Delayed Systemic Administration of PACAP38 Is Neuroprotective in Transient Middle Cerebral Artery Occlusion in the Rat

Dora Reglodi, MD; Aniko Somogyvari-Vigh, MD; Sandor Vigh, MD; Tamas Kozicz, MD, PhD; Akira Arimura, MD, PhD

Background and Purpose—Many substances have been shown to reduce brain damage in models of stroke, but mainly when given either before or shortly after the onset of ischemia. Delayed systemic administration of pituitary adenylate cyclase–activating polypeptide (PACAP) has been shown to attenuate the neuronal damage in the hippocampus in a model of global ischemia in rats. The present study examined the neuroprotective action of delayed systemic administration of PACAP38 in a model of transient focal ischemia produced by middle cerebral artery occlusion (MCAO) in rats.

Methods—We administered PACAP38 as an intravenous bolus (20 nmol/kg body wt) followed by an intravenous infusion for 48 hours using a micro-osmotic pump at a rate of 160 pmol/μL per hour, beginning 4, 8, or 12 hours after a 2-hour transient MCAO using a filament model. The size of the infarct was determined by examining 2-mm-thick brain sections stained with triphenyltetrazolium chloride, followed by image analysis. Control animals received intravenously 0.1% bovine serum albumin in 0.9% saline as a bolus and infusion at the same time intervals.

Results—The administration of PACAP38 beginning 4 hours after MCAO significantly reduced the infarct size by 50.88%. Treatment with PACAP38 starting 8 or 12 hours after the onset of ischemia did not result in a significant reduction of the infarct size, although infarct volumes tended to be smaller than in the control groups.

Conclusions—Systemic administration of PACAP38 should be clinically useful for reducing brain damage resulting from stroke even when administration is delayed for several hours. (Stroke. 2000;31:1411-1417.)

Key Words: cerebral infarction ■ middle cerebral artery occlusion ■ neuroprotection ■ neuropeptides ■ rats

During the last decade, dozens of new candidate therapeutic substances have been demonstrated to exert protection against ischemic brain damage in middle cerebral artery occlusion (MCAO) models. However, most of these agents are effective in animals only when administered either before or shortly after the onset of ischemia. The toxic side effects and the inability to cross the blood-brain barrier (BBB) are the major other reasons that most of the therapeutic agents proven to reduce infarct size in experimental animals have failed to ameliorate human stroke outcome.1,2

Morphological studies have demonstrated the temporal evolution of brain damage after the ischemic event.3–5 It is generally believed that 3 to 4 hours of focal cerebral ischemia is sufficient to attain a maximal infarction in rats and that recirculation or pharmacological intervention after this time provides little benefit.6 However, recent evidence suggests that the evolution of ischemic injury, especially in the penumbral regions, is more protracted in time than previously believed, and tissue damage may be influenced long after the induction of ischemia.2,7–10 A few reports have documented beneficial effects of different substances in rats even if treatment is delayed.7,8,11,12

Pituitary adenylate cyclase–activating polypeptide (PACAP) was first isolated from ovine hypothalami and occurs in 2 amidated forms, with 38 and 27 amino acid residues (PACAP38 and PACAP27, respectively).13,14 PACAP is a member of the secretin/glucagon/vasoactive intestinal peptide (VIP) family and has 68% sequence similarity with VIP, but its adenylate cyclase–stimulating activity has been shown to be 1000 to 10 000 times greater than that of VIP. PACAP has been demonstrated to act mainly as a hypophysiotropic hormone, neurotransmitter, and neuromodulator.15 PACAP has also been shown to have neurotrophic and neuroprotective effects at very low concentra-
PACAP has been proven to cross the BBB: 0.118% of an intravenous dose of PACAP38 is taken up by brain when expressed by percent intravenous per gram, which is 6 times the amount of morphine accumulated in the brain. This finding has raised the question of whether similar cytoprotection could be achieved in vivo even when PACAP38 is administered intravenously. In fact, PACAP38 has been shown to prevent the ischemic death of rat CA1 neurons when given either intracerebroventricularly or intravenously in a model of transient global ischemia, even if administration is delayed for 24 hours after the ischemic event. In a recent study it has been found that an intravenous bolus injection before the slow infusion is necessary to exert neuroprotection with PACAP38.

