Calbindin D28K Overexpression Protects Striatal Neurons From Transient Focal Cerebral Ischemia

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Background and Purpose—Increased intracellular calcium accumulation is known to potentiate ischemic injury. Whether endogenous calcium-binding proteins can attenuate this injury has not been clearly established, and existing data are conflicting. Calbindin D28K (CaBP) is one such intracellular calcium buffer. We investigated whether CaBP overexpression is neuroprotective against transient focal cerebral ischemia.

Methods—Bipromoter, replication-incompetent herpes simplex virus vectors that encoded the genes for cabp and, as a reporter gene, lacZ were used. Sprague-Dawley rats received bilateral striatal injections of viral vector 12 to 15 hours before ischemia onset. With the use of an intraluminal occluding suture, animals were subjected to 1 hour of middle cerebral artery occlusion followed by 47 hours of reperfusion. Brains were harvested and stained with X-gal (to visualize β-galactosidase, the gene product of lacZ). The number of remaining virally transfected, X-gal–stained neurons in both the ischemic and contralateral striata were counted and expressed as the percentage of surviving neurons in the ischemic striatum relative to the contralateral nonischemic striatum.

Results—Striatal neuron survivorship among cabp-injected animals was 53.5 ± 4.1% (n = 10) versus 26.8 ± 5.4% among those receiving lacZ (n = 9) (mean ± SEM; P < 0.001).

Conclusions—We conclude that viral vector–mediated overexpression of CaBP leads to neuroprotection in this model of central nervous system injury. This is the first demonstration that CaBP overexpression protects neurons in a focal stroke model. (Stroke. 2001;32:1028-1035.)

Key Words: calcium ■ calcium-binding protein ■ cerebral ischemia, focal ■ cerebral ischemia, transient ■ gene therapy

Intracellular calcium accumulation plays a significant role in mediating ischemic injury.12 Experimental studies have shown that limiting the amount of intracellular calcium accumulation in neurons, by blocking entry through either voltage-gated or glutamate-activated channels, decreases neuronal injury and infarct size. Neurons also possess endogenous means by which intracellular calcium levels are tightly controlled. The calcium-binding protein calbindin D28K (CaBP) is a member of the superfamily of calcium-binding proteins implicated in the regulation of intracellular calcium and is found in neurons throughout the nervous system.3 Whether CaBP plays a protective role against various central nervous system insults has been controversial. Some studies have shown that CaBP-containing neuronal populations are better able to survive ischemic4 and excitotoxic insults5 than those that lack CaBP. Astrocytes also express CaBP in response to various central nervous system insults.6,7 Mattson et al7 showed that tumor necrosis factors induce CaBP in astrocytes and subsequently render them resistant to acidosis and calcium ionophore exposure. Whereas such studies suggest a beneficial role of CaBP, others suggest that the presence of CaBP has nothing to do with resistance to central nervous system insults.8,9 To directly test the hypothesis that CaBP protects the cell from injury, it is now possible to selectively increase its expression by vector-mediated gene transfer.

We have previously developed a defective herpes simplex viral (HSV) amplicon vector to overexpress CaBP, primarily in neurons. Vector-mediated overexpression of CaBP reduced intracellular calcium accumulation and improved cultured hippocampal neuron survival against conditions of hypoglycemia and excitotoxicity.10,11 At the in vivo level, we have shown that gene transfer of other potentially neuroprotective proteins, including Bcl-2,12,13 glucose transporter (glut-1),14 and Hsp72,15 protects neurons from stroke16 and that vector-mediated CaBP overexpression protects hippocampal neu-
rons from toxicity induced by kainic acid (a glutamate agonist) and 3-acetylpyridine (an antimetabolite).17 By targeting striatal neurons with defective HSV vectors that encode the cabp gene, we overexpress CaBP to investigate whether CaBP may be neuroprotective in an experimental model of focal cerebral ischemia.

Materials and Methods

All animal protocols were approved by the institutional Administrative Panel on Laboratory Animal Care, and all procedures were followed according to their guidelines.

Materials

Male Sprague-Dawley rats were obtained from Charles River (Wilmington, Mass). Vero cells (African green monkey kidney cells; ATCC CCL81) were obtained from American Type Culture Collection (Rockville, Md). Lipofectamine was from Life Technologies (Gaithersburg, Md). X-gal (4-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) was from Molecular Probes (Eugene, Ore). HSV mutant d120 and cell line E5 were provided by Dr N.A. DeLuca (University of Pittsburgh, Pittsburgh, Pa).

