Recombinant Fv-Hsp70 Protein Mediates Neuroprotection After Focal Cerebral Ischemia in Rats

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Background and Purpose—This study investigated the effects of intravenous recombinant Fv-Hsp70 protein on infarction volume and behavior after experimental ischemic stroke.

Methods—Focal cerebral ischemia was produced by occluding the middle cerebral artery using the intraluminal suture technique. Rats subjected to 2 hours of focal ischemia were allowed to survive 24 hours. At 2 1/4 hours and 3 hours after onset of ischemia, Fv-Hsp70 recombinant protein (0.5 mg/kg) or saline was injected through the tail vein. Sensorimotor function and infarction volume were assessed at 24 hours after ischemia.

Results—Administration of Fv-Hsp70 after focal cerebral ischemia significantly decreased infarct volume by 68% and significantly improved sensorimotor function compared with the saline-treated control group. Western blots showed Fv-Hsp70 in ischemic but not in control brain; and Fv-Hsp70 suppressed endogenous Hsp70.

Conclusion—Fv-Hsp70 protected the ischemic brain in this experimental stroke model. (Stroke. 2010;41:538-543.)

Key Words: antibody ■ behavior ■ Fv ■ Hsp70 ■ ischemic stroke ■ rat ■ recombinant protein

Tissue plasminogen activator is the only medication approved by the Food and Drug Administration for treatment of acute ischemic stroke. Although effective, the limited time window for treatment and its complications limit its use. Thus, there is an unmet need for developing effective therapies for stroke.

Hsp70 is a molecular chaperone that protects cells against many types of stress. Neuroprotection by exogenously delivered Hsp70 has been demonstrated by many independent research groups using a variety of approaches, including viral transfection of the brain; intrathecal delivery of protein; delivery of naked RNA; and transgenic and other approaches using both in vitro and in vivo models. However, none of these methods is likely to be useful or successful in the clinical setting of acute stroke. To test the potential of Hsp70 for treating stroke, we searched for a reliable method for in vivo delivery of protein.

The plasma membrane and blood–brain barrier present formidable obstacles to delivery of therapeutic molecules to intracellular compartments. Effective molecular therapy depends on efficient intracellular delivery vehicles. The cell-penetrating single chain fragment of the anti-DNA antibody mAb 3E10 (Fv) is a molecular delivery vehicle that we have shown produces Hsp70 protein-mediated neuroprotection in vitro. Thus, this study examined the potential of intravenous administration of Fv-Hsp70 for treating stroke in vivo when given at clinically relevant times after experimental strokes in rats. We show that Fv-Hsp70 given at 2 1/4 and 3 hours after stroke enters ischemic but not control brain, markedly decreases infarct volume, and improves sensorimotor function.

Materials and Methods

Expression of Fv-Hsp70 Recombinant Protein in Pichia pastoris

Single-chain Fv antibody was derived from mAb 3E10. cDNA for the Fv fragment was ligated into the plasmid pPICZαA as previously described. Briefly, human Hsp70 cDNA was ligated into the Fv containing pPICZαA separated by Myc and His6 tags. The subsequent construct was electroporated into the X-33 methylotrophic yeast strain Pichia pastoris (Invitrogen, Carlsbad, Calif). Recombinant protein was purified from the medium using Ni-NTA agarose beads (Qiagen, Valencia, Calif) under sterile conditions. Protein was eluted from the column with elution buffer (50 mmol/L NaH₂PO₄, 300 mmol/L NaCl, 500 mmol/L imidazole, pH 8.0) in 5 1.5-mL aliquots. Aliquots were then exchange-dialyzed with Dulbecco phosphate-buffered saline (Mediatech, Manassas, Va) to remove the imidazole (with a final concentration of imidazole less than 2 mmol/L). Final sample volume was 1 to 3 mL with an Fv-Hsp70 concentration of approximately 0.5 mg/mL. Fv-Hsp70 protein was stored at 4°C and used within 24 hours.
Animals
Thirty-seven male Sprague-Dawley rats weighing 298 to 346 g (Charles River Laboratories; Hollister, Calif) were used in this study. The Institutional Animal Care and Use Committee at the University of California at Davis reviewed and approved the animal protocols in accordance with National Institutes of Health guidelines.

