Calcitonin Gene–Related Peptide Reduces Brain Injury in a Rat Model of Focal Cerebral Ischemia

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Background and Purpose  Calcitonin gene–related peptide is an endogenous vasodilating neuropeptide with a dense concentration in the trigeminocebrovascular system. It is hypothesized that depletion of this peptide contributes to delayed cerebral ischemia after subarachnoid hemorrhage and that an exogenous supply of calcitonin gene–related peptide will augment ischemic cerebral blood flow and reduce neuronal injury.

Methods  In this study we have investigated the effect of an intravenous infusion of calcitonin gene–related peptide (100 ng/kg per minute), started 1 hour before and continued throughout 4 hours of focal cerebral ischemia, on cerebral blood flow and the volume of brain injury in a rat model of middle cerebral artery occlusion.

Calcitonin gene–related peptide (CGRP) is a 37–amino acid peptide with a wide distribution throughout a variety of tissues, including the nervous system, that has particularly high concentrations in the cerebral vasculature.\(^1\) \(\alpha\)-CGRP is produced by alternative processing of the calcitonin gene product and predominate in the trigeminocebrovascular system\(^2\)–\(^6\) but differs by only three amino acids from \(\beta\)-CGRP, which is encoded by a separate gene\(^4\) and predominates in the periphery.\(^5\) The exact physiological role of these peptides and the reason for their differential expression remains unclear, but both are potent vascular smooth muscle relaxants, indicating a role in the control of vascular tone.\(^6\)

The cerebral circulation is supplied with a dense network of CGRP-like immunoreactive nerve fibers that originate in the trigeminal ganglia,\(^7\)–\(^8\) the dorsal upper cervical cord,\(^9\) and in the internal carotid miniganglia.\(^10\) CGRP is a strong vasodilator of cerebral arteries both in vitro\(^11\) and in situ,\(^12\) inducing relaxation via non–endothelium-dependent and endothelium-dependent mechanisms with an associated activation of adenylate cyclase.\(^13\) It has been hypothesized that the cerebrovascular CGRP neuronal system operates to restore normal cerebrovascular tone after vasoconstriction occurring during a pathological event, such as would occur with migraine or after subarachnoid hemorrhage (SAH).\(^12\) Patients suffering an SAH have high jugular venous CGRP levels,\(^14\) and the cerebral vasculature is depleted of this peptide if death occurs in the acute stages following the hemorrhage.\(^15\) A depletion of this peptide may contribute to the onset of delayed cerebral ischemia after SAH.

In anesthetised rats an intravenous infusion of CGRP does not affect cerebral blood flow (CBF) or systemic blood pressure below a threshold dose of 120 ng/kg per minute.\(^16\) At 100 ng/kg per minute intravenous CGRP has been shown to reverse the decrease in CBF that occurs after extracranial carotid artery dissection in the rats.\(^17\)

The aim of this study was to assess the effect on CBF and cerebral ischemia of 100 ng/kg per minute IV CGRP started 1 hour before middle cerebral artery (MCA) occlusion in the anesthetised rat. Our hypothesis was that exogenous CGRP would supplement the endogenous peptide, maximize cerebral vasodilatation, and reduce the extent of focal cerebral ischemia.

Methods  Twenty male Wistar rats (weight 339±9 g; mean±SD) were assigned to either control or treatment groups of 10 rats each. They were anesthetized with an intraperitoneal injection of fentanyl citrate (0.24 mg/kg), fluanisone (7.5 mg/kg), and midazolam (5.25 mg/kg), and anesthesia was maintained by intramuscular injections of fentanyl (0.1 mg/kg) and fluanisone (0.3 mg/kg) every 30 minutes and intraperitoneal midazolam (5.25 mg/kg) at 3 hours. A rectal thermometer was inserted, and body temperature was maintained at 37±2°C by a homeothermic operating table. The rats were paralyzed with...
intraVenous gallamine triethiodide (12 mg/kg) and ventilated via a tracheostomy. The left femoral artery was cannulated to monitor blood pressure and heart rate and to allow sampling for blood gas estimations. The left femoral vein was cannulated for the administration of CGRP (100 ng/kg per minute), or normal saline in the control groups, which was started 1 hour before and continued throughout 4 hours of left MCA occlusion.

MCA occlusion was achieved using an endovascular suture as described by Longa and his colleagues. A 3-0 monofilament nylon suture was introduced into the left internal carotid artery via an arteriotomy in the isolated external carotid artery to an average depth of 22 mm. This suture lodged in the narrow proximal segment of the anterior cerebral artery, producing occlusion of the origin of the MCA.

