate that vasodilatory responses mediated by cAMP and cGMP are intact, although the levels of cGMP in SAH vessels are reduced. Together, these observations suggest that an attenuation in the capacity of vessels to dilate in response to CGRP occurs during cerebral vasospasm, and this change in CGRP vasoactivity is a result of modifications prior to, or independent of, the elevation of cyclic nucleotide second messengers.

KEY WORDS • vasospasm • calcitonin gene–related peptide • basilar artery • cyclic adenosine monophosphate • cyclic guanosine monophosphate • rabbit

CEREBRAL VASOSPASM after subarachnoid hemorrhage (SAH) is a serious complication that occurs following the rupture of intracranial aneurysms.11 Despite substantial ongoing efforts, effective therapeutic strategies for managing the angiographic features of vasospasm have yet to be established in the clinic. It is therefore important to characterize precisely the function of known first and second messenger systems in cerebral vessels after SAH. Calcitonin gene–related peptide (CGRP) is one of the most potent vasodilators of cerebral vessels.3 This peptide is released from terminals of perivascular nerves and serves as a neurovascular signaling molecule in cerebral arteries.3,7 Calcitonin gene–related peptide activates specific receptors located on vascular smooth-muscle cells;10 cyclic nucleotide second messengers are key intermediaries in its vasodilatory actions.14,24 Several studies have implicated changes in the CGRP signaling system in the pathophysiology of cerebral vasospasm.2,4,8,18,20,22 The CGRP-like immunoreactivity around cervical vessels is less intense after SAH,5,18 which suggests that the amount of peptide available for release is reduced. The functional impact of SAH on CGRP–induced dilation, however, remains controversial. Edvesson and colleagues2 have presented evidence that CGRP–induced vasorelaxation is facilitated in rat basilar artery segments tested during the peak of SAH-induced vasospasm. In contrast, Nozaki and coworkers19,20 have demonstrated an attenuation of CGRP–induced vasorelaxation in canine basilar arteries at the peak of vasospasm. One of the central goals of the present study is to address this controversy by examining the responsiveness of CGRP during vasospasm in a rabbit model of SAH.
Another goal of this study is to evaluate the role of second messenger systems in the functional modification of CGRP vasoreactivity. Cyclic nucleotide second messengers play a prominent role in regulating cerebrovascular tone. Moreover, both cyclic 3',5'-adenosine monophosphate (cAMP) and cyclic 3',5'-guanosine monophosphate (cGMP) have been implicated as mediators of the vasodilatory responses to CGRP. It is plausible that alterations in one or both of these systems could underlie the modification in CGRP-induced dilation observed during cerebral vasospasm. The present study evaluates this issue.

Materials and Methods

Rabbit Model of Subarachnoid Hemorrhage

New Zealand White male rabbits, weighing 2.8 to 3.5 kg, were anesthetized by intramuscular injection of ketamine (25 mg/kg) and xylazine (2.5 mg/kg). Subarachnoid hemorrhage was induced in 72 rabbits (SAH group) as described in detail elsewhere and an additional 72 animals were used for the control (non-SAHAH) groups. Briefly, 2.5 ml of fresh, autologous, nonheparinized arterial blood was perfused intracardially through both femoral arteries. The basilar arteries were dissected free with the aid of a stereomicroscope after the brainstem was removed en bloc. Four basilar arterial rings, each 3 mm in length, were obtained from the rabbit model of subarachnoid hemorrhage.

