Time Course of Variations in Rabbit Cerebrospinal Fluid Levels of Calcitonin Gene-Related Peptide- and Substance P–Like Immunoreactivity in Experimental Subarachnoid Hemorrhage

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Background and Purpose Cerebral vasospasm after subarachnoid hemorrhage may result partially from the imbalance between vasodilator and vasoconstrictor factors. The vasodilator peptides substance P and calcitonin gene-related peptide contained in the trigeminovascular system are involved in the vasomotor phenomenon occurring after subarachnoid hemorrhage. The delayed arterial narrowing may reflect the time course of the release of these peptides. Therefore, we followed the time course of the changes in cerebrospinal fluid immunoreactivity of substance P and calcitonin gene-related peptide in a model of experimental subarachnoid hemorrhage.

Methods Cerebrospinal fluid samples were taken in the basal state and at 30 minutes, 24 hours, and 3 days after a single injection of 1 mL autologous arterial blood into the cisterna magna of rabbits using a percutaneous suboccipital route. Substance P-like and calcitonin gene-related peptide-like immunoreactivities were determined in centrifuged cerebrospinal fluid and plasma by use of enzyme immunoassay.

Results Early (30 minutes) after induced subarachnoid hemorrhage, there was a large increase in cerebrospinal fluid substance P-like immunoreactivity (P<.01) and calcitonin gene-related peptide–like immunoreactivity (P<.01). Arterial and hemorrhagic cerebrospinal fluid levels of substance P–like immunoreactivity were different (P<.03), indicating that the increased cerebrospinal fluid level did not result only from the blood contamination. Twenty-four hours after induced subarachnoid hemorrhage, the immunoreactivities of substance P and calcitonin gene-related peptide remained significantly higher than the basal level (P<.01). At day 3, both immunoreactivities had decreased to a level nonsignificantly different from the basal level.

Conclusions The early high values of the cerebrospinal fluid immunoreactivities for substance P and calcitonin gene-related peptide, apart from the contamination by arterial blood, probably resulted from the depletion of neurotransmitter peptides from the trigeminovascular fibers. (Stroke. 1994;25:160-164.)

Key Words • cerebrospinal fluid • subarachnoid hemorrhage • vasospasm • rabbits

It is now well documented that cerebral blood vessels are supplied by nerves containing the neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP), which originate mainly in the trigeminal ganglion. In vitro studies show that these peptides have vasodilator properties that seem to be implicated in many pathological circumstances such as vascular encephalic pain, postocclusive cerebral hyperemia, and subarachnoid hemorrhage (SAH). The latter pathological condition is frequently associated with the delayed arterial narrowing called cerebral vasospasm. The pathogenesis of this phenomenon is poorly understood, but it may be assumed that a contributing factor is the development of an imbalance between the vasoactive agents present, with a prevalent substance P–like immunoreactivity (P<.01) and calcitonin gene-related peptide–like immunoreactivity (P<.01). Arterial and hemorrhagic cerebrospinal fluid levels of substance P–like immunoreactivity were different (P<.03), indicating that the increased cerebrospinal fluid level did not result only from the blood contamination. Twenty-four hours after induced subarachnoid hemorrhage, the immunoreactivities of substance P and calcitonin gene-related peptide remained significantly higher than the basal level (P<.01). At day 3, both immunoreactivities had decreased to a level nonsignificantly different from the basal level.

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Materials and Methods

Animals Twenty-one male Fauve de Bourgogne rabbits weighing 2.5 to 3 kg were used in this study (SAH group, n=11; sham group, n=10).

SAH Procedure and CSF Sampling The rabbits were anesthetized with acepromazine (17 mg/kg IV) and sodium pentobarbital (20 mg/kg IV). Arterial blood
(2 mL) was sampled from the central artery of the ear, 1 mL for assay of the peptides and 1 mL to induce SAH. After the suboccipital region was shaved, a needle (0.9 mm in diameter) was inserted percutaneously into the cisterna magna. A small amount of control CSF (0.5 to 0.8 mL) was first withdrawn, then, without moving the needle, 1 mL of the homologous arterial blood was injected into the subarachnoid space. This injection was performed carefully over a period of 15 seconds to minimize the acute intracranial hypertension induced. Animals of the sham group were treated identically, except that they received 1 mL of artificial CSF (composition in mmol/L: NaCl 126, KCl 2.9, MgCl2 0.9, NaHCO3 18, CaCl2 1.2, glucose 5.4; gassed with 4% CO2/20% O2/76% N2, pH 7.3 to 7.4, temperature 37°C). The rabbits were then tilted head down for 3 minutes. Thirty to 40 minutes later, a second sample of CSF (0.4 to 0.8 mL) was taken. The third CSF sample was removed at 24 hours (n=5) or 3 days (n=5) after the injection, after which animals of the SAH group were decapitated under anesthesia. Gross anatomic verification confirmed, in all animals, the presence of a clot surrounding the basilar artery and the pterionl arch, both at 3 days the volume was sometimes reduced.

