Targeted hsp70.1 Disruption Increases Infarction Volume After Focal Cerebral Ischemia in Mice

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Background and Purpose—Heat-shock proteins (HSPs) are highly conserved proteins that are induced by a variety of stresses. HSP70 is a 70-kDa HSP family known to have cytoprotective effects against various insults. The role of HSP70 in cerebral ischemia remains to be elucidated in vivo.

Methods—To investigate the effect of reduced HSP70 levels on cerebral ischemia, focal cerebral ischemia by intraluminal occlusion of the middle cerebral artery was induced in hsp70.1 knockout mice. The expressions of hsp70.1 and hsp70.3 mRNAs and HSP70 protein were determined, and infarction volumes were measured and compared.

Results—Northern blots confirmed the absence of hsp70.1 mRNA expression in the knockout mice. The mean infarction volume was significantly larger in hsp70.1 knockout mice (92.5 ± 8.3 mm³) than in the wild-type mice (59.3 ± 8.9 mm³, P<0.001). Western blots showed increased HSP70 expression in the ischemic hemisphere in both knockout and wild-type mice, but HSP70 expression levels in knockout mice were significantly lower than those in their wild-type littermates. Immunohistochemistry did not show any significant differences between the knockout and wild-type animals and showed increased HSP70 immunoreactivity in the ischemic hemisphere, with predominance in the cerebral cortex, especially in the penumbra.

Conclusions—Our results suggest that hsp70.1 plays an important role in the early protection of the brain, at least after acute focal cerebral ischemia in mice. (Stroke. 2001;32:2905-2912.)

Key Words: cytoprotection ■ heat-shock proteins ■ ischemia ■ mice
glucose or oxygen deprivation, and simulated ischemia. Moreover, in vivo studies using transgenic mice have shown that the heart is protected by HSP70 from myocardial ischemic injury. In the nervous system, it has been reported that gene therapy with HSP72 improves neuron survival after focal cerebral ischemia; however, recent studies using transgenic mice are controversial. Overexpression of HSP70 resulted in a 24-hour improvement in hippocampal neuron survival, but the overall infarct size was not affected. Another study using HSP70-transgenic mice reported that there is no relationship between infarct size and hippocampal neuron survival. Recently, Rajdev et al showed that cerebral infarction after 6 hours of ischemia is significantly lower in transgenic mice overexpressing rat HSP70 than in wild-type mice. This inconsistency may be related to differences in the level of HSP70 overexpression or the transgenic strains used. Thus, although several studies have suggested that HSP70 may protect the brain from various insults, the role of HSP70 in cerebral ischemia in vivo requires further clarification. In addition, because hsp70.1 and hsp70.3 are at different loci but encode an identical protein, it has been suggested that the 2 genes may be separately expressed in different situations or with different patterns. However, studies on this topic are limited, especially in the brain.

In the present study, we used hsp70.1 knockout mice to further investigate the neuroprotective function of HSP70 in cerebral ischemia. The present study used a middle cerebral artery (MCA) occlusion/reperfusion model in hsp70.1(−/−) mice and their wild-type littermates to investigate the role of HSP70 in cerebral ischemic injury.

