Neuropeptide Y in the primate model of subarachnoid hemorrhage

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The cause of cerebral vasospasm after subarachnoid hemorrhage (SAH) remains unknown. Recently, an association between the potent vasoconstricting peptide, neuropeptide Y, and delayed cerebral vasospasm after SAH has been postulated. This was based on the findings of increased neuropeptide Y levels in the cerebrospinal fluid (CSF) and plasma after SAH in animals and humans. For this study, the primate model of SAH was used to assess the possible role of neuropeptide Y in delayed vasospasm after SAH. Fifteen cynomolgus monkeys underwent placement of a clot of either whole blood or red blood cells in the subarachnoid space around the middle cerebral artery (MCA). Sequential arteriography for assessment of MCA diameter and sampling of blood and CSF for neuropeptide Y were performed: before SAH (Day 0); 7 days after SAH, when signs of delayed cerebral vasospasm peak in this model and in humans; 12 days after SAH; and 28 days after SAH.

Subarachnoid hemorrhage did not evoke changes in CSF or plasma levels of neuropeptide Y. Nine monkeys had arteriographic evidence of vasospasm on Day 7, but no change in neuropeptide Y levels occurred in plasma or CSF. In addition, neuropeptide Y levels did not change, even after resolution of vasospasm on Day 12 or Day 28. Neuropeptide Y levels were substantially higher in CSF than in arterial plasma (p < 0.003 at each interval). No correlation was found between neuropeptide Y levels in CSF and in plasma. These results do not confirm a relationship between neuropeptide Y levels in the CSF or peripheral plasma and delayed cerebral vasospasm in SAH.

KEY WORDS • neuropeptide Y • vasospasm • cerebrospinal fluid • subarachnoid hemorrhage • cynomolgus monkey

For four decades neuroscientists have sought, unsuccessfully, to identify the agent(s) that cause delayed narrowing of cerebral vessels after subarachnoid hemorrhage (SAH). Recently, a new vasoactive peptide was isolated from the porcine brain65,66 that has been found to be one of the most widely distributed neuropeptides within and without the central nervous system in several species, including man.2,8,9,11,20,32,39,43,47,50,54,57,61,67 This 36-amino acid peptide, named neuropeptide Y after the tyrosine amine at the C terminus and tyrosine residue at the N terminus, is a potent direct and indirect constrictor of peripheral vessels34,47 and cerebral arteries in rats,19,69 cats,20,31 rabbits,1,19 and man.30,34 Demonstration of neuropeptide Y in the cerebral arteries of rats,69 cats,20 guinea pigs,70 and humans,4 and in the cerebrospinal fluid (CSF) of rabbits21 and humans61 suggests its involvement in the regulation of cerebrovascular tone. Increased CSF concentrations of neuropeptide Y in a rabbit model of SAH1 and in the CSF of patients with delayed neurological deficit after SAH,64 depletion of neuropeptide Y activity in arteries of the circle of Willis after SAH in dogs73 and rats,23 and an increase in plasma levels of neuropeptide Y in some patients after SAH,22 suggest a possible role of neuropeptide Y in the etiology of post-hemorrhagic vasospasm.

We used a primate model of SAH and cerebral vasospasm to establish whether changes in the CSF neuropeptide Y concentration are associated with the development of delayed vasospasm after SAH and to determine if there is a correlation between CSF and plasma neuropeptide Y concentrations.

Materials and Methods

The primate model of SAH developed by Weir and his group64 was used. Fifteen cynomolgus monkeys (10 males and five females), each weighing from 2.3 to 5.8
kg (mean ± standard deviation 4.1 ± 1.3 kg), underwent placement of whole blood (11 animals) or red blood cells suspended in an artificial collagen clot (four animals) around the right middle cerebral artery (MCA) (J Morgan, et al., unpublished data). The protocol was reviewed by the National Institute of Neurological Disorders and the Stroke Animal Care and Use Committee, and met National Institutes of Health guidelines for animal care.