All the CSF and blood samples, collected in the presence of EDTA, were centrifuged immediately after removal. Supernatants were mixed in polypropylene tubes with a small amount (10% of their volume) of a protease inhibitor mixture (0.5 mg/mL benzamidine, 0.17 mg/mL phenylmethylsulfonyl fluoride, and 0.36 mg/mL p-hydroxymercuribenzoate) and frozen (—20°C) until extraction.

**Extraction and Immunoassays for SP and CGRP**

The samples were assayed without knowledge of the experimental procedures.

**Extraction.** Plasma or CSF aliquots (360 µL) were acidified with 40 µL of 1N HCl and heated at 95°C for 10 minutes. After a centrifugation for 4 minutes at 4°C in a Beckman microfuge, supernatants were neutralized with 1N NaOH and immediately assayed for SP-LI and CGRP-LI. Experiments indicated that these extraction procedures allowed the recovery of more than 90% of the two peptide activities.

**Immunooassays.** SP-LI was measured by a competitive enzyme immunoassay that we developed and described previously.18,19 This assay uses a rabbit polyclonal anti-SP antibody, an SP-acetylcholinesterase conjugate (SP-AChE) as a tracer, and a microtiter plate coated with a mouse monoclonal antibody to rabbit immunoglobulins to separate free from bound tracer. Enzyme activity was measured by the Ellman method.20 Under our experimental conditions, the sensitivity of the assay (ie, concentration of unlabeled SP that displaced 50% of the tracer binding) was about 100 pg/mL, and the minimum detectable value (concentration of SP inducing a significant lowering—3 SD—of tracer binding) was 10 pg/mL. Several dilutions of each sample were routinely assayed, and a parallelism between the dilution curves of standards and sample curves was always observed.

CGRP-LI was assayed through a novel competitive enzyme immunoassay using, as for the immunoassay of SP, a peptide-AChE conjugate as the tracer and a microtiter plate coated with a monoclonal antibody to rabbit immunoglobulins as the separation method (unpublished data). Briefly, the tracer was prepared as follows: After introduction of thiol groups into CGRP by reaction of its primary amino groups with N-succinimidyl-S-acetylthioglycolic acid, thiolated CGRP was covalently coupled to AChE by use of the heterobifunctional reagent N-succinimidyl-4-(N-maleimidomethyl)-cyclohexane carbodiimide as previously described for other in vivo or in vitro coupling reactions.21,22 The antisera (anti-CGRP rabbit polyclonal antibodies from Peninsula, R.A.S. 6012) was used at a final dilution of 1:300 000. This antisera has been prepared against human CGRP-II, which is identical to rabbit CGRP in 35 of 37 amino acid residues (only amino acids in positions 1 and 35 are different). Considering that the cross-reactivity of the antiserum with rat α-CGRP, which also differs from human CGRP-II in the same positions, is 100% (Peninsula catalog), it is very likely that the cross-reactivity of the antiserum with rabbit CGRP is close to 100%. To improve the sensitivity of the assay, a preincubation of samples or standards with the antibodies was performed for 4 hours at 4°C in the wells of the microtiter plate before the tracer was added for a further 48-hour incubation period at 4°C. Under these experimental conditions, the sensitivity of the assay, as defined above, was around 500 pg/mL, and the detection limit was close to 100 pg/mL. The validity of the determination of CGRP-LI is supported by the fact that, as observed in the immunoassay for SP, different dilutions of the same sample provided identical results, indicating good parallelism between the standard curve, established with rat α-CGRP, and sample dilution curves. Furthermore, high-performance liquid chromatographic fractionation control experiments indicated that the immunoreactive material found in biological extracts is eluted in a single immunoreactive peak with a retention time identical to that of synthetic rat CGRP (data not shown).

**Data Analysis**

The CSF values of SP-LI and CGRP-LI of the various groups were compared by analysis of variance followed by a Dunnett test. For statistical tests, the values were converted to logarithms, since the distributions of the values within several groups were otherwise nongaussian. Plasma levels of SP-LI and CGRP-LI were compared with the CSF levels at 30 minutes by Student’s t test. P<.05 was considered a significant probability.

**Results**

The control levels in the CSF of SP-like and CGRP-like peptides were 0.7±0.3 and 3.0±0.9 ng/mL, respectively (n=21). These concentrations correspond to 5×10^-9 and 7×10^-10 mol/L, respectively. There was no significant difference between the SAH (n=11) and the sham groups (n=10); therefore, the SAH and sham values were pooled. The basal levels of SP-LI and CGRP-LI were about 25-fold higher in plasma than in CSF (Table).