In the present study we investigated the neuroprotective effect of PACAP38 in a transient MCAO model in rats. We observed that infarct size increased slowly during the first 12 hours and reached its maximum at 48 hours. On the basis of this observation and the aforementioned studies, we hypothesized that PACAP38 treatment started during the first 12 hours would save some brain tissue. To examine the effect of PACAP38, a bolus intravenous injection followed by a slow intravenous infusion was begun 4, 8, or 12 hours after the insult.

Materials and Methods
Animal Preparation
Adult male CD rats weighing 275 to 300 g were purchased from Charles River Laboratories and were housed under diurnal lighting conditions. The animals were quarantined for at least 7 days before the experiment. Before the experiment, the rats were placed in individual cages and fasted overnight but allowed free access to water. Animal housing, care, and application of experimental procedures were in accordance with institutional guidelines under approved protocols.

The rats were anesthetized with halothane in a mixture of 70% nitrous oxide and 30% oxygen. Anesthesia was induced with 3% halothane and subsequently maintained with 1% halothane delivered with a face mask. Body temperature was maintained in the normal range (36.5°C to 37.5°C) with a heating lamp and a heating pad during the operation. Temperature was monitored with a rectal probe and kept in the normal range during the first 6 hours after MCAO. According to our previous in vitro and in vivo studies,22 this surgical insult. The left middle cerebral artery (MCA) was occluded by the intraluminal suture technique described by Longa et al.27 Briefly, the left carotid region was exposed through a midline cervical incision. The external carotid and the common carotid arteries were ligated with a 3-0 suture. The pterygopalatine branch of the internal carotid artery was also ligated to prevent incorrect insertion of the occluder filament. A 5-cm length of a 4-0 monofilament nylon suture (Ethilon; Ethicon Inc), whose tip had been rounded by heating near a flame and coated with poly-L-lysine (Sigma), was introduced from the carotid bifurcation into the internal carotid artery until a mild resistance was felt (18 to 19 mm), thereby occluding the origin of the MCA. The surgical procedure required approximately 10 minutes. After surgery, anesthesia was discontinued, and the rats were allowed free access to food and water.

Recirculation of MCA was established 2 hours after MCAO by gentle withdrawal of the suture until the rounded tip reached the insertion point where a resistance was felt (under the same anesthetic conditions as surgery).

Neurological Evaluation
The neurological evaluations were performed 2 hours after MCAO according to the methods described by Bederson et al.28 and Memezawa et al.30 Animals with successful occlusion of the MCA showed gait disturbances with circling or walking to the right (corresponding to grade 3 by Bederson et al.). Rats showing circling also always showed forelimb flexion, thorax twisting, decreased pain reflexes of the right forelimb, and decreased resistance to lateral push. Our preliminary studies are in agreement with those of Memezawa et al.; rats that displayed these latter signs, but without circling, did not have a satisfactory, reliable occlusion. Therefore, animals without circling or walking to the right were excluded from further experiments. Including those rats would have increased the variance in both the control and the treated groups and would have led to misinterpretation of the data. Some animals developed convulsive behavior, which would also influence the infarct size independently of the ischemic damage; therefore, those animals were also excluded.