Generation of HSV Vectors

The construction of ampiclons po22βgal4cabp and po22βgal4s and viral vectors has been described elsewhere.10,18 Briefly, the ampiclon plasmid po22βgalαcabp contained the rat cabp gene and the Escherichia coli lacZ gene under the control of the HSV α4 and α22 promoters, respectively.10 The HSV oriS and the “a” sequence provide the necessary cis-signals for replication and packaging of the ampiclon DNA po22βgalα4s, which is identical to po22βgalαcabp except that it lacks the cabp sequence, was used to generate control vectors. Vectors were generated by transfection of po22βgalαcabp or po22βgalα4s into E5 cells with the use of lipofectamine according to the manufacturer’s protocol. Twenty-four hours after transfection, the cells were superinfected with helper virus d120 (HSV-1 strain KOS)19 at multiplicities of infection of 0.03 or 0.1. The cells were harvested when 100% cytopathic effect developed. Stocks were further purified by centrifugation at 1800 × g for 10 minutes, and the supernatants were spun at 70 000 × g for 18 hours. The resulting pellets were resuspended in PBS. The ampiclon plasmids were denoted with the prefix “p,” and the defective vectors thus generated were denoted with the prefix “v.” The titters of helper virus were determined on E5 cells with a standard plaque assay. The titters of ampiclon vectors were determined on Vero cells by quantifying the number of β-galactosidase (β-gal, the gene product of lacZ)–expressing cells. For po22βgalαcabp (CaBP-expressing vector), titers were as follows: 1.1 × 103 infectious particles per milliliter, with helper virus titer of 3.1 × 105 plaque-forming units per milliliter (ratio of vector to helper virus was 1:2.8). For po22βgalα4s (control vector), titers were 1.3 × 104 infectious particles per milliliter, with helper virus titer of 9.7 × 108 plaque-forming units per milliliter (ratio of vector to helper virus was 1:7.5).

Vector Delivery

Animals were anesthetized with xylazine (5 mg/kg) and acepromazine (4 mg/kg) given intraperitoneally, followed by 1.5% to 2% halothane by face mask, and placed in stereotactic frames. Injection sites were identified over each hemisphere. Coordinates for striatal injections (ischemia experiments) from bregma were as follows: anteroposterior = 0, mediolateral = 3.5 mm with 2 injection sites at dorsoventral = 5 and 6 mm. Coordinates for hippocampal injections were as follows: anteroposterior = −3.8 mm, mediolateral = 2.0 mm, and dorsoventral = 1.8 mm. Each injection consisted of 3 μL of vector.

Coexpression From the Bipromoter Vectors

To verify coexpression of both CaBP and β-gal from the bipromoter vector, Vero cells and brain sections from the ischemia experiments were used. Vero cells were transfected with CaBP-expressing vector or control vector. Twelve hours later, cultures were fixed with acetone/methanol (3:1; −20°C). Adjacent brain sections from representative animals subjected to focal cerebral ischemia (one injected with the CaBP-expressing vector and the other injected with the control vector) were fixed in 3% paraformaldehyde and 20% sucrose for 24 hours. Brain sections (30 μm) were cut on a cryostat and allowed to dry. Sections were treated with Proteinae K (1:2 dilution, Dako) for 15 minutes, then washed in PBS.

After they were blocked in 10% normal horse serum containing 2.5% Triton-X 100, cultures and sections were incubated in primary antibodies against β-gal (mouse monoclonal; 1:1000 dilution for Vero cells and 1:250 dilution for brain sections; Sigma) for 1 hour at room temperature. After they were washed in PBS, cultures and sections were incubated for 1 hour in fluorescein-conjugated goat anti-mouse antibody (1:150; Vector FL-2000). The cultures and sections were washed and blocked in 10% normal goat serum. The secondary primary antibody against CaBP (rabbit polyclonal; 1:1000 dilution for Vero cells [gift of K. Baimbridge]; 1:200 dilution for brain sections [Cappell]) was applied for 1 hour at room temperature, then washed. The second secondary antibody (Vero cells: rhodamine-conjugated horse anti-rabbit antibody; 1:100; brain sections: Texas Red–conjugated goat anti-rabbit; 10 μg/mL) was applied for 1 hour, then washed. Cultures and brain sections were then viewed and photographed under an Olympus epifluorescence microscope.