Preparation of the Basilar Artery and Tension Recording

Two days after blood injection the SAH animals were sacrificed and ring strips of the basilar artery were prepared as follows. The rabbits were anesthetized by intramuscular injection of ketamine (50 mg/kg) and xylazine (5 mg/kg), after which they were orally intubated, paralyzed with a single intravenous injection of pancuronium bromide (0.08 mg/kg), and ventilated using a dual-phase control respirator. While the heart was still beating, each animal was perfused intracardially with 100 ml of aerated (95% O2/5% CO2) modified Krebs' solution (in mM: NaCl, 120; KCl, 4.5; MgSO4, 1.0; CaCl2, 2.5; H2PO4, 1.0; NaHCO3, 27.0; KH2PO4, 1.0; CaCl2, 2.5; dextrose, 10.0) at room temperature. Exsanguination was achieved by severing both femoral arteries. The basilar arteries were dissected free with the aid of a stereomicroscope after the brainstem was removed en bloc. Four basilar arterial rings, each 3 mm in length, were obtained from each animal and prepared according to established methods. Each ring was placed in two-L shaped, silicon-coated rods in an organ bath with a 10-ml working volume and placed in an isometric tension setup. Isometric tension was recorded using a force-displacement transducer, a polygraph, and a chart recorder. The resting tension was adjusted to 400 mg and the preparations were allowed to equilibrate at 37°C for 1 hour prior to any drug treatment. Similar preparative steps were undertaken for vessels from non-SAHAH animals. All vessels were tested initially for their responses to treatment with 40 mM KCl. If consistent contractions were ob-served in two consecutive treatments with elevated KCl, the vessels were used for drug testing.

Drug Preparation and Treatment

Nitroglycerin (GTN) was obtained at a concentration of 5 mg/ml. Stock solutions of forskolin were made by dissolving the drug in dimethyl sulfoxide. The CGRP was dissolved in double-distilled water. The drugs were further diluted in modified Kreb’s solution before use and the final volume of a given drug-containing solution was less than 0.2 ml. Drugs were added directly to the 10-ml organ baths. In experiments in which multiple concentrations of a single drug were tested, the compound was added to the bath in a cumulative manner.

Assays for cAMP and cGMP

Calcitonin gene-related peptide (1, 10, and 100 nM), forskolin (1, 10, and 100 nM), or GTN (0.1 and 10 μM) was added for 3 minutes to the organ bath after the arterial strips had been equilibrated for 1 hour at 37°C. The reaction was halted by soaking the arterial strips in ice-cold acidic ethanol (1 ml 1 N HCl:100 ml ethanol). The strips were then homogenized in the same solution using a glass homogenizer. The homogenate was centrifuged and the pellet was used for the assay of protein levels after air-drying. The supernatant was dried by heating it to 50°C under a stream of air. Samples were assayed for cAMP or cGMP using standardized enzyme immunoassay kits. Absorbance at wavelengths of 405 nm for cAMP and 450 nm for cGMP was determined using an automated microplate reader.

Supplies and Equipment

The dual-phase control respirator, model 683, was obtained from Harvard Instruments, South Natick, Massachusetts. The force-displacement transducer, model FT03D, and the polygraph, model 79D, were supplied by Grass Instruments, Quincy, Massachusetts. Soltex Corporation, Sun Valley, California, provided the chart recorder model 3418. The automated microplate reader, model EL311SX, was obtained from Bio-Tek Instruments, Winooski, Vermont.

Nitroglycerin was supplied by Solo Pak Laboratories, Inc., Elk Grove Village, Illinois. All other drugs used in the experiment were obtained from Sigma Chemical Company, St. Louis, Missouri. Assays for cAMP and cGMP were performed using enzyme immunoassay kits provided by Cayman Chemicals Ann Arbor, Michigan, and Amersham Corporation, Arlington Heights, Illinois, respectively.

Statistical Analysis

The values presented in this study are expressed as means ± the standard error of the means. Differences between means within each experiment were evaluated by analysis of variance. If significant differences were demonstrated, the two-tailed Student’s unpaired t-test was used to determine which pairs of means were significantly different. Probability values less than 0.05 were considered significant.

Results

Tension Measurement Studies

In these studies we examined changes in the isometric tension of ring strips of rabbit basilar arteries in response to treatment with signaling molecules. We then compared responses in vessels prepared from non-SAHAH and SAHAH animals.

Effects of SAH on Contractions Induced by Potassium or Serotonin.

The treatment of ring strips of basilar arteries with a high concentration of KCl (40 mM) provoked contractions with peak values of 1.94 ± 0.48 g (seven arterial ring strips) and 1.45 ± 0.40 g (five arterial ring strips) in vessels from non-SAHAH and SAHAH rabbits, respectively. Although the magnitude of contractions in response to KCl was lower in vessels from SAHAH animals, this effect did not achieve statistical significance.