Drug Administration
The rats that showed positive neurological signs at 2 hours after MCAO were randomly divided into control and PACAP-treated groups. Administration of PACAP38 (Polypeptide Laboratories) started 4 (n=20), 8 (n=15), or 12 (n=15) hours after MCAO. The operative conditions were the same as for the MCAO surgery. A micro-osmotic pump with a pumping rate of 1 μL/h (Alza Corp) was filled with PACAP38 dissolved in 0.1% BSA (Sigma). The filling and operational instructions given by Alza were closely followed. Briefly, pumps were prepared 12 hours before intravenous cannulation and were soaked in physiological saline at 37°C for 12 hours. The pump was attached to Micro-Renathane tubing (type MRE-040) (Braintree Scientific Inc). The tubing was examined after 12 hours for the presence of bubbles. Those pumps that had bubbles were not used for implantation because of the uncertain fluid conductivity. Three centimeters of the tubing was inserted into the internal jugular vein so that the tip of the tubing was located in the right atrium. The pump was implanted in the subcutaneous space of the nuchal region. The infusion rate was 160 pmol PACAP38 per microliter per hour, and an additional bolus injection of 5 nmol/rat PACAP38 was given intravenously (into the penile vein). These doses were chosen on the basis of our previous in vitro and in vivo studies. This surgical procedure also took approximately 10 minutes to be completed. Control groups received 0.1% BSA in 0.9% saline as a bolus injection and infusion 4 (n=11), 8 (n=15), or 12 (n=11) hours after MCAO under the same operative conditions as the PACAP-treated animals.

Calculation of the Infarct Volume
All rats were decapitated under anesthesia 48 hours after MCAO. Brains were removed rapidly and cooled in 98% isoamyl alcohol.
Physiological Parameters in Rats After a 2-Hour MCAO and a 2-Hour Reperfusion

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Before</th>
<th>5 Minutes</th>
<th>30 Minutes</th>
<th>1 Hour</th>
<th>3 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.384±0.02</td>
<td>7.384±0.01</td>
<td>7.368±0.03</td>
<td>7.32±0.02</td>
<td>7.375±0.01</td>
</tr>
<tr>
<td>PO&lt;sub&gt;2&lt;/sub&gt;, mm Hg</td>
<td>154±9</td>
<td>154±7</td>
<td>125±10</td>
<td>122±16</td>
<td>145±3</td>
</tr>
<tr>
<td>PCO&lt;sub&gt;2&lt;/sub&gt;, mm Hg</td>
<td>36.5±2.4</td>
<td>36.5±1.8</td>
<td>35.7±4.2</td>
<td>44.5±8.1</td>
<td>45.4±2.7</td>
</tr>
<tr>
<td>Blood pressure, mm Hg</td>
<td>122±9</td>
<td>111±12</td>
<td>108±8</td>
<td>116±16</td>
<td>110±9</td>
</tr>
<tr>
<td>Plasma glucose, mg/dL</td>
<td>97±10</td>
<td>95±9</td>
<td>93±5</td>
<td>92±10</td>
<td>126±24</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>44.8±3.2</td>
<td>40±1.8</td>
<td>43.8±4.2</td>
<td>40.1±3.5</td>
<td>39.9±2.8</td>
</tr>
</tbody>
</table>

| Intravenous PACAP38 (5 nmol) | | | | | |
| pH | 7.392±0.01 | 7.381±0.02 | 7.368±0.03 | 7.388±0.01 | 7.386±0.02 |
| PO<sub>2</sub>, mm Hg | 145±8 | 147±11 | 140±12 | 138±14 | 148±4 |
| PCO<sub>2</sub>, mm Hg | 33.9±1.8 | 34.3±2.5 | 34.2±2.1 | 35.4±4.0 | 48.2±3.4 |
| Blood pressure, mm Hg | 118±10 | 86±6* | 105±9 | 108±11 | 110±8 |
| Plasma glucose, mg/dL | 86±13 | 85±11 | 91±12 | 100±12 | 144±26 |
| Hematocrit, % | 43.2±4.1 | 44.7±3.2 | 46.4±5.4 | 38.1±2.7 | 38.9±1.9 |

Values are mean±SEM. *P<0.05 compared with the group treated with saline.

Results

There was no significant difference in body weight, temperature, or mortality between the groups. Body temperature rose in each animal 15 to 20 minutes after MCAO, but it could be maintained within the normal range by cooling the animals. Only 1 rat showed elevated body temperature despite cooling; this rat was excluded from further experiment. One or 2 rats died in each group, mostly during the first 24 hours. Premature deaths were mainly caused by cerebral hemorrhage or extreme cerebral edema. Two rats in the 4- and 8-hour PACAP-treated groups, 1 rat in the 4-hour control group, and 3 rats in the 12-hour control group developed convulsions during the first 24 hours. Hemorrhage, which was not fatal, occurred in 2 rats of the 8-hour and 1 rat in the 12-hour PACAP-treated groups and 2 rats in the 8- and 12-hour control groups. All animals with convulsions or hemorrhage were excluded from further evaluation. There was no significant difference in pH, PCO<sub>2</sub>, PO<sub>2</sub>, blood glucose concentration, and hematocrit between the control and PACAP-treated animals. Only the mean arterial pressure dropped by 30 mm Hg after the bolus injection of PACAP, but it gradually returned to normal levels after 30 minutes (Table).