Surgical Procedure

The procedure is generally the same as that described elsewhere.44 Prior to surgery the monkeys received atropine sulfate (0.05 mg/kg), sodium thiopental (25 mg/kg), dexamethasone (0.7 mg/kg), cefazolin (500 mg), and intramuscular ketamine (10 mg/kg). They were intubated and ventilated with N2O:O2 (1:1), and 0.5% to 1% isoflurane was used as the anesthetic agent. The expired PaCO2 level was maintained at approximately 40 mm Hg by ventilatory control and was confirmed by arterial blood gas level measurements. A right frontotemporal craniotomy was performed under asptic conditions, the dura mater was opened, and with sharp dissection the sylvian fissure was entered. The arachnoid over the proximal portion of the MCA and internal carotid bifurcation was opened. A clot consisting of 2.5 to 5 ml whole blood or red blood cells in a collagen clot was placed around the right MCA.

Arteriography

To assess vasospasm, cerebral arteriograms were performed 2 to 4 days before surgery and on Days 7 and 12 postoperatively. For arteriography, monkeys were anesthetized with intramuscular ketamine (10 mg/kg) and xylazine (Rompun, 1 mg/kg). A femoral artery cutdown was performed under asptic conditions and a No. 3 (animals weighing < 5 kg) or No. 4 (animals weighing > 5 kg) French polyethylene catheter was advanced, under fluoroscopic control, to the right internal carotid artery. Contrast medium (0.5 to 0.75 ml Conray 60%) was injected by hand. The filming sequence was 2 films/sec for 3 seconds then 1 film/sec for 6 seconds. All filming was carried out with a magnification factor of 2. Subtractions of the anteroposterior projections were made. The presence of vasospasm was defined by comparing the results of cerebral angiography of the right MCA before surgery to the results of arteriography on Days 7 and 12 after surgery. A computerized image analysis system* was used to measure the area of the proximal 14 mm of the right MCA using an anteroposterior projection (A Zauner, et al., unpublished data). The animals were assigned to one of two groups, those with vasospasm and those without vasospasm, according to whether the pre- and postoperative arteriographic measurements of the right MCA indicated narrowing of the vessel lumen (defined as > 25% reduction of measured area in the anteroposterior view).

Blood and CSF Sampling

Blood and CSF were sampled on Days 0, 7, 12, and 28. Heparinized blood samples from an arterial line were transferred to tubes with aprotinin (Trasylol, 0.25 mg/ml) and centrifuged at 1200 G for 10 minutes. The plasma was then quickly removed, frozen in dry ice, and stored at −70°C for up to 2 weeks. Cerebrospinal fluid samples were collected via a percutaneous puncture of the cisterna magna at the time of anesthesia for cerebral arteriography, except the Day 0 samples, which were obtained during surgery through direct access to the CSF cisterns. The CSF was rapidly transferred to tubes with Trasylol (0.25 mg/1 ml CSF), frozen in dry ice, and stored at −70°C for up to 2 weeks.

Neuropeptide Y Radioimmunoassay

After thawing, plasma and CSF samples (2 ml) were extracted with ice-cold acid ethanol (1:2). After 2 hours of incubation at 4°C, the samples were centrifuged for 20 minutes at 2000 G and the supernatants were transferred to prechilled polyethylene tubes and evaporated to dryness in a concentrator. The dry extracts were redissolved in 0.8 ml of ice-cold assay buffer (0.1% Triton X-100, 0.05 M NaCl, 0.5% bovine serum albumin, and 0.01% sodium azide, pH 7.4) and subjected to radioimmunoassay using neuropeptide Y antiserum.† Synthetic porcine neuropeptide Y in serial dilutions of 7.3 to 940 fmol/tube was used to construct a standard curve for the assay. After 24 hours of incubation at 4°C with 100 μl of neuropeptide Y antiserum, [125I]-radiolabeled synthetic neuropeptide Y was added (6000 cpm at 100 μl/tube) as a tracer, followed by another 24 hours of incubation. Then, 100 μl of goat antirabbit gamma globulin and 100 μl of normal rabbit serum were added to separate free and antibody-bound tracer. After 2 hours of incubation at room temperature, the samples were centrifuged for 20 minutes at 2000 G and the supernatants were discarded. The radioactivity of [125I]-neuropeptide Y in the remaining pellets was then determined. The assay sensitivity was 15 fmol/tube, the intra-assay coefficient of variation was 5%, and the interassay coefficient of variation was 10%. Levels of neuropeptide Y immunoreactivity in plasma and CSF samples are expressed as means ± standard deviation in pmol/liter.

Statistical Analysis

For statistical analysis the paired and unpaired t-test, analysis of variance (ANOVA), and correlation coefficients were used. Significance was accepted at p < 0.05.