The maximum contraction elicited by serotonin (5-HT) was markedly augmented in vessels from SAHAH rabbits (Fig. 1). The sensitivity of the vessels to 5-HT was also enhanced as indicated by the shift to the left in the dose–response curve for the SAHAH vessels (Fig. 1). Mean effective concentration (EC50) values for 5-HT were 0.41 ± 0.16 μM (seven arterial ring strips) and 0.017 ± 0.004 μM (five arterial ring strips) in vessels from non-SAHAH and SAHAH rabbits, respectively; this difference was statistically significant (p < 0.05).
The vasorelaxant effects of CGRP were tested by applying doses of the peptide in a cumulative fashion to vessels precontracted with 5-HT. To achieve similar plateau levels of precontraction, vessels from non-SAH rabbits were treated with 10 μM 5-HT, whereas vessels from SAH animals were treated with 0.3 μM 5-HT. Calcitonin gene–related peptide (0.1–100 nM) reversed 5-HT–induced contraction in a concentration-dependent manner in vascular strips obtained from both non-SAH and SAH rabbits. However, the relaxing effect of CGRP was shown to be attenuated after SAH. The concentration–response curve was significantly shifted to the right in strips obtained from SAH rabbits (Fig. 2 left).

Effects of SAH on Vasorelaxation Induced by Forskolin and GTN. Vasorelaxation responses to activators of cyclic nucleotides were tested as described above. Forskolin (0.1–100 nM), an activator of adenylate cyclase,21 elicited concentration-dependent relaxation in strips precontracted with 5-HT. At concentrations of 10 and 30 nM, forskolin-induced vasorelaxation was significantly greater in the SAH group than in the non-SAH group (Fig. 2 center). Nitroglycerin (0.01–30 μM), an activator of guanylate cyclase,9 elicited concentration-dependent relaxation in both groups of vascular strips. In contrast to the other vasorelaxants, GTN-induced relaxation did not differ significantly between vessels from non-SAH and SAH groups (Fig. 2 right). A summary of the mean inhibiting concentration (IC50) values of the vasorelaxing agents is presented in Table 1.

Cyclic Nucleotide Assays

Modifications in vascular responses after SAH could be
Effects of SAH on vascular responses to CGRP, cAMP, and cGMP

the result of alterations in the generation of cyclic nucleotide second messengers. In these experiments we examined resting levels of cyclic nucleotides and changes in levels of cyclic nucleotide in response to activation with the signaling molecules in vessels from non-SAH and SAH animals.

**Effects of CGRP and Forskolin on cAMP Levels.** Resting levels of cAMP did not differ between vessels from non-SAH and SAH animals. The effects of CGRP (1–100 nM) and forskolin (1–100 nM) on cAMP synthesis were examined by treating vessels with a single concentration of these compounds for 3 minutes. Both CGRP and forskolin increased the cellular concentration of cAMP in a concentration-dependent manner (Fig. 3). There were no significant differences between non-SAH and SAH groups in their responses to CGRP or forskolin. When compared at the same concentrations (10 and 100 nM), production of cAMP was significantly higher in the forskolin-treated groups than in the CGRP-treated groups of vessels.

**Effects of CGRP and GTN on cGMP Levels.** Resting levels of cGMP were reduced in SAH vessels (Fig. 4). In general, CGRP exerted no significant effect on the cGMP levels in vessels from non-SAH or SAH animals. At a concentration of 1 nM, CGRP elicited a slight, but significant, increase in cGMP levels in SAH; however, this was the only case in which CGRP significantly modified cGMP levels (Fig. 4 upper). In contrast, GTN (10 μM) elicited significant increases in cGMP levels in vessels from both groups of animals (Fig. 4 lower). No significant differences in the magnitude of GTN-induced cGMP generation were observed between vessels from non-SAH and SAH animals.

