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

Substantial knowledge has emerged concerning the cellular and molecular events underlying neuron death after cerebral ischemia, allowing the design of gene therapies targeting various aspects of the ischemic cascade. We and others have shown that herpes simplex viral (HSV) vectors expressing Bcl-2, HSP72, glucose transporter, and calbindin D28K delivered before ischemia reduce neuron death. More clinically relevant is our demonstration that vectors expressing Bcl-2 or HSP72 also improve striatal neuron survival when delivered up to 2 hours after insult. No protection was observed when vector was delivered at 5 hours after ischemia onset. Thus, it would be beneficial to find a way to prolong the narrow therapeutic window for gene therapy after cerebral ischemia.

Intraischemic hypothermia can protect against cerebral damage for months after ischemia onset. We and others previously showed that delayed mild hypothermia protects against injury in a focal ischemia model if begun within 2 hours of ischemia onset. Here we assessed whether mild postischemic hypothermia can extend the temporal therapeutic window for gene therapy neuroprotection with the antiapoptotic gene bcl-2. Since cytochrome c is released from mitochondria into the cytosol after ischemia and activates caspase 9 and 3 leading to apoptosis, we also determined whether Bcl-2 plus postischemic hypothermia reduces cytochrome c release.

Materials and Methods

Generation of Plasmids

HSV vectors were generated as previously described. The amplicon plasmid pα22bβgalo4bcl-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 6.3×10^3; control vector, 5.8×10^3. 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

Received April 28, 2003; final revision received September 3, 2003; accepted October 14, 2003.

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recover for 50 minutes and then were reanesthetized, and hypothermia (rectal temperature of 33°C, corresponding to brain temperature of 33°C \(^\circ\)) 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 rewarmed were achieved within 10 to 15 minutes. To confirm, rats were rewarmed on a heating pad under a lamp. Both cooling and rewarmed were achieved within 10 to 15 minutes. To confirm

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Figure 1. Surgical procedures.

![Figure 1](image)

**Physiological Variables Before, During, and After Stroke**

<table>
<thead>
<tr>
<th>Groups (n=3/Group)</th>
<th>Normothermia Group</th>
<th>Hypothermia Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before Ischemia</td>
<td>During Ischemia</td>
</tr>
<tr>
<td><strong>MABP</strong></td>
<td>93.6±2.4</td>
<td>84.3±9.7</td>
</tr>
<tr>
<td>pH</td>
<td>7.41±0.02</td>
<td>7.39±0.01</td>
</tr>
<tr>
<td><strong>PaCO</strong> (_2)</td>
<td>43.90±0.93</td>
<td>38.76±1.76</td>
</tr>
<tr>
<td><strong>PaO</strong> (_2)</td>
<td>134.9±4.96</td>
<td>127.8±3.23</td>
</tr>
<tr>
<td>Rectal temperature</td>
<td>37.1±0.06</td>
<td>37.1±0.05</td>
</tr>
<tr>
<td>Brain temperature</td>
<td>36.6±0.05</td>
<td>36.7±0.05</td>
</tr>
</tbody>
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\( ^* \) During hyperthermia.
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 β-gal were present in all control vector–treated animals and in normothermic/Bcl-2 animals. In contrast, the percentage of β-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, β-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, posts ischemic hypothermia may only transiently delay ischemic cell death unless protracted periods of hypothermia are used. Neuroprotective 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 in normothermic rats, perhaps because the vector was 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. 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 (33C-Bcl-2) vs normothermia Bcl-2 (37C-Bcl-2); **P < 0.001, hypothermia Bcl-2 (33C-Bcl-2) vs hypothermia control (33C-control) and normothermia control (37C-control).
effects of hypothermia. 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.\textsuperscript{34-37} In reports that demonstrated that isoflurane reduced the infarct caused by focal ischemia, isoflurane was applied during rather than after ischemia.\textsuperscript{38-39} It is unknown whether postischemic isoflurane provides protection. The effect of differences in postischemic isoflurane exposure would likely be small relative to the protective effect of hypothermia. Although we cannot completely exclude an influence of isoflurane, we conclude that hypothermia prolongs the therapeutic time window for gene therapy.

Conclusion

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

Acknowledgments

This work was supported by National Institutes of Health, National Institute of Neurological Disorders and Stroke grants P01 NS37520 (Drs Steinberg and Sapolsky), R01 NS 27292 (Dr Steinberg), R01 40516 (Dr Yenari), and R01 NS32848 (Dr Sapolsky); Tobacco-Related Disease Research Program for the State of California (Dr Sapolsky); the Adler Foundation (Dr Sapolsky); and American Heart Association grant 006091Y (Dr Yenari). We thank Beth Hoyte and David Schaal for assistance in preparing the manuscript.

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Stroke. 2004;35:572-577; originally published online January 15, 2004;
doi: 10.1161/01.STR.0000110787.42083.58

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