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![Graph showing mean neuropeptide Y levels in plasma and cerebrospinal fluid](image)

**Results**

Substantial vasospasm (>25% reduction in the anterior-posterior area of the proximal 14 mm of the right MCA) occurred in nine of the 15 monkeys 7 days after placement of clot. During the experiment, plasma and CSF neuropeptide Y levels were stable. The mean plasma levels of neuropeptide Y for all animals on Days 0, 7, 12, and 28 were 47 ± 13, 52 ± 10, 44 ± 8, and 39 ± 4 pmol/liter, respectively. Mean CSF concentrations on the same days were 120 ± 33, 108 ± 52, 106 ± 19, and 112 ± 17 pmol/liter (Fig. 1). When all animals were considered, the differences in neuropeptide Y levels in plasma or CSF on Days 0, 7, 12, and 28 were not significant (ANOVA, p > 0.1).

**Correlation of Vasospasm and CSF and Plasma Neuropeptide Y Levels**

In animals with vasospasm on Day 7, plasma neuropeptide Y levels were similar to the levels found in monkeys without vasospasm (unpaired t-test, p = 0.76) (Table 1). Furthermore, the plasma levels in the group with vasospasm on Day 7 were unchanged from the control levels (paired t-test, p = 0.06), as were the levels in the group without vasospasm (paired t-test, p = 0.78) (Table 1).

The CSF levels of neuropeptide Y did not change in association with vasospasm (Table 1). The difference between monkeys with vasospasm and without vasospasm was insignificant, although the mean level of neuropeptide Y in the CSF of monkeys with vasospasm on Day 7 was slightly higher (120 ± 65 vs. 90 ± 19 pmol/liter; unpaired t-test, p = 0.34). Similarly, there was no statistically significant difference in CSF neuropeptide Y concentrations in the group of monkeys with vasospasm on Day 7 and in the same monkeys on Day 0 (paired t-test, p = 0.66).

In all nine monkeys with cerebral vasospasm, arterial narrowing (a decrease in the anterior-posterior area of the right MCA of <25% compared to the baseline measurement) had resolved by Day 12. The neuropeptide Y levels in the CSF and plasma were unaffected by the resolution of vasospasm (p > 0.1 for comparisons of plasma and CSF neuropeptide Y on Days 12 and 28 to Day 7).

**Influence of the Clot Type on Neuropeptide Y**

Comparison of neuropeptide Y levels in plasma and CSF between the groups of animals with whole blood and red blood cells demonstrated no differences (ANOVA, p > 0.2). Additionally, there was no significant change in the CSF neuropeptide Y concentrations from basal levels at any sampling time in animals that received either whole blood or red blood cells (ANOVA, p > 0.1).

**Correlation of Plasma and CSF Neuropeptide Y in Primates**

The neuropeptide Y levels in CSF were almost threefold higher than in plasma in all groups at all times (Table 1 and Fig. 1). The difference between neuropeptide Y levels in plasma and CSF was highly significant at all sampling times (paired t-test, p < 0.003). Furthermore, there was no correlation of simultaneous values in CSF and plasma, nor was there a correlation between changes in CSF levels and changes in plasma levels (R² = 0.014, correlation coefficient 0.117).

**Discussion**

**Neuropeptide Y**

The discovery of neuropeptide Y, 65 the determination of its amino acid structure, 65 its messenger ribonucleic acid isolation from human phaeochromocytoma, and the subsequent synthesis of its complementary deoxyribonucleic acid 65 evoked an intensive search for its physiological role. Immunohistochemical studies revealed a wide distribution of neuropeptide Y-like im-
munoreactivity in the nervous system of mammals.\textsuperscript{1,2,8-10,17,21,31,32,39,47,49,50,57,61,67,71} Neuropeptide Y is one of the most abundant peptides identified in several areas of the central nervous system, including the hypothalamus,\textsuperscript{9,7} pituitary gland,\textsuperscript{48} limbic system,\textsuperscript{15} cortex,\textsuperscript{13} epine
dyma,\textsuperscript{47} and brain stem.\textsuperscript{9} It has also been demonstrated in the peripheral sympathetic nervous system of the cardiovascular system,\textsuperscript{5,7,7,46} the gastrointestinal system,\textsuperscript{26} the kidney,\textsuperscript{30} and the adrenal medulla,\textsuperscript{4} and in other endocrine organs (pancreas, thyroid gland, gonads, and pineal gland).\textsuperscript{46,72} Thus, it is widely spread throughout the central and peripheral adrenergic system.\textsuperscript{44} Neuropeptide Y exerts its effect through the sympathetic nervous system. It coexists with noradrenaline in sympathetic nerve endings\textsuperscript{1,2,12,26,37,62} and is coreleased with norepinephrine by sympathetic stimuli.\textsuperscript{15,42,43,55}