Vector Specificity

Two additional animals were injected with control vector (po22βgalα4s) into the stratum or hippocampus, as described above. Brain sections were prepared and stained with X-gal (a chromogenic substrate for β-gal) and cresyl violet. Adjacent sections were double immunofluorescence labeled as described above. Antibodies against neuronal (monoclonal anti–microtubule-associated protein 2 [MAP2]; 1:3000; Sigma) or astrocyte (glial fibrillary acidic protein; 1:1000; Sigma) markers were applied, followed by antibodies against β-gal. The number of double- and single (β-gal only)–labeled cells were counted from the 3 brain sections centered on the injection site. Counts of cells from the contralateral, nonischemic striata among 8 representative animals included in this study (5 CaBP-expressing vector injected, and 3 control vector injected) were determined, and the percentage of neuronlike cells was computed from the total number of X-gal–positive cells.

Time Course of Reporter Gene Expression

To determine the time course of gene expression from the vectors, striata were injected and brain sections were harvested 4 or 12 hours or 1, 2, or 7 days later. After they were postfixed in 3% paraformaldehyde/20% sucrose solution for 1 to 2 days, 25-μm frozen sections in the coronal plane were taken at 100-μm increments 0.5 mm anterior and 0.5 mm posterior to the infusion sites. Sections were costained with X-gal to identify vector-infected neurons expressing β-gal, then counterstained with cresyl violet. The number of X-gal–positive cells was counted over 10 sections as described subsequently. Animals subjected to transient focal ischemia were injected 12 to 15 hours before middle cerebral artery (MCA) occlusion because peak expression from the vectors coincided with this time point (Figure 4).

Transient Focal Cerebral Ischemia Model

Rats weighing 280 to 300 g were anesthetized with face mask with 2% halothane plus oxygen and air supplied in a ratio of 0.2:0.8 L/min. Once surgical levels of anesthesia were attained (assessed by absence of hind leg withdrawal to pinch), halothane was decreased to 1% to 1.5%, and anesthetic levels were reassessed every 15 minutes throughout the remainder of the procedure. Either CaBP-expressing or control vectors were directly injected bilaterally into the striata of
rats. The striatum was chosen for injection because this region consistently shows signs of injury in this model. Animals were allowed to recover; 12 to 15 hours later, they were reanesthetized with halothane. Ischemia was induced with an occluding intraluminal suture. A cervical midline incision was made, and the left carotid artery and branches were isolated. The common carotid artery, external carotid artery, and pterygopalatine artery were identified and ligated. An aneurysm clip was placed on the proximal internal carotid artery, while an arteriotomy was made on the distal common carotid artery. An uncoated 30-mm-long segment of 3-0 nylon monofilament suture with the tip rounded by a flame was inserted into the arteriotomy. The aneurysm clip was removed, and the suture was advanced under direct visualization into the internal carotid artery approximately 19 to 20 mm from the bifurcation to occlude the ostium of the MCA.

The occluding suture was kept in place for 60 minutes. At the end of the ischemic period, the suture was removed, and the surgical incisions were closed. The animal was allowed to recover, then transported to the intensive care unit at the animal facility for postoperative monitoring. Forty-eight hours later, the animal was euthanatized with a barbiturate overdose, then perfused transcardially with heparinized saline followed by 3% paraformaldehyde. Brain sections were prepared for histological analysis.

**Cell Counts**

Sections were fixed in 3% paraformaldehyde/20% sucrose and reacted with X-gal to identify vector-infected neurons expressing β-gal and cresyl violet. The number of transfected neurons was counted at ×40 magnification by an investigator blinded to treatment. While these vectors tend to infect neurons, the vector can also be taken up by ependymal and endothelial cells. Other small, nonspecific parenchymal cells take up the vector as well. Therefore, cells were counted only if they (1) were contained within the striatum, (2) were X-gal positive, and (3) possessed characteristic neuronal morphology (possessed processes, were larger in size, and cell body diameters were 15 to 25 μm). The number of surviving cells was expressed as the ratio of positive blue neuronlike cells in the ischemic striatum compared with the contralateral nonischemic striatum.