Our studies on the evolution of infarct size 4, 8, 12, 24, 48, and 72 hours after MCAO showed that the infarct size gradually increased and the individual response to the ischemic injury was very different during the first 12 hours. At 24 hours the infarct size reached the maximum, whereas no significant difference could be demonstrated between the infarct sizes after 24, 48, or 72 hours. The variation was the smallest in the 48-hour group. We chose to kill the animals after 48 hours because of the reliable and reproducible infarct size. These results are summarized in Figure 1.

When the treatment started 4 hours after the MCAO, the infarct volumes of the PACAP-treated group and the control.
group were 10.38±2.02% and 21.13±4.02%, respectively (Figure 2). This was a 50.88% reduction compared with the control group and was highly significant (P<0.01). The effect can be clearly seen in the representative photographs (Figure 3) of the 6 TTC-stained brain slices from the 4-hour control and PACAP-treated groups. In the PACAP-treated group, the unstained areas are located mainly in the lateral part of the caudoputamen and the surrounding cortical areas, which constitute the core of the infarct.3,30 The anterior and medial parts of the striatum, as well as large areas in the cortex, remained unaffected compared with the control group. An analysis of the infarcted areas for each 2-mm coronal section from the control group and the group treated with PACAP38 starting 4 hours after MCAO (Figure 4) showed that the most affected sections were the middle sections (3 to 5), which represent most of the territory of the MCA. The reduction of the infarct size with treatment starting 4 hours after MCAO is also most pronounced in the middle sections, whereas the reduction is not significant in the first section.

When the treatments started 8 hours after the MCAO, the infarct volumes in the PACAP-treated and in the control groups were as follows: control group, 19.69±2.79%; group treated with PACAP38 starting 8 hours after MCAO, 15.3±2.93% (Figure 2). This is a substantial (22.3%), but not statistically significant (P=0.14), reduction in infarct volume. The reduction in the size of the infarct in the group treated with PACAP38 starting 12 hours after MCAO was also not statistically significant. The infarct size of the PACAP-treated group was 18.75±3.31%, whereas that of the control group was 21.22±1.73% (Figure 2). This is an 11.64% reduction in infarct size, which again did not reach significance (P>0.05).

These results convincingly demonstrate that PACAP38 is neuroprotective when the administration starts within 4 hours after the ischemic insult. Although the reduction in infarct volume was not statistically significant when administration started 8 or 12 hours after the onset of ischemia, infarct sizes tended to be smaller than those of the corresponding control groups.

**Discussion**

Our study demonstrated that systemic administration of PACAP38 effectively reduces infarct volume in a rat model of focal ischemia when administration begins 4 hours after MCAO. Infarct volumes still tended to be smaller when treatment started 8 or 12 hours after MCAO, although the reductions were not statistically significant.

Premature mortality was evenly distributed among all groups, indicating that the administration of PACAP38 did not contribute to the mortality. Mortalities were mainly due to brain edema or cerebral hemorrhage. Convulsions after transient MCAO can be a complicating factor that may lead to a type of cell damage not related to ischemia, and therefore...
they can result in misinterpretation of the data.\textsuperscript{31} We observed convulsions in some rats during the first 24 hours, regardless of the treatment and the animal’s core temperature. Those animals were excluded from further evaluations. Subarachnoid hemorrhage can also occur as a complication of the MCAO experiments. A recent study\textsuperscript{34} has reported that subarachnoid hemorrhage is more common than previously believed. Hemorrhage causes further cerebral damage,\textsuperscript{35} and therefore animals with hemorrhage were also excluded. The occurrence of hemorrhage also showed no relation to the treatment. Fluctuations in the size of ischemic areas suggest that there is a considerable variability in individual responses. Individual variations in MCAO experiments have been reported by many others\textsuperscript{4,34,36,37} and have been attributed mainly to differences in the arterial patterns and collateral blood flow, present even within the same strain, size, sex, and age.