**TABLE 1**

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<tr>
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<th>Non-SAH</th>
<th>SAH</th>
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<tr>
<td>CGRP (nM)</td>
<td>1.29 ± 0.27 (11)</td>
<td>11.84 ± 4.69† (11)</td>
</tr>
<tr>
<td>forskolin (nM)</td>
<td>6.56 ± 2.28 (12)</td>
<td>2.51 ± 0.47 (10)</td>
</tr>
<tr>
<td>GTN (nM)</td>
<td>44.5 ± 7.7 (12)</td>
<td>84.4 ± 21.5 (11)</td>
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* Values (in nanomoles) are expressed as the mean ± standard error of the mean. Numerals within parentheses indicate the number of arterial ring strips tested. SAH = subarachnoid hemorrhage; CGRP = calcitonin gene–related peptide; GTN = nitroglycerin.
† p < 0.05 compared to arteries from non-SAH animals.

Fig. 3. Bar graphs showing the concentration-dependent effects of calcitonin gene–related peptide CGRP (upper) and forskolin (lower) on the production of cyclic 3′,5′-adenosine monophosphate (cAMP) in strips of basilar arteries obtained from rabbits without (non-subarachnoid (non-SAH), open bars) and with (SAH, shaded bars) SAH. Vertical bars indicate the standard error of the means. Six to nine rabbits were used in the CGRP study and seven to 10 in the forskolin study.

Fig. 4. Bar graph showing the effects of calcitonin gene–related peptide (CGRP) (upper) and nitroglycerin (GTN) (lower) on the production of cyclic 3′,5′-guanosine monophosphate (cGMP) in strips of basilar arteries obtained from rabbits without (non-subarachnoid (non-SAH), open bars) and with (SAH, shaded bars) SAH. *p < 0.05; **p < 0.01. Vertical bars indicate the standard error of the means. Seven rabbits were used in the CGRP study and six in the GTN study.
Discussion

Functional modification of cerebrovascular messenger systems after SAH has attracted increasing attention. The interest in this issue derives from three basic facts: 1) cerebral vasospasm is the most critical clinical complication that occurs after SAH; 2) the mechanism for vasospasm remains controversial; and 3) an adequate therapeutic treatment for ameliorating the angiographic manifestation of cerebral vasospasm is not available in the clinic. A clear characterization of the functional roles of first and second messengers in the cerebrovasculature is essential for understanding the pathophysiology of cerebral vasospasm after SAH. Several recent studies have evaluated the role of the CGRP signaling system following SAH. The levels of CGRP-like immunoreactivity are reduced after SAH but eventually recover. The functional impact of reduced levels of immunoreactivity may be that less peptide is available for release or that more CGRP is released continuously during vasospasm. The impact of SAH on the functional sensitivity of the CGRP system has also been a topic of investigation. Edvinsson and colleagues have presented evidence that CGRP-induced relaxation is enhanced slightly during peak vasospasm in a rat model of SAH. In contrast, Nozaki, et al., have shown that the relaxant effects of CGRP are attenuated during peak vasospasm in a canine model of SAH. The results of the present study are in accord with the observations of Nozaki, et al.,. In a rabbit model of SAH, CGRP-induced vasorelaxation was attenuated during peak vasospasm. Thus, a shift to the right of the dose–response curve for CGRP was observed during peak vasospasm in both canine and rabbit models.

The discrepancy between the current observations and those of Edvinsson and associates could be the result of several experimental variables. One obvious explanation for the divergent outcomes is that different species (rat vs. rabbit) were used in the two studies. This explanation is somewhat unlikely given the findings of Nozaki, et al., in which a decreased responsiveness to CGRP was also observed in dogs. Despite the consistency between observations in the rabbit and dog models of SAH, a species-specific difference cannot be ruled out as a possible explanation for the different outcome in the rat studies.