The peripheral vasoconstrictive properties of neuropeptide Y \textit{in vitro}\textsuperscript{34} and \textit{in vivo}\textsuperscript{40,66} have been defined. Constrictive action \textit{in vitro} is by direct action on the smooth-muscle cells,\textsuperscript{5} whereas the pressor action of neuropeptide Y \textit{in vivo} is by potentiation of vasoactive catecholamines.\textsuperscript{1,19,34,72,74} Three mechanisms of neuropeptide Y action at the neuroeffector junction occur: (1) direct postsynaptic action\textsuperscript{19-21} through inhibition of 3',5' cyclic adenosine monophosphate synthesis,\textsuperscript{5,35} which produces vasoconstriction; (2) potentiation of the postsynaptic response of norepinephrine;\textsuperscript{38,74} and (3) presynaptic inhibition of norepinephrine release from sympathetic nerves.\textsuperscript{28} In spite of the many recent advances, the physiological and pathophysiological roles of neuropeptide Y remain unclear.\textsuperscript{26,53,71}

\textbf{Neuropeptide Y and Vasospasm}

It has been proposed that neuropeptide Y, because of its vasoconstrictive action,\textsuperscript{1,6,18,21,42,53,63,66,69} its influence on cerebral blood flow (CBF),\textsuperscript{8,63,69} its existence in the wall of cerebral vessels,\textsuperscript{8,18,20,31,38,70} its presence in the CSF\textsuperscript{11,47,64} and plasma\textsuperscript{2,3,4,2,4,4,4} and its increased levels in the CSF following SAH,\textsuperscript{1} may be a causative agent of cerebral delayed vasospasm after SAH.\textsuperscript{13} Several experiments in animals and observations in humans support this theory. In a rabbit model of SAH, McDonald, et al.,\textsuperscript{47} observed a dramatic increase in CSF neuropeptide Y concentration after SAH. Additionally, depletion of the neuropeptide Y stores in cerebral arteries\textsuperscript{33,47} and the increase in its level in CSF\textsuperscript{46} and plasma\textsuperscript{23} in patients after SAH seemed to confirm its involvement in cerebral delayed vasospasm.

However, certain of the observations purported to link neuropeptide Y and cerebral vasospasm may not be relevant for consideration of delayed cerebral vasospasm after SAH. Although the vasoconstrictive effect of neuropeptide Y was initially observed after intracarotid injection of the neuropeptide,\textsuperscript{3,16} peptides exert their action on vessels from an extraluminal, rather than luminal, side of the artery.\textsuperscript{68,69} The conclusions of neuropeptide Y involvement in cerebral vasospasm by other investigators were based on the observation of increased levels of neuropeptide Y in the CSF rather than in peripheral blood.\textsuperscript{1,54} The principal aim of the current study was to clarify the association, if any, of neuropeptide Y in the production of delayed cerebral vasospasm in the primate model of SAH.

\textbf{Neuropeptide Y Concentration in CSF}

The depletion of neuropeptide Y in the arterial wall after SAH\textsuperscript{33,31} and the increase in CSF after SAH\textsuperscript{1,47,64} suggest neuropeptide Y involvement in delayed vasospasm.\textsuperscript{1} However, in our primate model of SAH, in which nine of 15 monkeys developed moderate to severe narrowing of the MCA, there were no changes in the CSF level of neuropeptide Y after SAH and there was no association of the levels of neuropeptide Y in CSF with the presence of vasospasm.

Several factors might account for the differences between our results and those of prior studies. One is the timing of CSF sampling. After SAH, neuropeptide Y is released acutely from MCA storage sites.\textsuperscript{33,71} This acute vasospasm\textsuperscript{39} is important for initial arterial constriction to terminate hemorrhage.\textsuperscript{62} In the rabbit model of SAH, CSF samples were obtained on Day 3 after SAH,\textsuperscript{1,47} a time when arterial wall neuropeptide Y stores are known to be depleted.\textsuperscript{33} Therefore, the observed higher levels of neuropeptide Y in CSF may be related to the acute phase of the SAH. We sampled CSF on Day 7 after SAH, when functionally significant vasospasm develops in primates and humans\textsuperscript{24,40,72} (also J Morgan, et al., and A Zauner, et al., unpublished data).

