Mild Postischemic Hypothermia Prolongs the Time Window for Gene Therapy by Inhibiting Cytochrome c Release

Heng Zhao, PhD; Midori A. Yenari, MD; Robert M. Sapolsky, PhD; Gary K. Steinberg, MD, PhD

Background and Purpose—We showed previously that Bcl-2 overexpression with the use of herpes simplex viral (HSV) vectors improved striatal neuron survival when delivered 1.5 hours after stroke but not when delivered 5 hours after stroke onset. Here we determine whether hypothermia prolongs the therapeutic window for gene therapy.

Methods—Rats were subjected to focal ischemia for 1 hour. Hypothermia (33°C) was induced 2 hours after insult and maintained for 3 hours. Five hours after ischemia onset, HSV vectors expressing Bcl-2 plus β-gal or β-gal alone were injected into each striatum. Rats were killed 2 days later.

Results—Striatal neuron survival of Bcl-2–treated, hypothermic animals was improved 2- to 3-fold over control-treated, hypothermic animals and Bcl-2–treated, normothermic animals. Neuron survival among normothermic, Bcl-2–treated animals was not different from control normothermics or control hypothermics. Double immunostaining of cytochrome c and β-gal demonstrated that Bcl-2 plus hypothermia significantly reduced cytochrome c release.

Conclusions—Postischemic mild hypothermia extended the time window for gene therapy neuroprotection using Bcl-2 and reduced cytochrome c release. (Stroke. 2004;35:572-577.)

Key Words: cerebral ischemia ■ gene therapy ■ hypothermia ■ ischemia, focal ■ proto-oncogene proteins c-bcl-2

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From the Departments of Neurosurgery (H.Z., M.A.Y., R.M.S., G.K.S.), Biological Sciences (H.Z., R.M.S.), and Neurology and Neurological Sciences (M.A.Y., R.M.S., G.K.S.), Stanford University, Stanford, Calif.

Correspondence to Gary K. Steinberg, MD, PhD, Department of Neurosurgery, Stanford University School of Medicine, 300 Pasteur Dr R200, Stanford, CA 94305-5327. E-mail gsteinberg@stanford.edu

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Materials and Methods

Generation of Plasmids

HSV vectors were generated as previously described. The amplicon plasmid pα22b βgal4bcl-2 contained the human bcl-2 gene and the Escherichia coli lacZ gene under the control of the HSV α4 and α22 promoters, respectively. A control vector, designated α4s, which contains lacZ gene alone, was also generated. Amounts of infectious vector particles in each injection were as follows: Bcl-2 vector, 4.6 to 6.3x10^1; control vector, 5.8x10^1. Vector:helper virus ratios ranged from 1:4 to 1:5 for Bcl-2 and 1:5 for control.

Surgery

The Stanford University Administrative Panel on Laboratory Animal Care approved all animal procedures. We based the design of the current experiment on our previous time course study in which Bcl-2 overexpression improved neuronal survival when delivered 1.5 hours but not 5 hours after stroke. Figure 1 presents a diagram of the surgical procedure. Sprague-Dawley rats (weight, 290 to 350 g) were anesthetized with 5% isoflurane, then isoflurane was decreased to 1.5% to 3.0% for the remainder of the procedures. Rats were placed on a heating/cooling blanket to maintain rectal temperature between 37°C and 38°C. There were 4 groups: (1) normothermic rats given control vector (n=11); (2) normothermic rats given Bcl-2 vector (n=11); (3) hypothermic rats given control vector (n=11); and (4) hypothermic rats given Bcl-2 vector (n=10). The left middle cerebral artery (MCA) was occluded by inserting an intraluminal 3-0 nylon monofilament suture through the common carotid artery to the branch point of the MCA. After 1 hour of ischemia, the suture was withdrawn. Normothermic rats were allowed to recover for 3.5 hours and then were reanesthetized. Hypothermic animals were allowed to
recover for 50 minutes and then were reanesthetized, and hypothermia (rectal temperature of 33°C, corresponding to brain temperature of 33°C)13,14 was induced and maintained for 3 hours. Cooling was achieved by spraying alcohol onto the rat and cooling it with a fan. Rats were rewarmed on a heating pad under a lamp. Both cooling and rewarming were achieved within 10 to 15 minutes. To confirm that key physiological variables did not differ between groups during surgery, mean arterial blood pressure (MAPB), arterial pH, and PaO₂ and PaCO₂ were measured (by methods described previously15) in 2 groups of animals (n=3 per group) that were treated exactly the same as the rats in the normothermic and hypothermic groups, except that no vector was delivered. Additionally, to verify a close correlation between brain and rectal temperature in these animals, a small burr hole was drilled to permit insertion of a 33-gauge thermocouple temperature probe to measure brain temperature in the nonischemic hemisphere. The probe was inserted 4.5 mm into the striatum.