CBF was measured using the hydrogen clearance technique, via two platinum electrodes (diameter, 0.125 mm) in each hemisphere placed to an intradural depth of 1 mm, through two burr holes sited over the lateral parietal skull at the bregma (anterior site) and 6 mm posterior to this (posterior site). Before CBF measurement the electrodes were allowed to stabilize for 1 hour and were polarized 400 mV positive relative to a silver/silver chloride reference electrode placed subcutaneously in the anterior abdominal wall. Hydrogen was introduced into the ventilator circuit through a parallel gas input as a 20% mixture with air and oxygen to achieve brain tissue saturation. The electrical currents recorded from the cortical electrodes were sampled, digitized, and displayed graphically using computer software developed in our laboratory. The cessation of hydrogen administration produced clearance curves that were analyzed during the 45- to 105-second interval to estimate CBF at the four electrode sites. Repeated measurements of CBF were made once during each hour of the experiment.

Blood pressure, pulse rate, and body temperature were continuously monitored; blood gas analysis was performed half-hourly, and CBF estimations were calculated hourly. All animals remained normoglycemic, and ventilation parameters were altered appropriately to achieve brain gas tensions within normal limits.

Each rat that underwent 4 hours of MCA occlusion was then heparinized (2000 U/kg IV), and a midline thoracotomy was performed to gain access to the ascending aorta. The occulsive nylon suture was then removed from the internal carotid artery, and the animals were killed by exsanguination followed by perfusion-fixation with neutral formalin at 90 mm Hg. After 24 hours of immersion in formalin the brain was removed from the skull and cut into blocks to be processed through a series of formalin/alcohol/chloroform solutions and embedded in polyester wax. Coronal sections 7 μm thick were cut on a microtome, mounted on albumin-coated glass slides, and baked for 12 hours before staining with hematoxylin and eosin. In eight sections from each brain the extent of cerebral ischemia was determined with light microscopy and marked onto a corresponding section from a stereotactic atlas. The marked ischemic area was cut out and weighed for each section, and the volume of cerebral ischemia was calculated by integration. All data were analyzed using unpaired t tests with 95% confidence levels.

**Results**

There were no significant differences in basic physiological parameters (mean arterial pressure, heart rate, arterial gas tensions, and blood sugar) between the treatment and control groups (Table 1).

After preparatory carotid dissection but before MCA occlusion, CGRP increased CBF in both hemispheres from $85±3$ to $105±5$ mL/100 g per minute (mean±SEM; $t=3.524, P<.005$), which accords with previous findings. After MCA occlusion, CBF fell by 82% in the ischemic hemisphere of the control rats, with blood flows below 25 mL/100 g per minute, which is the ischemic threshold for rat neurons. In the CGRP group MCA occlusion reduced CBF by only 60% in the ischemic hemisphere, with flows maintained above the critical 25 mL/100 g per minute threshold throughout the 4-hour ischemic period (Table 2 and Fig 1).

Intravenous CGRP reduced the volume of ischemic brain injury from $234±19$ to $102±2$ mm$^3$ (mean±SEM; $t=4.469, P<.001$), a reduction of 57%. In the cortex the volume of ischemic neuronal injury decreased by 53% (191±13 to 89±19 mm$^3$; $t=4.461, P<.001$) and in the

| TABLE 1. Average Physiological Variables During the 4-Hour Period of Middle Cerebral Artery Occlusion |
|-------------------------------------------------|-----------------|-----------------|
| Variable                         | Control       | CGRP            |
| MAP, mm Hg                       | 100±2         | 102±2           |
| HR, bpm                          | 399±7         | 397±8           |
| $\text{PO}_2$, kPa                | 20±1          | 21±1            |
| $\text{PCO}_2$, kPa               | 5.1±0.1       | 4.8±0.2         |
| pH                               | 7.33±0.01     | 7.35±0.02       |

CGRP indicates calcitonin gene-related peptide; MAP, mean arterial pressure; HR, heart rate; and bpm, beats per minute. Data are expressed as mean±SEM.

| TABLE 2. Cerebral Blood Flow at the Anterior and Posterior Electrode Sites in Middle Cerebral Artery Territory for Treatment and Control Groups, Before and After Middle Cerebral Artery Occlusion |
|-------------------------------------------------|-----------------|-----------------|
| Variable                         | Control       | CGRP            | Statistical Values |
| Preocclusion                      | 85±3          | 105±5           | $t=3.52, P<.005$  |
| Postocclusion                     |               |                 |                  |
| Ischemic hemisphere              |               |                 |                  |
| Anterior                          | 13±2          | 32±2            | $t=6.92, P<.0001$|
| Posterior                         | 17±3          | 39±3            | $t=5.19, P<.0001$|
| Normal hemisphere                |               |                 |                  |
| Anterior                          | 87±5          | 109±11          | $t=1.97, P>.05$  |
| Posterior                         | 69±4          | 75±6            | $t=0.85, P>.45$  |

CGRP indicates calcitonin gene-related peptide. Data are expressed as milliliters per 100 grams per minute, mean±SEM, average values during 4 hours of ischemia.
basal ganglia by 70% (42±10 to 13±6 mm³; \( t = 2.539, P < .05 \)) (Fig 2).