Another factor relates to the different experimental models of SAH. Neuropeptide Y concentrations in the CSF of rabbits increase early after SAH,\textsuperscript{1,46,47} but in humans during the same interval (Days 3 to 5 after SAH) CSF neuropeptide Y concentrations are low.\textsuperscript{64} Moreover, in the rat model, SAH depleted neuropeptide Y arterial stores in one study\textsuperscript{31} but did not affect the level of neuropeptide Y-like immunoactivity in the cerebral arteries in another.\textsuperscript{18}

In the only study that demonstrated higher CSF neuropeptide Y levels after SAH in humans,\textsuperscript{64} the increase occurred several days after SAH. The neuropeptide Y increase, however, was related to delayed neurological deficit and not to vasospasm demonstrated by arteriography. However, cerebral ischemia stimulates the central sympathetic nervous system, producing an increase in CSF catecholamine.\textsuperscript{12,21,62} Therefore, it is expected that it would also produce an increase in CSF neuropeptide Y levels. Moreover, neuropeptide Y-like immunoreactivity disappears from neocortex and striatum after transient cerebral ischemia.\textsuperscript{29} Thus, the delayed neurological deficits produced by cerebral ischemia may have been responsible for the increase in CSF neuropeptide Y concentrations in the patients described by Suzuki, et al.\textsuperscript{64} None of the monkeys in our experiment developed delayed neurological deficits that were apparent on daily examination.

It is worthwhile to underline here the difference between the effects of ischemia and SAH on the distri-
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duption of neuropeptide Y storage. Cerebral ischemia does not produce a depletion of neuropeptide Y in cerebral arteries28 but does deplete neuropeptide Y in cerebrospinal fluid20,21. However, SAH depletes storage of neuropeptide Y in the arterial wall22,13,71 and probably diminishes cerebral storage as well.1,47

Plasma Neuropeptide Y Level
Allen, et al.,8 first discovered that intra-arterial neuropeptide Y decreased CBF in the rat. This was confirmed by Edvinsson, et al.21 who related the diminished CBF to potent vasoconstriction of the MCA and of the pial arteries by neuropeptide Y in the plasma. However, Tuor, et al.,68 who repeated Allen’s experiment, was unable to confirm the influence of an intra-carotid bolus of neuropeptide Y on CBF. They concluded that a peptide that produces prolonged and marked CBF changes must gain access to the abluminal side of the blood-brain barrier. This observation was supported by experiments in vitro, which showed vasoconstriction of the MCA and the pial arteries20,21,30 evoked by neuropeptide Y weak or no vasoconstriction after parenteral infusion.4,5,3

In the only report on the plasma level of neuropeptide Y after SAH in humans, Edvinsson, et al.,21 found a correlation between neuropeptide Y levels and vasospasm only in patients with higher blood flow velocity in the MCA, but not for all patients with vasospasm detected by transcranial Doppler ultrasound. Our results, which demonstrate no change in peripheral levels of neuropeptide Y in primates with delayed cerebral vasospasm after SAH, indicate that delayed arteriographically observed vasospasm is not related to changes in plasma neuropeptide Y concentration.

Relationship of CSF vs. Plasma Concentrations of Neuropeptide Y
Neuropeptide Y-like activity is detectable in human plasma22,24 and in CSF.11,14 The neuropeptide Y concentration in plasma rises after stimulation of the sympathetic system.5,41,42,54 The level of neuropeptide Y in the CSF is influenced by release of this neuropeptide from the cerebral arterial wall18 and/or from the brain in situations (such as cerebral ischemia29 and SAH30) that are known to stimulate potently the sympathetic nervous system.51,52 In our experiment, there was no correlation between CSF and plasma concentrations of neuropeptide Y, and changes in these concentrations in CSF and plasma were unrelated. This finding suggests that the production and release of neuropeptide Y to CSF and plasma are regulated by different, unrelated, mechanisms.

The significantly higher levels of neuropeptide Y in CSF than in plasma confirm the active release of the peptide to the subarachnoid space from the brain and arteries21,66 or other unknown sources,1 but any physiological or pathophysiological role of neuropeptide Y in the CSF remains unclear.

References
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