Bcl-2 or control vectors were injected bilaterally into the striata 5 hours after ischemia onset. Coordinates for the 2.5-μL injections were as follows (from bregma): anteroposterior=-0.13, mediolateral=3.5 mm, with 2 injection sites at dorsoventral=5 and 4 mm. Animals were killed 48 hours later with an overdose of halothane, and 30-μm coronal brain sections were prepared and analyzed as previously described.2,6,7 X-gal/cresyl violet–stained sections were prepared. Infarcts were measured from 1 section at the bregma, normalized to the entire ipsilateral striatum.

**Laser-Scanning Microscopy**

Double-fluorescence confocal microscopy was performed to detect whether Bcl-2 plus hypothermia inhibited cytochrome c release. (It is not possible to use Western blotting to address this issue because of the small number of transfected neurons.16) One of every 3 consecutive sections was stained with X-gal and counterstained with cresyl violet to determine whether transfection was successful. Seven to 10 slices adjacent to slices that were positive for X-gal were selected for immunostaining. For double labeling of cytochrome c and β-gal, primary antibodies of purified mouse anti-cytochrome c antibody (1:500, catalog No. 556432, PharMingen) and rabbit anti-β-galactosidase (1:200, code No. 55976, ICN Biomedicals, Inc) were used. Secondary antibodies were Cy3-conjugated donkey anti-mouse IgG (1:200, Jackson ImmunoResearch) and fluorescein isothiocyanate–conjugated donkey anti-rabbit IgG (1:200, Jackson ImmunoResearch). Negative controls, in which the primary antibodies were omitted, were run in parallel. Sections were coverslipped and examined under a LSM510 confocal laser-scanning microscope (Carl Zeiss).

**Cell Counts**

We have shown that the bipromoter vector coexpresses transgene and reporter gene similarly2,6,7. Thus, we compared the percentage of surviving transfected X-gal–positive neurons (relative to the number in the contralateral striatum) in Bcl-2–treated and control animals.6,8 Individual X-gal–positive neurons were counted over successive sections in each hemisphere using criteria similar to those in our previous studies,6,8 and the number in the ischemic hemisphere was expressed as a percentage of the number in the nonischemic hemisphere.

β-gal–positive neurons and double-labeled (β-gal with cytochrome c) neurons were counted using morphological criteria similar to those used for the X-gal–stained neurons. Since there were no cytochrome-c–positive neurons in the nonischemic striatum, only those in the ischemic striatum were counted. The number of double-labeled neurons was determined from consecutive sections and expressed as a percentage of the total number of β-gal–positive neurons.

**Statistical Analysis**

One-way ANOVA followed by Student-Newman-Keuls post hoc tests were used. Data are presented as mean±SEM.

**Results**

**Physiological Variables**

Rectal and striatal temperatures differed as planned between normothermic and hypothermic rats (n=3 per group; Table). Brain temperature was equal to rectal temperature during hypothermia and was 0.4°C to 0.6°C below rectal temperature during normothermia. There were no significant differences in other physiological parameters (arterial pH, PaCO₂, PaO₂, MAPB) between groups before, during, or after ischemia (Table).