Materials and Methods

Animals

We produced mice bearing a null allele of hsp70.1 by using a gene-targeting technique described previously. In brief, a murine genomic clone of the hsp70.1 locus was cloned from a λ FixII phage library prepared from 129/Sv embryonic stem (ES) cells with the use of a human hsp70 cDNA probe and then characterized by Southern blot analysis and DNA sequencing. The targeting vector contained a 7.5-kb NotI-XhoI fragment from the 5′ promoter and a regulatory region of the hsp70.1 gene as the long arm, a 1.9-kb neomycin-resistance gene, an 0.8-kb NotI-SmaI fragment derived from the hsp70.1 exon as the short arm, and a 3.4-kb fragment containing 2 copies of the herpes simplex virus thymidine kinase gene (Figure 1A). The overall strategy for hsp70.1 gene targeting was to replace the promoter and some of the coding sequences with a phosphoglycerate kinase promoter with neomycin resistance gene, an 0.8-kb NotI-Smal fragment derived from the hsp70.1 exon as the short arm, and a 3.4-kb fragment containing 2 copies of the herpes simplex virus thymidine kinase gene (Figure 1A). The overall strategy for hsp70.1 gene targeting was to replace the promoter and some of the coding sequences with a phosphoglycerate kinase promoter with neomycin resistance gene (PGK-neo) expression cassette. Ten micrograms of NotI-linearized targeting vector was electroporated into E14/BK4 ES cells, and correctly targeted clones were selected with G418 (0.2 mg/mL, Life Technologies) and FIAU (200 μmol/L, Syntex) in DMEM medium. Southern blot analysis using a 520-bp 3′ UTR genomic probe. N indicates NotI; H, HindIII; B, BamHI; X, XhoI; E, EcoRI; and TK, thymidine kinase. B, Representative Southern blot analysis of genomic DNA from hsp70.1 mouse embryonic fibroblast. Mouse embryonic fibroblasts were prepared from 13.5-day postcoital embryos. Genomic DNA was digested with BamHI, EcoRI, and HindIII and probed with the 3′ UTR fragment that is specific for hsp70.1.
biopsies. Mice were genotyped by isolating tail DNA and digesting it with BamHI for Southern blot analysis (Figure 1B). For PCR genotyping, the following 2 primer sets were used: (1) forward primer a (5'-AGGAGCTGACCCCTAA CAGC-3') and reverse primer a' (5'-GTCTGGCGGATGTCTC-3'), annealing to the deleted part of genomic sequences, and (2) forward primer b (5'-CGAGATCAGCCAGCTGTTCC-3'), located within the PGK promoter in the neomycin-resistance cassette, and reverse primer c (5'-AGGAGCTGACCCTTAA CAGC-3'), annealing to the genomic sequences in the '3' arm homologous region. A 500-bp PCR fragment was generated from the wild-type allele with primers a and a', and a 1250-bp fragment was generated from the targeted allele with primers b and c. When the hsp70.1 knockout mice were produced, the targeted disruption of hsp70.1 was found not to be lethal, because the ratio of hsp70.1 allele with primers a and a' to hsp70.1/ knockout and wild-type mice were compared by using the Mann-Whitney U test (mean±SD).

Immunohistochemistry

After the heart was perfused with 4% paraformaldehyde in PBS, the brain tissues were embedded in paraffin blocks and sectioned at 4-μm thickness. Sections, mounted on slides, were deparaffinized and exposed to 3% hydrogen peroxide in methanol (3% H2O2 ratio 3:1). For blocking, sections were incubated in 3% BSA, followed by 10% normal goat serum in PBS for 30 minutes. Anti-HSP70 antibody (1:500, HSP70 W27 catalogue No. sc-24 mouse monoclonal IgG2a, Santa Cruz Biotechnology Inc) was used in a humidified chamber at 37°C for 30 minutes. Slides were then washed 3 times with PBS, secondary antibody (DAKO Corp) was applied at 37°C for 30 minutes, and the slides were washed with 50 mmol/L Tris-HCl (pH 7.6) and treated with diaminobenzidine chromogen.

Preparation of Brain Protein Extracts and Western Blot Analysis

Mice were euthanized by cervical dislocation, and the brains were quickly removed and dissected into right and left hemispheres. These were then placed on ice in 10 vol cold homogenization buffer (50 mmol/L Tris and 120 mmol/L NaCl, pH 7.4) to which protease inhibitors (complete Mini, GIBCO) had been freshly added. The tissue was then homogenized and stored at −70°C. Protein concentrations were determined by using the Bradford method (Bio-Rad). Protein extracts from brain tissue (20 μg) were separated by SDS-PAGE. Protein separation was performed in 10% polyacrylamide-amine with 0.05% bis-acrylamide. The proteins were then transferred to cellulose membrane, and the blots were probed with anti-HSP70 antibody. Signals were detected by enhanced chemiluminescence (Supersignal, Pierce). Film autoradiograms were exposed from 1 second to 30 minutes. The films were scanned with a GS-700 imaging densitometer (Bio-Rad), and the results were quantified by using the Multi-Analyist software program (Bio-Rad). Each blot was probed for α-tubulin as an internal control to ensure equivalent protein loading and protein integrity. Relative optical densities were obtained from each mouse, and the results were compared by using the unpaired Student t test. Statistical significance was accepted at a value of P<0.05.