Because the extent of vector-mediated infection is limited to only a few hundred striatal neurons within a 0.5-mm radius of the injection site, it is not expected that overall infarct size would be affected. On the other hand, improvement in vector-infected striatal neuron survival could be explained by smaller infarcts in one group compared with the other. To confirm that improved striatal neuron survival was not due to an imbalance between groups with regard to the severity of ischemia, the cresyl violet–stained sections were visually inspected for injury. Brains were assigned a numeric score on a scale of 0 to 3, with 0 representing no damage and 3 representing severe damage with involvement of the striatum and surrounding cortex. Animals with no visible damage (score of 0) were excluded from the analysis. Furthermore, infarct sizes were computed from regions that failed to stain with cresyl violet from the centermost section of the infarct. This section reflected the anatomic level with the maximum amount of ischemic damage and was also centered on the injection site.

**Statistical Analysis**

Standard statistical methods were used to analyze data. Differences between groups were determined with Student’s t test. Nonparametric tests (ie, Mann-Whitney) were used to compare differences between CaBP-expressing and control groups with respect to numeric infarct scores. Statistical significance was determined at the \( P<0.05 \) level. All data are presented as mean±SEM.
Results

Coexpression From Bipromoter Vectors

Twelve hours after transfection of cultured Vero cells, double immunofluorescence staining showed that the CaBP-expressing vector coexpressed both CaBP and β-gal (Figure 1A and 1B), whereas the control vector only expressed β-gal (Figure 1C and 1D). Similarly, vector transgene expression in ischemic brain showed that the CaBP-expressing vector expressed both proteins, whereas the control vector was positive only for β-gal (Figure 2A and 2B). Faint CaBP staining was present in nontransfected cells, suggesting that endogenous CaBP was expressed in many neurons. However, CaBP immunofluorescence in the CaBP-expressing vector-transfected cells was higher than that of nontransfected cells (Figure 2A). Faint CaBP immunofluorescence was also observed in the ischemic striatum of a control vector–injected brain (Figure 2C), but transfected cells were only positive for β-gal (Figure 2D).

Vector Specificity

Double labeling with neuronal markers and β-gal showed that many transfected cells were neurons (Figure 3). Within the hippocampus, 81±5% of all β-gal–labeled cells were neurons, and within the striatum, 44±2% of all β-gal–positive cells were neurons. Among nonneuronal cells, approximately 9% were astrocytes (hippocampus), and other cells were indeterminate. Transfected neurons could also be distinguished from nonneuronal cells by morphology, and our criterion for cell counting corresponded to this. Within the 8 nonischemic striata of animals included in this study, 52±3% of all X-gal–positive cells could be identified as neurons. Therefore, the X-gal method could be used to assess transgene expression in neurons, provided that a strict morphological criterion was used.

Time Course of Vector Expression

Consistent with our prior studies,12,13,16 gene expression from the control vector began within 4 to 6 hours after striatal injection, and peak expression occurred at approximately 12 hours, with 53±12 X-gal–positive cells per brain section. The number of X-gal–positive cells declined thereafter, with approximately 20 to 30 positive cells per brain section by 2 to 3 days. By 7 days after injection, only a few positive cells remained (Figure 4).

Protection Against Focal Ischemia

A total of 34 animals were studied. Ten were excluded because of no infarct or because infarction was not contained within the region of vector expression (5 receiving CaBP-expressing vector and 5 receiving control vector). The incidence of infarcts in this model is likely due to the relatively brief duration of ischemia; however, the relatively short occlusion time was used to ensure translation of the transgenes by 48 hours. Another 5 were excluded because they died before the end of the observation period (4 received

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**Figure 2.** Coexpression from the bipromoter vectors in ischemic striata. Forty-eight hours after ischemia onset and 63 hours after injection of the CaBP-expressing vector, micrographs of the ischemic striatum show several CaBP-positive cells (A). Some cells have higher immunofluorescence than others (A, arrows). These latter cells are also β-gal positive (B, arrows). Micrographs of the ischemic striatum injected with the control vector show faint CaBP staining in cells within the ischemic striatum (C, arrowheads), suggesting that there is some endogenous CaBP in ischemic tissue. However, none of these cells are positive for β-gal (D, arrow).
CaBP-expressing vector, and 1 received control vector). Most of these animals died because of subarachnoid hemorrhage that originated at the site of the occluding suture; therefore, the cause of death was most likely due to technical considerations in the stroke model and not the treatment itself. Among the 19 animals included in the final analysis, infarct grades were no different between treated and untreated groups, implying that the severity of ischemia was similar between groups (mean infarct scores for CaBP-expressing vector [n = 10], 1.9 ± 0.3; control vector [n = 9], 2.3 ± 0.2; P = 0.46). Infarct sizes (percentage of ipsilateral hemisphere) from the same animals were also not significantly different (CaBP-expressing vector, 57.8 ± 10%; control vector, 55 ± 5.7%; P = 0.83).