Very substantial modifications of the CSF SP-LI and CGRP-LI were induced by the experimental SAH but not by the sham injection. The time course of the SP-LI and CGRP-LI in the CSF for SAH animals is represented in the Figure, which shows that the changes in concentration of the two peptides in the CSF samples were closely related. Data are expressed as the mean±SEM and summarized in the Table.

Thirty minutes after the experimental SAH, the second CSF sample showed a significant increase in the SAH group, about 50-fold for SP-LI and 18-fold for CGRP-LI (corresponding to about 2×10^-7 and 1×10^-7 mol/L, respectively). There was no significant variation in the sham group. Comparison with the basal level in the plasma showed that SP-LI, but not CGRP-LI, was significantly higher in the CSF (P<.03). However, a plot of plasma SP or CGRP concentrations versus CSF SP or CGRP concentrations for each rabbit showed no correlation between plasma and CSF values: for SP, r=.027, P=.96; for CGRP, r=.36, P=.63. Twenty hours after the induced SAH, the SP-LI and CGRP-LI in the CSF remained significantly raised despite a partial return toward control levels, but at day 3, they were not significantly different from control. At neither of these times were the values of the sham group significantly different from control.
SP-LI and CGRP-LI in the Plasma and the CSF Before and After Induced SAH

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<tr>
<th></th>
<th>Substance P</th>
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<td>Basal Level</td>
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<td>CSF, 24 Hours</td>
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<td>Sham</td>
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<tr>
<td>(n=21)</td>
<td>13.6±2.2</td>
<td>0.7±0.3</td>
<td>1.2±0.3 NS</td>
<td>3.0±0.9</td>
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<tr>
<td>SAH</td>
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<td>1.2±0.4 NS</td>
<td>5.2±1.2 NS</td>
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<tr>
<td>(n=21)</td>
<td>13.6±2.2</td>
<td>0.7±0.3</td>
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<td>33.9±15.1 NS</td>
<td>9.8±5.8 NS</td>
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<td>4.9±1.3 NS</td>
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SP-LI indicates substance P-like immunoreactivity; CGRP-LI, calcitonin gene-related peptide-like immunoreactivity; CSF, cerebrospinal fluid; and SAH, subarachnoid hemorrhage.

Values are expressed as mean±SEM (ng/mL).

Discussion

The aim of this work was to study the effects of the arterial blood in the subarachnoid space on the CSF level of SP-LI and CGRP-LI and to evaluate possible effects resulting from the increase of the intracranial pressure caused by the injection. The lack of significant variation in the CSF concentrations of SP-LI and CGRP-LI of the sham group strongly suggests that the intracranial pressure effects were of negligible importance for peptide release under the present conditions. SAH is a frequent pathological cause of severe increase of intracranial pressure by alteration of the intracranial volume buffering capacity and CSF absorption. In experimental conditions, this parameter is modulated by the volume of injected blood. Our procedure of induced SAH was different from that of most investigators in that we injected a single, relatively small amount of arterial blood (1 mL). This volume was added shortly after the first CSF sample (0.5 to 0.8 mL) was removed, so the variation of intracranial pressure was due to the addition of only about 0.2 to 0.5 mL real injected volume. Experimental or pathological elevation of intracranial pressure has been shown to increase cerebral blood flow, which could be interpreted as resulting from the stimulation of trigeminal fibers innervating the dura mater, the venous sinuses, and the cerebral arteries, because it is well established that stimulation of the trigeminal vascular system induces cerebral vasodilation by antidromic release of the vasodilator peptides SP and CGRP. The lack of significant increases in CSF peptide immunoreactivity in sham animals might be explained by the relatively small liquid volume increase (not exceeding about 25%).

In contrast to the sham animals, the CSF of blood-injected animals contained extremely high concentrations of SP-LI and CGRP-LI at 30 minutes and 24 hours but nonsignificantly different levels compared with control at 3 days. In view of the high plasma concentrations of these two reactivities (Table), how much of the increase in CSF levels was due to simple addition of peptide-rich plasma? In the case of the SP-LI, it is clear that the early CSF levels greatly exceeded the plasma levels, so a large majority of the excess SP in the CSF at 30 minutes must have come from a nonplasmatic source. In the case of the CGRP-LI, the situation is less clear-cut, since the plasma and CSF concentrations were not significantly different. However, after the CSF sample is withdrawn, the remaining volume of CSF can be estimated as about 1.5 mL, since the rate of production of CSF is known to be about 0.5%/min, corresponding to about 10 µL/min, giving a total control CSF volume of 2 mL. A simple calculation shows, therefore, that the concentration of CGRP-LI in CSF excluding that added by the plasma (1 mL) would be about 39 ng/mL, assuming mixing of CSF with the blood. This may not be a totally valid assumption, but the value is so far above the basal CSF level that a simple plasma-dilution explanation seems untenable. Finally, the absence of correlation between plasma and CSF (at 30 minutes) concentrations of peptide is in accordance with this interpretation.