Another potential explanation for the differences between the current study and the study of Edvinsson and colleagues is that the vessels under study were precontracted in a somewhat different manner. Both studies used 5-HT to precontract basilar artery segments. However, different degrees of precontraction were probably obtained in the two studies. As shown in the present study and elsewhere, the contractile response to a given concentration of 5-HT differs significantly between vessels from non-SAH and SAH animals. Consequently, a fixed concentration of 5-HT elicits a greater contractile response in vessels from SAH animals than in vessels from non-SAH animals (Fig. 1). In their study, Edvinsson and colleagues examined the vasodilator effects of CGRP under conditions in which both groups of vessels were precontracted with 1 μM 5-HT. It is therefore likely that CGRP-induced contractions were tested under conditions in which different degrees of pharmacological precontraction had been established in the two groups of vessels. The present study circumvented this complication by first determining the dose–response relationships for 5-HT–induced contractions and then selecting concentrations of 5-HT that elicited precontractions of a similar magnitude in the two groups of vessels. Under these conditions, relaxations induced by CGRP were shown to be attenuated in SAH vessels; this finding is similar to the attenuation of CGRP-induced relaxations observed in canine vessels precontracted with prostaglandin F2α.

The mechanism(s) underlying the observed attenuation of CGRP-induced relaxation could theoretically involve modifications in any or all of the steps from receptor occupation to muscle-cell contraction. In the present study we evaluated the possibility that alterations in the generation of cyclic nucleotide second messengers contribute to this effect. The generation of cAMP in response to direct activation of adenylate cyclase by forskolin did not differ between vessels from non-SAH and SAH animals. This finding indicates that the capacity for cAMP production is intact during cerebral vasospasm. Moreover, the relaxant response to forskolin was actually facilitated after SAH. This suggests that a direct change in cAMP-mediated relaxation is not responsible for the attenuation of CGRP-induced vasodilation. Finally, the capacity of CGRP to elevate cAMP levels did not differ significantly between vessels from non-SAH and SAH animals. Together these findings suggest that the reduction of CGRP-induced vasorelaxation is not due to a reduction in the ability of CGRP to stimulate cAMP production or to a general attenuation of cAMP-mediated relaxation responses.

Another second messenger postulated to participate in CGRP-mediated vasodilation is cGMP. The response of the cGMP-generating system to SAH has been investigated previously in some detail. Kim and associates have provided evidence that resting levels of cGMP are reduced during cerebral vasospasm in a canine model of SAH. This is consistent with the reduction in resting levels of cGMP in the rabbit SAH model described in our current report. This reduction in the resting levels of cGMP could reflect an attenuation of the release of endothelium-derived relaxing factor; endothelial injury, which is known to occur after SAH, is consistent with this possibility. Another possible explanation for lower cGMP levels after SAH is that the response of smooth muscle to activation of soluble guanylate cyclase is reduced. Kim and associates have provided evidence indicating that the responsiveness of the cGMP generating system is attenuated during vasospasm. Direct stimulation of cGMP production by nitric oxide was attenuated after SAH in the canine model of SAH. Our present results are not in accord with this aspect of the work by Kim and associates. The production of cGMP in response to treatment with GTN did not differ significantly between vessels from non-SAH and SAH rabbits. Moreover, vasorelaxation elicited by GTN did not differ between vessels from non-SAH and SAH rabbits. These findings indicate that although resting levels of cGMP are reduced in the basilar artery of the SAH rabbit, the capacity to generate cGMP directly and to elicit cGMP-mediated vasorelaxation is relatively intact.

The role of cGMP in vasorelaxation that is elicited by CGRP remains to be clarified in this system. The levels of cGMP in rabbit basilar arteries were not significantly

J. Neurosurg. / Volume 83 / September, 1995
Effects of SAH on vascular responses to CGRP, cAMP, and cGMP

affected by CGRP under most treatment conditions. The general lack of a significant elevation of cGMP levels in vessels treated with CGRP suggests that cGMP is not a crucial intermediary of CGRP-induced vasorelaxation in rabbit basilar arteries.

Conclusions

The evidence presented here indicates that CGRP-induced vasorelaxation is attenuated after SAH. This fundamental modification in a potent vasodilatory system could contribute directly to the spastic constriction of cerebral vessels after SAH. In addition, our findings indicate that modifications in two cyclic nucleotide second messenger systems, that of cAMP and cGMP, are unlikely to be responsible for the alteration in the physiological impact of CGRP. Future studies will be directed toward identifying the mechanisms responsible for attenuating vasodilatory function during cerebral vasospasm.

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References


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