Striatal neuron survival was improved 2- to 3-fold among animals receiving CaBP-expressing vector compared with animals receiving control vector. The percent survivorship of striatal neurons transfected with the CaBP-expressing vector was 53.5 ± 4.1% of the X-gal–positive neurons in the control striatum, while among control vector–treated animals, only 26.8 ± 5.4% of the X-gal–positive striatal neurons remained (P < 0.001) (Figures 5 and 6). Absolute numbers of remaining striatal neurons showed a significant 3-fold increase with CaBP overexpression compared with the control group (P < 0.01). Within nonischemic striata, the number of X-gal–positive neurons was similar between groups (Table).

Discussion

This study is the first to demonstrate that CaBP overexpression protects neurons in a focal stroke model. Excessive cytosolic calcium is known to potentiate ischemic injury by activating a variety of damaging proteases and phospholipases and increasing the generation of free fatty acids and reactive oxygen species.1,2 Calcium accumulates within ischemic neurons by entry through voltage-gated or ligand-gated receptors, as well as by intracellular release by the endoplasmic reticulum and mitochondria.1 Several experimental studies have shown that blocking calcium entry, either via voltage-gated calcium channels or glutamate receptor antagonists, protects against ischemia and ischemia-like insults.2-21 Limiting intracellular calcium accumulation by administering various chelators has also been shown to protect neurons against excitotoxicity24,25 and experimental stroke.24 We now show that by altering endogenous calcium-buffering systems, striatal neurons can be made more resistant against ischemia by overexpressing CaBP, arguing for a neuroprotective role of this protein.

Earlier work examining CaBP expression after various central nervous system insults showed that CaBP immunoreactivity decreases as cells die,26 leading some to postulate that the presence of CaBP correlated with neuronal resistance to calcium-mediated injury. In addition, CaBP-containing neurons4,5 or astrocytes induced to express CaBP7 were more resistant to excitotoxic and ischemia-related injury than neurons that lacked CaBP. However, CaBP is normally found within CA1 and dentate granules of the adult rat hippocampus. Because CA1 hippocampal neurons are particularly vulnerable to ischemia and dentate granule cells are notably resistant,3,8 others believed that CaBP may have nothing to do with neuroprotection. In fact, some reports found that the presence of CaBP did not correlate with enhanced survival.

Figure 3. The defective herpes viral vector transfects neurons. Double immunofluorescence labeling of a neuron transfected with the control vector (βgalα4s) shows colocalization of β-gal (A) and MAP2 (B).
against ischemic and excitotoxic insults. Although ischemia is frequently associated with decreased protein synthesis, increases in CaBP mRNA have been detected in brains 6 to 12 hours after kainic acid administration and forebrain ischemia. However, endogenous CaBP levels may not be present in sufficiently high quantities to buffer toxic intracellular calcium levels. To directly determine whether CaBP serves a protective role against calcium-mediated insults, we artificially expressed it at sufficiently high levels and confirmed that it does indeed confer ischemic resistance. There are also reports of protection with CaBP overexpression in other models of neurodegeneration. Another group used retroviral vectors and found that gene transfer of cabp protected cultured neurons from neurotoxicity in a model of motor neuron disease by decreasing intracellular calcium rises. Conversely, they also found that decreasing CaBP expression with antisense oligonucleotides reversed this protection. Kindy et al showed that adenoviral vectors expressing CaBP protected hippocampal CA1 neurons against forebrain ischemia in gerbils. Our recent work also showed that at the in vivo level, CaBP overexpression protected against kainic acid–induced excitotoxicity and mitochondrial insults.