Our results can therefore be best explained by an effect of the injected blood on the trigeminal fibers around cerebral blood vessels, releasing large quantities of SP and CGRP. This is compatible with studies showing that SAH causes depilation of trigeminal nerves, as suggested by the reduced density of the SP-LI and CGRP-LI in perivascular nerves in the subacute phase of SAH. This was recently suggested to be due to a direct effect of erythrocytes on the nerve fibers. It cannot be excluded that the synthesis of these peptides is also stimulated, further increasing their CSF concent-
trations. The frequent colocalization of these peptides in the trigeminovascular nerves probably explains the essentially identical variations in CSF SP-LI and CGRP-LI observed. Finally, the external jugular vein level of CGRP-LI has been found to be increased in correlation with cerebral vasospasm after SAH.31

The present results thus show that bleeding in the subarachnoid space results in a marked increase in the SP-LI and CGRP-LI in the CSF, caused by both trigeminal nerve stimulation and the blood contamination. The time course of the variations in the peptide levels shows that the most intense period of release is within the first 24 hours, perhaps within a few hours. The literature on the in situ and in vitro reactivity of cerebral arteries to SP and CGRP suggests that the CSF concentrations measured are within the vasoactive range. For example, feline pial arteries in situ dilated substantially to SP concentrations of 10−9 to 10−6 mol/L,32 and the pD2 for isolated human cerebral arteries was reported as 8.7.33 Likewise, in vitro pD2 values for CGRP of cerebral arteries of various species ranged from 8.3 to 9.4, with a maximum response at 10−7 mol/L.3,34 Thus, according to our findings, the CSF peptide concentrations varied between levels inducing half-maximal (basal values) and maximal (30-minute values) relaxation. This means that the peptide-induced dilator tone would increase substantially after the blood injection. Cerebral blood flow was not measured in the present experiments, but data in humans suggest that a hyperemic phase may occur by day 3 after SAH, preceding a vasospasm.35 In a previous study with the same model, we found that the reactivity of arteries removed shortly (10 minutes) after the injection of blood was highly potentiated for two endogenous constrictors, serotonin and uridine triphosphate.36 The high concentrations of CSF dilator peptides (maximally effective concentrations) present at this time can thus be expected to substantially limit the degree of vasoconstriction during the first few hours or even cause a vasodilatation at 24 hours to 3 days.

At 3 days the peptide levels are largely back to normal, and this subacute period generally corresponds to the beginning of a vasospasm, although events may occur by a little slower in humans. The time course of the imbalance between the vasoactive agents in CSF may explain the kinetics of cerebral vasospasm occurring after SAH: (1) the exhaustion of the vasodilator peptide reserves (SP and CGRP) contained in the distal trigeminovascular fibers and the subsequent fall-off of CSF concentrations to, probably, a very low level may lead to progressive domination of the various vasoconstrictor activities released by SAH, resulting in a spasm maximal at less than 1 week;37 (2) later, however, these vasoactive peptides are synthesized once again by the nerve cell bodies and transported by the fast axoplasmic flow (normally 300±100 mm/d) to the distal part of the nerves. The time necessary for the vasodilator peptides to return to normal levels is probably several days, corresponding to the terminal phase of the cerebral vasospasm. This lapse of time may be even longer if the fast axonal transport is impaired by the SAH. These considerations obviously do not exclude the probability of parallel but more or less opposite changes in the CSF concentration of vasoconstrictor factors.

The present results are thus in agreement with other clinical and experimental data1,25,38 supporting the idea that the trigeminovascular system plays a protective role against the arterial narrowing occurring after SAH. Further study of the dynamics of the release of dilator peptides will no doubt improve our understanding of the development of cerebral vasospasm.

Acknowledgment

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References

It is generally believed that SAH—be it aneurysmal or experimental—causes a brief period of vasospasm, which is then followed by a period in which the vessels are of normal caliber. The authors propose an interesting explanation for this period of normalcy before vasospasm sets in, namely, that the vasoconstrictive properties of blood components are being balanced by the vasodilator effects of SP-LI and CGRP-LI released from trigeminal nerve endings around blood vessels. I do not believe that these findings can be used in the near future to prevent vasospasm after SAH, nor do I concur with the authors’ possible explanation for the disappearance of vasospasm, namely, that the levels of SP-LI and CGRP-LI in the nerve endings are restored after some time to counteract the vasoconstriction again. Nevertheless, since the absence of vasospasm in the first few days after SAH has been thought of more as a period in which vasospasm is developing rather than as a period in which there is still a balance between constriction and dilatation, this paper leads us away from the trodden paths and encourages reassessment of the whole process of vasospasm.

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