Middle Cerebral Artery Occlusion
Middle cerebral artery occlusion (MCAO) was produced using the intraluminal suture technique.8 Briefly, rats were anesthetized with 3% isoflurane and maintained on 1.5% isoflurane with 100% oxygen. The right common carotid artery was exposed through a ventral midline incision. To occlude the middle cerebral artery, a 3-0 monofilament nylon suture with the tip rounded by heat was inserted into the external carotid artery and advanced into the internal carotid artery approximately 20 to 23 mm beyond the carotid bifurcation until mild resistance was felt. Rectal temperature was maintained at 36.6°C to 37.5°C with a heating blanket throughout the procedure. Rats subjected to 2 hours of focal ischemia followed by 22-hour reperfusion were allowed to survive 24 hours. Behavior was assessed immediately after surgeries and only rats demonstrating circling contralateral to the MCAO were included in the study.

Administration of Fv-Hsp70 Recombinant Protein
Fv-Hsp70 recombinant protein or saline was injected twice through the tail vein. The first injection was performed at 15 minutes after reperfusion (2¼ hours after onset of ischemia), and the second injection was performed at 1 hour after reperfusion (3 hours after the onset of ischemia). Each dose of Fv-Hsp70 protein was 0.5 mg/kg. Two separate protein injections were performed to increase the amount of protein in blood and prolong its action within the 2¼- to 3-hour therapeutic window. The control group was injected with the same volume of 0.9% sodium chloride at the same time points.

Infarct Volume Measurement
Twenty rats were used for the measurement of infarction volume. Brains were removed from euthanized rats and were cut into 2-mm thick coronal slices with the aid of an acrylic brain matrix (Zivic Instruments, Pittsburgh, Pa). Slices were placed into a 2% solution of 2,3,5-triphenyltetrazolium chloride and then warmed in a 37°C chamber for 10 minutes. Slices treated with paraformaldehyde (4%) were refrigerated at 4°C for 48 hours before digitization on a flatbed scanner at 1200 dpi.

Infarct area of each section was measured by a blinded investigator using National Institutes of Health ImageJ software9 and total infarct volumes were calculated. Ipsilateral and contralateral hemisphere volumes were also determined to correct for edema, which can artificially affect the infarct volume.10 The formula used to calculate the edema-corrected infarct volume was: corrected infarct volume = contralateral volume/ipsilateral volume × infarct volume.

Behavior Testing
Behavior was assessed by a blinded investigator at 24 hours after onset of ischemia in the same animals that were used for the measurement of infarction volume. The vibrissae-induced forelimb placement test was used to assess sensorimotor function.11 This test was specifically chosen because it requires intact vibrissae sensory and forelimb motor cortical function,12 and both vibrissae sensory and forelimb motor cortex lie squarely within the middle cerebral artery vascular territory. Briefly, the rat was held parallel to the edge of a bench and one set of whiskers was brushed against its edge. Movement of the front forelimb, ipsilateral to the whisker stimulation, was monitored as a response and recorded as successful if the paw was placed on the bench surface with or without pad contact. The percentage of successful forelimb placements over 10 trials was calculated for forelimbs ipsilateral and contralateral to the side of stroke.

Immunohistochemistry
After the measurement of infarction volume, the brain slices were used for immunohistochemistry. Coronal sections 50 μm thick were cut in a cryostat (−20°C). Primary antibody for Hsp70 (monoclonal, 1:200 dilution; BioVision; Mountain View, Calif) was incubated with the sections overnight at 4°C. A biotinylated horse antimouse IgG (1:200 dilution; Vector Labs; Burlingame, Calif) secondary antibody was used. The antibody complex was detected using ABC reagent and a substrate solution of H2O2 and diaminobenzidine according to the manufacturer's instructions (Vector Labs). Nonspecific labeling was assessed by omitting the primary antibody.

Western Blot Analysis
Brains from 9 rats were treated as previously described for Western blot analysis.15 Fifty micrograms of protein isolated from the hemisphere ipsilateral to MCAO was loaded into each lane. Membranes were probed overnight at 4°C with rabbit polyclonal anti-Hsp70 (1:4000; Stressgen; Victoria, BC Canada) antibody. Primary antibody was detected using horseradish peroxidase-conjugated antirabbit IgG (Bio-Rad; Hercules, Calif). The signal was detected using the Pierce electrochemiluminescent detection system (Thermo Scientific; Rockford, Ill).

Physiological Parameters
Eight rats were used for the measurement of physiological variables. Physiological variables were measured at 4 time points: (1) before injection; (2) the first injection; (3) the second injection; and (4) 24 hours after focal cerebral ischemia. Either saline or Fv-Hsp70 recombinant protein was injected. Heart rate, breathing rate, and SpO2 were measured using the MouseOx Murine Pulse Oximeter System (Starr Life Sciences Corp; Oakmont, Pa). Cerebral blood flow was measured using a Laser Doppler Monitor (Perimed Inc; Ventura, Calif).13

Statistical Analysis
Data are reported as mean±SEM. t tests were performed when comparing 2 groups. One-way analysis of variance was performed with a Fisher least significant difference post hoc test when comparing variables for multiple groups/time points. A probability value of <0.05 was considered statistically significant.