**Overexpression of Bcl-2 Plus Hypothermia Protects Against Neuron Loss After Ischemia**

Occlusion of the MCA for 1 hour caused a focal infarct within the medial and lateral striatum. Striatal infarct sizes did not differ across groups. Infarct size was not altered by gene transfer because only a limited number of neurons were transfected. Delivery of vectors 5 hours after ischemia onset resulted in local expression in the striatum (Figure 2). Since the number of transfected neurons in nonischemic hemispheres was similar in hypothermic and normothermic rats,
temperature did not alter transfection efficiency. Neuron survival in normothermic/control rats did not differ from that in hypothermic/control or normothermic/Bcl-2 rats (Figure 3). This suggests that neither hypothermia nor Bcl-2 alone improved neuron survival 2 days after stroke. In contrast, survival of striatal neurons infected with Bcl-2 in hypothermic rats was improved 2- to 3-fold relative to hypothermic/control rats ($P<0.01$, ANOVA) and normothermic/Bcl-2 rats ($P<0.01$, ANOVA; Figure 3). This suggests a synergistic effect of Bcl-2 and hypothermia.

**Overexpression of Bcl-2 Plus Hypothermia Inhibits Cytochrome $c$ Release**

There was no cytochrome $c$ immunoreactivity in the contralateral striatum (Figure 4) or in control tissue in which primary antibody was eliminated (data not shown). Many neurons labeled with cytochrome $c$ and $\beta$-gal were present in all control vector–treated animals and in normothermic/Bcl-2 animals. In contrast, the percentage of $\beta$-gal–positive neurons that were positive for cytosolic cytochrome $c$ was significantly lower among hypothermic/Bcl-2 rats than in other groups (Figure 5). In the contralateral cortex, $\beta$-gal–stained neurons did not colocalize with cytochrome $c$.

**Discussion**

This is the first demonstration that postischemic hypothermia prolongs the time window for neuroprotection by gene therapy. In previous studies Bcl-2 overexpression improved neuron survival when delivered 1.5 but not 5 hours after stroke. We now show that hypothermia extends the window for Bcl-2 gene therapy to 5 hours after ischemia onset.

Although intraischemic hypothermia provides long-term protection against cerebral ischemia, postischemic hypothermia may only transiently delay ischemic cell death unless protracted periods of hypothermia are used. Neurprotective agents may be coupled with short-term hypothermia to enhance its protective effects and to avoid the adverse effects of long-term hypothermia. For example, 3 hours of immediate postischemic hypothermia (30°C) combined with MK-801 (a noncompetitive $N$-methyl-$D$-aspartate antagonist) increased CA1 neuron survival over either treatment alone. Similar findings were reported when postischemic hypothermia was combined with either an anti-inflammatory cytokine or a free radical scavenger.

Intraischemic hypothermia protects against cell damage by lowering metabolism and energy demand, inhibiting glutamate release, and preventing dysfunction of the blood-brain barrier. Recently, intraischemic hypothermia has been shown to decrease caspase-3 expression and cytochrome $c$ release after focal ischemia, although one study showed no change in Bcl-2, Bax, or caspase-3 expression. In contrast, mild hypothermia increased Bcl-2 expression after global ischemia. Therefore, the mechanism of hypothermic protection may depend on the nature and severity of the insult.