Among the physiological parameters, only blood pressure showed a significant difference between the control and the PACAP-treated animals: the intravenous bolus injection of 5 nmol of PACAP38 caused a prompt fall of the mean arterial blood pressure, which returned to the preinjection level 30 minutes later. This effect of PACAP has been demonstrated earlier\textsuperscript{15}; the peptide is well known to relax vascular smooth muscle. It is possible that intravenous administration of PACAP38 causes dilation of cerebral arteries and increases cerebral blood flow, which could be beneficial for the treatment of MCAO.\textsuperscript{36} However, this cannot account for the neuroprotective effect observed in this study because, according to most studies, altering the blood pressure and cerebral blood flow beginning 4 hours after the ischemic insult has no proven beneficial effects on infarct size.\textsuperscript{1,2,9} In addition, our preliminary experiments showed that an intravenous bolus injection of PACAP38 alone did not alter the infarct size after MCAO even though it lowered the mean arterial blood pressure for 5 to 30 minutes. A bolus injection of the peptide was given before the slow infusion because there is a PACAP binding protein in the circulation of rats.\textsuperscript{23} It is possible that binding of PACAP38 to the binding protein would nullify the action of the peptide. A bolus injection of PACAP38 was used to saturate the binding sites of the circulating binding protein. However, a bolus injection might not be necessary in humans, since human plasma does not bind PACAP.\textsuperscript{23}

The present results provide an additional in vivo demonstration of the neuroprotective effect of PACAP38. Since its discovery, the neurotrophic and neuroprotective effects of PACAP have been extensively investigated in vitro. These studies have been summarized in a recent review article.\textsuperscript{15} For example, PACAP has been shown to enhance neuronal cell survival\textsuperscript{19,20} and to prevent apoptotic cell death in cultured cerebellar granule cells.\textsuperscript{17} PACAP has also been found to protect cultured rat cortical neurons against glutamate-induced cytotoxicity\textsuperscript{18} and dopaminergic neurons against 6-hydroxydopamine–induced cytotoxicity.\textsuperscript{41} Dramatic neuroprotection has been observed in cultured hippocampal neurons at femtomolar concentrations against gp120-induced neuronal cell death.\textsuperscript{16} A recent study has reported neuroprotection by PACAP at femtomolar concentrations in a lipopolysaccharide-induced neurotoxicity model.\textsuperscript{20} These studies indicate that PACAP is neuroprotective in various pathophysiological conditions, at nanomolar to subpicomolar concentrations.

PACAP has been demonstrated to readily cross the BBB.\textsuperscript{21} This raised the question of whether a systemic administration of PACAP would be neuroprotective in vivo as well. Indeed, its neuroprotective effect has been shown in a rat model of global ischemia.\textsuperscript{22} The typical histopathological lesion in global ischemia is delayed selective neuronal loss,\textsuperscript{9} particularly in the most vulnerable CA1 region of the hippocampus. PACAP38 has been found to prevent this selective neuronal death in CA1 by either intracerebroventricular (1 pmol/h) or intravenous (16 to 160 pmol/h) infusion. Intravenous PACAP38 has been found to be effective even if infusion begins 24 hours after ischemia. The doses necessary to exert neuroprotection in global ischemia have been calculated from previous in vitro studies and by the effectiveness of transport across the BBB.\textsuperscript{22} In our model of focal ischemia, the same doses were used. Most compounds investigated as possible treatments for stroke in animal models are maximally effective when given as an initial bolus followed by a constant intravenous infusion.\textsuperscript{2} In a recent study it has been found that such an administration pattern is necessary to obtain neuroprotection with PACAP38.\textsuperscript{23} Our bolus injection of 20 nmol/kg body wt is most probably able to saturate the binding protein for PACAP38 in the circulation and to cause a rapid increase in plasma PACAP38 to the level appropriate for neuroprotection.\textsuperscript{23}