Results
Physiological Parameters
Compared with baseline, heat rate, breathing rate, cerebral blood flow, SpO2, and temperature did not significantly differ after either Fv-Hsp70 or saline injection at any time point. Cerebral blood flow was significantly decreased (P<0.05) at 24 hours after ischemia compared with 15 minutes after reperfusion (first injection of either Fv-Hsp70 or saline). Compared with the control group, heart rate, breathing rate, cerebral blood flow, SpO2, and temperature did not change significantly (P>0.05) after Fv-Hsp70 protein injection at any time point (Table).

Fv-Hsp70 Reduced Infarct Volume
Fv-Hsp70 protein injection significantly decreased infarct volumes. Average infarct volume, as determined by 2,3,5-triphenyltetrazolium chloride staining (Figure 1A), was 65.91±27.31 mm3 in the Fv-Hsp70-treated rats, which was significantly (P<0.05) less than the saline-treated control rats, which had an average infarct volume of 208.62±42.59 mm3 (Figure 1B). Thus, the infarct volume was markedly decreased by approximately 68% after Fv-Hsp70 injection.
Fv-Hsp70 Improved Neurological Behavior

Fv-Hsp70 protein injection significantly improved behavioral function on the forelimb placement test (Figure 1C). Forelimb placement in response to whisker stimulation was used to measure the sensorimotor deficit after MCAO. Forelimb placement of the unimpaired side was near perfect for both the Fv-Hsp70 and control groups with 97.8% and 100.0% successful placements, respectively (data not shown). Table. Physiological Parameters in Animals Injected With Fv-Hsp70 Protein or Saline After Focal Cerebral Ischemia (n=4 per Group)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>Before Injection</th>
<th>First Injection</th>
<th>Second Injection</th>
<th>24 Hours After Ischemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breathing rate, breaths/min</td>
<td>Control</td>
<td>64.8±1.7</td>
<td>67.0±1.9</td>
<td>64.3±2.2</td>
<td>68.7±4.4</td>
</tr>
<tr>
<td></td>
<td>Fv-Hsp70</td>
<td>68.8±3.1</td>
<td>73.8±3.9</td>
<td>74.8±4.4</td>
<td>70.7±4.9</td>
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<tr>
<td>Heart rate, beats/min</td>
<td>Control</td>
<td>398.0±9.4</td>
<td>428.0±11.8</td>
<td>430.0±14.4</td>
<td>380.3±11.0</td>
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<tr>
<td></td>
<td>Fv-Hsp70</td>
<td>396.0±18.6</td>
<td>399.8±5.8</td>
<td>423.8±13.7</td>
<td>403.3±6.2</td>
</tr>
<tr>
<td>SpO2, %</td>
<td>Control</td>
<td>99.0±0.1</td>
<td>98.9±0.2</td>
<td>99.1±0.1</td>
<td>98.7±0.1</td>
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<tr>
<td></td>
<td>Fv-Hsp70</td>
<td>99.0±0.1</td>
<td>98.7±0.1</td>
<td>97.9±0.5</td>
<td>98.7±0.1</td>
</tr>
<tr>
<td>Temperature, ºC</td>
<td>Control</td>
<td>37.1±0.2</td>
<td>36.9±0.1</td>
<td>36.9±0.2</td>
<td>37.3±0.3</td>
</tr>
<tr>
<td></td>
<td>Fv-Hsp70</td>
<td>37.1±0.2</td>
<td>37.0±0.2</td>
<td>37.2±0.1</td>
<td>37.4±0.1</td>
</tr>
<tr>
<td>Cerebral blood flow (perfusion unit)</td>
<td>Control</td>
<td>151.0±5.5</td>
<td>173.4±8.8*</td>
<td>153.4±5.7</td>
<td>116.3±10.6*</td>
</tr>
<tr>
<td></td>
<td>Fv-Hsp70</td>
<td>125.8±16.5</td>
<td>148.5±22.1†</td>
<td>124.2±10.9</td>
<td>102.3±13.1†</td>
</tr>
</tbody>
</table>

*P<0.05 compared with each other in the control group.
†P<0.05 compared with each other in the Fv-Hsp70 group using analysis of variance followed by Fisher least significant difference method. There is no significant difference of any parameters between the 2 groups at the same time points.