**Discussion**

Our results demonstrate that pretreatment with intravenous CGRP markedly reduces the fall in CBF in this model of focal cerebral ischemia and reduces the volume of ischemic neuronal injury by 57%. The extrapolation of results and conclusions from animal stroke models to the clinical setting is fraught with difficulty, but the model of focal cerebral ischemia we have used is one of the best compromises available for the following reasons.

The endovascular suture technique of MCA occlusion produces a reproducible volume of ischemic neuronal injury (234±64 mm³, mean±SD) and has a number of advantages over direct methods of occlusion. The nylon suture technique is minimally invasive, producing an occlusion at the origin of the MCA that can be confirmed by observing the change in a hydrogen clearance curve in progress at the time of occlusion, and it avoids the problems of a craniectomy, such as loss of cerebrospinal fluid and alteration of intracranial fluid dynamics.

The use of the hydrogen clearance technique to measure CBF has distinct advantages over other techniques in allowing the simultaneous determination of quantitative local CBF at multiple sites repeatedly during the same experiment. The spreading depression injury from electrode implantation can lower CBF by 30% to 70% in acute experiments, but our use of small electrodes (0.125 mm in diameter) placed to an intradural depth of 1 mm minimizes this effect. The technique only provides CBF data from a small volume of tissue around the electrode and does not provide a complete picture of MCA blood flow; however, the standardization of electrode placement allows repeatable comparisons of CBF within groups of experiments.

Our histological assessment of neuronal injury is based on changes identifiable by light microscopy after 4 hours of MCA occlusion, but it is possible that additional tissue is subjected to injury beyond this time. The ischemic neurons are shrunken, angulated, and densely stained and are readily differentiated from healthy neurons, allowing the quantification of ischemic territory before the development of infarction. A major advantage of analyzing ischemic damage acutely is that key physiological variables, such as systemic arterial blood pressure and blood gas status, are monitored intensively right up to perfusion fixation, eliminating any secondary cerebral insults that may occur in recovery experiments. However, if permanent focal ischemia is extended for 24 hours, more of the ischemic penumbra may be recruited into the infarct, and CGRP may not reduce the ultimate infarct volume to the same extent.

It has been previously reported that exogenous intravenous CGRP reverses the fall in global CBF after carotid dissection, and the present results concur with these findings. The 10% fall in global CBF is presumably a result of global vasocostriction caused by activation of the cerebrovascular sympathetic system. Intravenous CGRP reverses this fall and, in the presence of an intact blood-brain barrier, must be achieving this effect through an endothelium-dependent process, perhaps linked to the release of nitric oxide.

In the ischemic MCA territory the cerebral vasculature will be dilated as a result of local hypoxia, hypercarbia, low flow, and the putative release of endogenous vasodilators such as CGRP. We hypothesized that the exogenous administration of CGRP would potentiate vasodilatation and improve the ischemic microenvironment. The CBF threshold for ischemic neuronal injury in rats is 25 ml/100 g per minute, and in the two sites measured in the ischemic hemisphere, pretreatment with CGRP maintained CBF above this level and reduced the volume of neuronal injury, supporting our hypothesis. Maximal dilatation of the origin of the MCA in the presence of relative MCA occlusion would improve blood flow, particularly at proximal sites, and could explain the differential benefit seen in the basal ganglia compared with the cortex. Alternatively, the basal ganglia may be more resistant to ischemic injury than cortex and derive greater benefit from the better maintained flow with CGRP.

Animal models of focal cerebral ischemia that achieve absolute occlusion of the MCA by ligation or diathermy produce a core of ischemia based on the caudate-putamen. The lack of an anastamotic supply to this region renders it severely compromised with an absolute proximal MCA occlusion, but in our model the volume of deep ischemic neuronal damage is reduced by...
70% (P<.05) by the infusion of CGRP. Although we have no data on deep CBF, CGRP is presumably achieving an increased CBF in the proximal MCA, either by maximally dilating its origin or improving the anastomotic supply from anterior and posterior cerebral arteries.

In patient groups at high risk of cerebral ischemia, such as those suffering an SAH from an intracranial aneurysm or undergoing carotid endarterectomy, the potential benefit of this peptide is considerable. A recent clinical trial of intravenous CGRP in SAH involved giving the agent after the onset of delayed cerebral ischemia; although a trend to improvement occurred, the trial was too small to show a statistically significant improvement in outcome. Further research in the laboratory is needed to delineate the therapeutic window for this peptide, and current evidence warrants a clinical trial of sufficient size using prophylactic CGRP after SAH has occurred but before the development of delayed cerebral ischemia.

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