Vector-mediated CaBP overexpression has been previously shown to alter neuronal synaptic responses, consistent with a calcium-buffering function. In our prior in vitro studies, the CaBP-expressing vector has also been demonstrated to reduce intracellular calcium responses. Consistent with the notion that calcium overload is damaging, we then found that CaBP overexpression protected neurons from various ischemia-like insults such as hypoglycemia and excitotoxin exposure, including N-methyl-D-aspartate, kainate, and glutamate. This protection could be observed even if the transfection occurred up to 30 minutes after insult. However, CaBP did not protect against cyanide toxicity, suggesting that the protective effects are not effective against mitochondrial toxins or that CaBP cannot protect against such severe insults. Interestingly, at the in vivo level, we did find hippocampal neuron protection against 3-acetylpyridine, a different mitochondrial toxin that damages neurons by uncoupling electron transport. This latter study suggests that the lack of protection against cyanide was more likely due to the insult severity. We now show that gene transfer–mediated CaBP overexpression in striatal neurons improves survival against transient focal cerebral ischemia. Given that ischemic injury is due to a variety of pathological processes, including excitotoxicity and mitochondrial disruption, our present results corroborate our previous findings.

In contrast to our findings, Klapstein et al found that CaBP-deficient mice were resistant to forebrain ischemia, suggesting that CaBP might play a detrimental role in ischemic injury. These animals displayed improved electro-
physiological parameters and higher CA1 cell counts. In addition, there was less terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining in the remaining hippocampal neurons, suggesting that these mice were also more resistant to apoptotic death. Interestingly, there did not appear to be any differences in the levels of other calcium-binding proteins, such as parvalbumin, in this strain. The reasons for this discrepancy are not clear; however, there may be unforeseen alterations in other systems in lifelong CaBP-deficient animals to explain the observed neuroprotection. On the other hand, some investigators have reported that intracellular calcium buffering may be detrimental. Abdel-Hamid and Baimbridge found that artificial calcium buffers potentiated excitotoxicity in cultured hippocampal neurons by paradoxically increasing cytosolic calcium levels. There may also be differences in the regulation or other actions of artificial buffers unrelated to calcium influx through voltage-gated ion channels. The investigators proposed that calcium chelators in this setting were particularly detrimental when excitotoxin exposure was prolonged and could be due to such factors as loss of cell volume regulation or other actions of artificial buffers unrelated to cytosolic calcium levels. There may also be differences in the susceptibility of neurons to ischemic injury within the hippocampus, compared with the striatum, where we altered CaBP expression.

We show that a gene therapy approach to stroke treatment is possible; however, it is still limited by the number of neurons our vector is capable of transfecting. Because we are able to transfected only a few hundred cells, we were not able to alter overall infarct size using this approach. Other groups have applied gene transfer techniques by overexpressing a diffusible substance within the brain parenchyma or within ependymal cells. Betz et al. used an adenoviral vector to overexpress interleukin-1 receptor antagonist in ependymal cells 5 days before permanent MCA occlusion. They found that treated rats had smaller infarcts than those receiving a control vector. Similar findings were recently reported by Kitagawa et al., who overexpressed glial cell–derived neurotrophic factor in the cortex using an adenoviral vector. Others have applied gene therapy to cerebral blood vessels with the hope of improving cerebral hemodynamics or blood vessel integrity.

It should also be noted that we began gene transfer before MCA occlusion so that maximum expression from the vectors would coincide with the onset of ischemia. Given that this approach would have limited clinical relevance, future studies should determine whether cabp gene transfer could be applied after insult. Certainly, this would depend on the gene being expressed and whether its late expression might be expected to provide protection. In fact, we previously showed that gene transfer of the antiapoptotic protein Bcl-2 could protect striatal neurons when administered 90 minutes after stroke onset. Given that expression from these vectors begins 4 to 6 hours after injection and peaks 12 to 15 hours later, this would suggest a particularly long therapeutic window for this protein. Whether this applies to CaBP has yet to be determined for ischemia, although in our prior in vivo study, vector was delivered immediately after toxin administration. Finally, the issue of viral vector safety should be mentioned. While our initial studies have shown that this strain of replication-incompetent HSV does not cause cytotoxicity in rodent brains, this has not been systematically studied in humans and should be investigated before the initiation of clinical studies. In fact, our laboratories are currently investigating the possibility of ex vivo gene transfer to human brain tissue.

In the meantime, we show that intracellular calcium reduction with CaBP overexpression is a potential target for stroke treatment. Several recent clinical studies have examined various inhibitors of calcium entry; however, none of these studies were successful mainly because of the untoward psychomimetic and hemodynamic side effects. Therefore, intracellular calcium reduction may prove to be an alternate approach for stroke treatment.

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