Figure 1. Effect of Fv-Hsp70 protein on the infarct volume and neurological behavior in adult male rats after focal cerebral ischemia. The infarct was produced by occluding the middle cerebral artery (MCA) for 2.0 hours using a suture. Either saline or Fv-Hsp70 recombinant protein (0.5 mg/kg) was infused intravenously at 15 minutes and 1 hour after reperfusion, that is, at 2½ hours and 3.0 hours after the onset of focal ischemia. A, 2,3,5-triphenyltetrazolium chloride staining in rat brain 24 hours after focal ischemia in saline-injected and Fv-Hsp70-injected animals. B, Infarct volumes in rat brain 24 hours after focal ischemia. Data for each treatment group are represented as a separate box. The large box represents the 25th and 75th percentiles. The whiskers demonstrate the 5th and 95th percentiles. Means are indicated by the small squares. *P<0.015. C, Percentage of successful forelimb placements of rats after stimulation of ipsilateral vibrissae over 10 trials. Placement was unsuccessful for nearly all trials in the saline treated rats, whereas Fv-Hsp70-treated rats successfully responded with correct placement at a significantly higher rate. Placement scores for the right forelimb were near perfect for both groups. *P<0.038.
shown). However, forelimb placement of the impaired side was much lower for the saline-treated rats (5.5%\% vs 3.7%). The Fv-Hsp70 rats had a statistically significant improvement in the success rate for placement of their impaired forelimb at 31.1%\% vs 11.5% \((P<0.05; \text{Figure 1C})\).

**Fv-Hsp70 Inhibited Endogenous Hsp70 Protein Expression**

Western blot analyses showed that the endogenous 70 kD Hsp70 was induced at 24 hours in the brains of rats subjected to MCAO and injected with saline (Figure 2, lane 1). Unexpectedly, there was suppression of the endogenous 70 kD Hsp70 protein normally induced by MCAO in the brains of animals subjected to MCAO and who received the Fv-Hsp70 protein (Figure 2, lane 2). Endogenous 70 kD Hsp70 was not induced in the brains of sham-operated rats (Figure 2, lane 3). Although the 110 kD Fv-Hsp70 recombinant protein was not detected, a protein of approximately 210 kD (Figure 2, lane 2) was detected in one third of the brains from the rats subjected to MCAO and who received Fv-Hsp70 protein. This protein is probably a dimer of 110 kD Fv-Hsp70 recombinant protein. Immunohistochemistry of brains of animals subjected to MCAO and who received Fv-Hsp70 demonstrated many Hsp70-positive cells (Figure 3A). These cells stained with an anti-Hsp70 antibody probably represent Fv-Hsp70 immunostained cells (Figure 3A) because the endogenous Hsp70 was suppressed by Fv-Hsp70 (Figure 2, lane 2). The Fv-Hsp70 immunostained cells included neurons and glia (Figure 3A) found in the “penumbral cortex” adjacent to the infarction (Figure 3B).

**Discussion**

The results demonstrate that intravenous administration of recombinant Fv-Hsp70 protein protects against focal cerebral ischemia. This is achieved, at least in part, by reducing cell death as measured by infarct volumes, which improves sensorimotor function compared with saline-injected control MCAO animals.

**Exogenous Fv-Hsp70 Protects Against Focal Ischemic Injury**

The mechanism by which Fv-Hsp70 recombinant protein protects against ischemic injury appears to involve exogenous Fv-Hsp70 rather than endogenous Hsp70. Hsp70 has been demonstrated to provide protection against in vivo cerebral ischemia using viral, transgenic drugs and other means of delivery.\(^1\)\(^2\)\(^3\)\(^4\)\(^6\)\(^14\)

A recent in vivo study has provided evidence that Hsp70 provides protection through its effects on apoptosis because it showed that the C-terminal portion of Hsp70 was as protective as the full-length molecule.\(^13\) This is an important observation because the N-terminal portion of Hsp70 is the ATP-binding portion that is essential for protein refolding, whereas the C-terminus is the peptide binding, antiapoptotic portion of the Hsp70 molecule.\(^15\) Because the full-length Hsp70 was used in the current study, it is possible that the refolding functions and/or the antiapoptotic functions accounted for the neuroprotection. Future studies are needed to address the alternative mechanisms.