Unfortunately, few studies have clarified the protective mechanisms of postischemic hypothermia. One recent study
showed that postischemic hypothermia only delayed neutrophil accumulation and microglial activation, which may account for the lack of persistent protection. However, our laboratory demonstrated that hypothermia inhibits leukocyte infiltration as late as 7 days and inflammatory cell generation of inducible nitric oxide synthase, nitric oxide, and peroxynitrite (ONOO•). Delayed cooling attenuates neuronal nitric oxide synthase expression to a greater extent than intraischemic hypothermia. It is still unclear why we found synergistic effects of Bcl-2 and hypothermia. Transgene expression from HSV vectors requires several hours, with peak expression occurring approximately 12 hours after delivery to brain. Bcl-2, originally characterized as an antiapoptotic protein, can also block necrosis. Bcl-2 has various roles within cells, including increasing mitochondrial calcium uptake, blocking Bax translocation, and inhibiting cytochrome c release. We have recently shown that overexpression of Bcl-2 decreased cytochrome c release when delivered before focal ischemia. In the current study Bcl-2 overexpression did not significantly inhibit cytochrome c release when delivered 5 hours after ischemia onset. In contrast, Bcl-2 plus hypothermia significantly reduced cytochrome c release. Our previous study demonstrated that intraischemic hypothermia reduced cytochrome c release at 5 but not 24 hours after stroke. Similarly, in this study, although hypothermia itself did not inhibit cytochrome c release 48 hours after stroke, it may have delayed the onset of cytochrome c release. This delay could allow the late expression of Bcl-2 to block cytochrome c release and protect against neuronal death. Hypothermia in the current study may also have inhibited or delayed some other aspects of cell death, such as intracellular calcium accumulation, generation of free radicals, or caspase activation. This inhibition or delay could allow Bcl-2 time to block these detrimental events and protect against neuronal death.

Hypothermic rats experienced postischemic isoflurane anesthesia for approximately 3 hours longer than normothermic rats, which may have influenced the outcome beyond the

Figure 4. Double-immunofluorescent staining of cytochrome c (Cyto C) and β-gal 48 hours after MCA occlusion. The top panels show a double-stained neuron in the ischemic striatum of a hypothermic/control vector–treated rat. The middle panels show that a β-gal–positive neuron is not colocalized with cytosolic cytochrome c in the ischemic striatum of a hypothermic/Bcl-2 rat. The bottom panels show no cytosolic cytochrome c staining in the nonischemic (NI), contralateral striatum of hypothermic/control animal.

Figure 5. Percentage of β-gal–positive neurons that were also positive for cytochrome c (number of double-labeled cells divided by number of β-gal–positive cells × 100). Cytochrome c release was inhibited in rats treated with Bcl-2 plus hypothermia relative to those treated with Bcl-2 and normothermia or rats treated with control vector (normothermic and hypothermic).

*P < 0.05, hypothermia Bcl-2 (33°C-Bcl-2) vs normothermia Bcl-2 (37°C-Bcl-2); **P < 0.001, hypothermia Bcl-2 (33°C-Bcl-2) vs hypothermia control (33°C-control) and normothermia control (37°C-control).
effects of hyperthermia. However, isoflurane did not influence any of the hemodynamic or physiological variables, nor did it alter brain temperature in this study (Table). Additionally, whether isoflurane itself protects against cerebral ischemia is not clear. Many reports disagree, and some argue that it provides little protection or even worsens ischemic damage.34–37 In reports that demonstrated that isoflurane reduced the infarct caused by focal ischemia, isoflurane was applied during rather than after ischemia.38,39 It is unknown whether posts ischemic isoflurane provides protection. The effect of differences in posts ischemic isoflurane exposure would likely be small relative to the protective effect of hyperthermia. Although we cannot completely exclude an influence of isoflurane, we conclude that hyperthermia prolongs the therapeutic time window for gene therapy.

Conclusion

Brief, mild posts ischemic hyperthermia prolonged the temporal therapeutic window for Bcl-2 gene therapy from 1.5 to 5 hours, and Bcl-2 plus hyperthermia blocked cytochrome c release 48 hours after ischemia onset. These data demonstrate a synergistic effect of hyperthermia and Bcl-2 overexpression, suggesting a potential clinical application of combined hyperthermia and gene therapy.

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