The exact mechanism for the neuroprotective effect of PACAP38 is not known. As mentioned above, PACAP can exert its neurotrophic action by direct and indirect mechanisms. In vitro, PACAP is neurotrophic at nanomolar to picomolar concentrations when there are no glial cells in the culture, but at low picomolar to femtomolar concentrations when glial cells are included.\textsuperscript{15,20,22} The direct neurotrophic effect is considered to be mediated by stimulation of the adenylate cyclase/protein kinase A signaling pathway. However, the concentration of PACAP38 in the brain in this study is unlikely to have exceeded a subpicomolar or low picomolar concentration,\textsuperscript{21} which is not sufficient to stimulate adenylate cyclase.\textsuperscript{16} Therefore, it is likely that the neuroprotection observed in this in vivo study was mediated indirectly, perhaps by the enhanced expression and release of neurotrophic factors from astrocytes.\textsuperscript{15,19,42} as has been demonstrated for VIP.\textsuperscript{43}

The data presented here suggest a promising therapeutic use for PACAP in cerebral ischemia. Among its practical advantages are that it readily crosses the BBB\textsuperscript{21} and is effective at extremely low concentrations. Many potential therapeutic agents that were effective in animal models have failed during clinical trials because of their toxicity.\textsuperscript{1} PACAP is a naturally occurring peptide that has not been shown to have toxic side effects at this low dosage.\textsuperscript{15} More important, the most promising feature of PACAP is the apparently long therapeutic window, since it is effective even when administration is delayed for 4 hours after ischemia. Several reports have described the time course of neuronal damage after an ischemic event.\textsuperscript{3,44} Neuronal necrosis progresses from the core of the lesion toward the initially less severely damaged
penumbral zone, which contains potentially salvageable neurons. The period available for rescuing penumbral regions is longer than for the ischemic focus, and rescue attempts have been directed mainly toward the penumbra. Although there are large discrepancies between different studies, many authors agree that the window of opportunity for effective therapeutic intervention is only a few hours. Most of the successful attempts to reduce infarct size have occurred when the treatment started either before or very shortly after the onset of ischemia. The clinical usefulness of such therapeutic agents is very limited because of the time interval between the recognition of the symptoms and the possible beginning of a treatment in stroke patients. According to recent investigations, the time window for therapeutic intervention may extend far beyond a few hours, and the evolution of ischemic injury, especially in the penumbral regions, is more protracted in time than previously believed. A few studies offer some hope that even delayed treatment may have beneficial effects and tissue damage can be influenced long after the ischemic event. In our study PACAP38 reduced infarct size when administration started 4 hours after MCAO, and infarct volumes still tended to be smaller even if treatment started 8 and 12 hours after MCAO, although this reduction was not significant. Changing the experimental conditions or combining PACAP with other neuroprotective substances may result in PACAP being effective after even longer delays. However, the pharmacological profiles of transient and permanent focal ischemia are clearly different. Therefore, it will be critical to determine whether delayed systemic administration of PACAP38 can also reduce the volume of the infarct in permanent occlusion of the MCA.

In summary, systemic administration of PACAP38 in focal ischemia may be a promising therapeutic agent in stroke management. PACAP38 decreased the infarct volume in a MCAO model in rats even when administration began as late as 4 hours after the onset of ischemia. This phenomenon needs to be investigated further to extend the window of therapeutic opportunity.

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References
The article by Reglodi et al shows that the systemic administration of PACAP38 in focal ischemia suggests that PACAP represents an attractive candidate for development as an acute stroke drug. However, at least two issues require further study in this regard. First, the current data were obtained in an ischemia/reperfusion model. There are several examples of drugs (notably, free radical scavengers) that are effective in reperfusion but not in permanent ischemia models. Because permanent ischemia represents an important variety of clinical stroke, this issue requires further exploration. Finally, Reglodi et al found that PACAP lowered blood pressure. Because even a mild degree of systemic hypotension may counteract beneficial cytoprotective effects in the critical first few hours after stroke, this issue will require further study as well.

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