Because the entire Fv-Hsp70 construct was neuroprotective, it is difficult to know whether the Fv portion of the construct provided any protection. We doubt Fv is protective because administration of Fv to neurons in vitro did not protect against hydrogen peroxide toxicity or against oxygen-glucose deprivation.\(^7\) However, future in vivo studies will need to compare Fv-Hsp70 with Fv alone.

**Endogenous Hsp70 Induction Is Suppressed After Fv-Hsp70 Administration**

One of the intriguing findings of this study was the inhibition of endogenous Hsp70 expression after Fv-Hsp70 protein
administration. Heat shock genes have functional heat shock elements in their promoters that are bound and activated by heat shock factor proteins (HSF, a family of transcription factors) to initiate Hsp transcription after heat shock or stress.\textsuperscript{16–18} HSF is normally bound to Hsp90 in the resting cell. With the presence of denatured proteins, Hsp90 dissociates from HSF and binds the abnormal proteins.\textsuperscript{19} The HSFs are freed, form trimers, and are activated. The activated trimers act at the heat shock element of Hsp70 to initiate Hsp70 transcription. Once Hsp70 is induced, it binds to denatured proteins to prevent further protein denaturation in collaboration with other chaperones.\textsuperscript{20}

Administration of a drug that binds Hsp90, like geldanamycin, stimulates the transcription of heat shock genes. Once Hsp70 protein is produced in large amounts, it binds to HSF in the transcriptional domains to suppress endogenous Hsp70 protein production.\textsuperscript{21} Here we show that exogenous Hsp70 suppresses the induction of endogenous Hsp70. We propose that Fv-Hsp70 binds to HSF, and prevents HSF, from binding to heat shock elements in the Hsp70 promoter, thereby preventing induction of endogenous Hsp70 mRNA and protein after MCAO. Thus, exogenous Fv-Hsp70 protein provides neuroprotection despite suppression of endogenous Hsp70 production.

We postulate that the reason for the marked protection afforded by exogenous Fv-Hsp70 relates to timing. The endogenous Hsp70 protein is not induced until approximately 4 to 6 hours after brain ischemia and is not maximal until 24 hours after ischemia. In contrast, exogenous Fv-Hsp70 protein was given at 2 1/4 and 3 hours after brain ischemia in this study. The exogenous Fv-Hsp70 likely protects because it peaks in the brain shortly after administration at 2 1/4 and 3 hours, whereas endogenous Hsp70 protection is probably not maximal until 24 hours after ischemia. It is possible that Fv-Hsp70 protection might be improved by giving it at even earlier times (eg, 1 to 2 hours). This was not tested here and would not be practical clinically in most cases, at least at the present time.

**Using Fv Fragment to Deliver Hsp70 Into the Brain**

The Fv antibody, mAb 3E10, was developed and used to deliver biologically active recombinant proteins to living cells in vitro and in vivo.\textsuperscript{22,23} These studies have demonstrated that the Fv-Hsp70 construct penetrated COS-7 cells and primary rat neurons and protected both cells from oxidative stress. These results provide evidence that Fv is capable of delivering a functional protein to neurons and indicate its potential in the development of Hsp70 protein therapy. Because we did not detect any Fv-Hsp70 in the brains of sham animals, this would suggest that despite the ability of Fv proteins to gain entry into many types of cells, it requires alterations of the blood–brain barrier for large amounts of Fv-Hsp70 to enter the brain. With an intact blood–brain barrier it is unlikely that much intravascular Fv-Hsp70 enters the normal brain based on the data here.

Of major interest is the question of how Fv gains entry into brain cells at all. Recent work by Hansen and colleagues has shown that Fv enters cells through a nucleoside salvage transporter, ENT2.\textsuperscript{24} The fact that the ENT2 transporter is highly expressed in the brain makes it possible that Fv might be particularly useful for delivery of proteins into the brain in a variety of acute neurological diseases, including stroke. In addition, the fact that very little Fv-Hsp70 entered the normal brain, but large amounts entered the ischemic brain, could suggest that the ENT2 transporter is activated by ischemia and that this leads to selective upregulation of ENT2 function and transport of nucleosides and Fv-Hsp70 into the ischemic brain. Thus, this could provide a disease and stroke-specific uptake of Fv-Hsp70 so that Fv-Hsp70 would not be taken up into normal brain and affect normal brain function but would be taken up into the ischemic brain and help it survive.

**Summary**

Intravenous administration of recombinant Fv-Hsp70 protein decreased infarct volume and improved sensorimotor function after focal cerebral ischemia. Fv-Hsp70 protein enters the ischemic but not the normal brain and suppresses endogenous Hsp70.

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**Disclosures**

None.

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