Preparation of Total RNA and Northern Blot Analysis

The right and left hemispheres were frozen immediately in liquid nitrogen after dissection and stored at −70°C. Total RNA was prepared by homogenizing the brain tissues in an acid guanidinium thiocyanate solution and extracted with phenol and chloroform, as previously described.27 The final RNA pellet was dissolved in diethyl pyrocarbonate–treated H2O. Ten micrograms of RNA was separated by electrophoresis on denaturing agarose gels and subsequently transferred to a nylon membrane (Amersham Pharmacia Biotech). Membranes were hybridized by using hsp70.1- or hsp70.3-specific probes labeled with [32P]dCTP (Amersham Pharmacia Bio-tech) with a random-priming DNA labeling kit (Amersham Pharmacia Biotech). The following 2 primer sets (accession No. M35021) were used to produce the following hsp70.1 mRNA-specific probes: forward primer (5'-TGCATCTGGATCTCGCTTG; start point, 2779) and reverse primer (5'-GCGTGTGACATGTCATGCA; start point, 3290). For the hsp70.3 mRNA-specific probe (accession No. M76613), the following primer sets were used: forward primer (5'-CTGCTAGGAGACATGAT; start point, 2990) and reverse primer (5'-GGCGATCGTGAATGGAA; start point, 3217). Hybridization was performed at 63°C for 20 hours in a hybridization solution containing 0.2 mol/L Na2HPO4 (pH 7.2), 7% SDS, 1% BSA, buffered 4% paraformaldehyde, and the infected and total hemispheric areas of each section were traced and measured by using an image analysis system (Image-Pro Plus, Media Cybernetics). The infarction volumes of the hsp70.1 knockout and wild-type mice were compared by using the Mann-Whitney U test (mean±SD).

Lee et al Neuroprotective Role of HSP70 2907

Identification of Genotypically Pure Lines

To confirm that the genotypically pure lines were used in the experiments, we analyzed the genotypes of C57BL/6J and Balb/c mice. DNA was extracted from individual tail biopsies by using the QIAamp DNA Mini Kit (Qiagen). PCR analysis was carried out under the following conditions. For PCR genotyping, the following 2 primer sets were used: (1) forward primer a (5'-GTCCTGGCCGATGTCTC-3') and reverse primer a' (5'-AGGAGCTGACCCTTAA CAGC-3'), annealing to the genomic sequences in the '3' arm homologous region. A 500-bp PCR fragment was generated from the wild-type allele with primers a and a', and a 1250-bp fragment was generated from the targeted allele with primers b and c. When the hsp70.1 knockout mice were produced, the targeted disruption of hsp70.1 was found not to be lethal, because the ratio of hsp70.1 allele with primers a and a' to hsp70.1/ knockout and wild-type mice were compared by using the Mann-Whitney U test (mean±SD).

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and 1 mmol/L EDTA. Autoradiography was performed with a Bio-
Imaging Analyzer BAS 1000 (FUJI Photo Film).

**Results**

Targeted deletion of the hsp70.1 gene did not produce any
remarkable effect on the natural development of the mice. In
terms of phenotype, no remarkable differences were observed
between the knockout and wild-type mice: they were all
black, and their sizes were similar. Gross morphology of the
brains from the knockout mice was indistinguishable from
that of their wild-type littermates, and the carbon black
perfusion study did not reveal any notable differences be-
tween the cerebrovascular structures of the 2 groups.

Physiological parameters were measured in 4 knockout and
4 wild-type mice, as shown in the Table. None of the
physiological parameters of the knockout and the wild-type
mice were found to be significantly different before, during,
or after the operation.

**Infarction Volume**

In the present study, most of the hsp70.1 knockout mice were
unable to survive 24 hours after a 2-hour MCA occlusion.
First, 6 hsp70.1 knockout mice that underwent 2-hour occlu-
sion and 24-hour reperfusion died from extensive brain
infarction and swelling. Even 4 hours after reperfusion, the
brain sections of the hsp70.1 knockout mice, compared with
those of their wild-type littermates, showed extended ische-
mic regions, as evidenced around the central core of the
ischemia. The infarction covered a wide area, from the basal
ganglia to the cerebral cortex, in knockout mice but was
mainly confined to the basal ganglia in wild-type mice
(Figure 2A).

The infarction volume was measured and compared by
using a transient focal ischemia model involving 2 hours of
MCA occlusion and 4 hours of reperfusion. Quantitatively,
the infarction volume in the hsp70.1 knockout mice was 30%
greater than that of their wild-type littermates (value for
knockout mice was 92.5±8.3 mm³; *P<0.001 versus wild-
type [59.3±8.9 mm³]) (Figure 2B).

**HSP70 Protein Expression**

Immunohistochemistry showed increased HSP70 immuno-
reactivity in the infarcted area in both groups (Figure 3).
HSP70 was preferentially localized to the basal ganglionic
and cortical neurons in the left hemisphere, and cortical
immunoreactivity was more relatively strong. In particular,
compared with the results of TTC staining, the cortical
immunoreactivity was denser in the penumbral areas. In
the contralateral hemisphere, both groups showed scant
HSP70 immunostaining.
Western blots showed increased HSP70 expression in the ischemic hemisphere in both wild-type and hsp70.1 knockout mice (Figure 4A and 4B). However, HSP70 expression levels in hsp70.1 knockout mice were significantly lower than those in their wild-type littermates (P<0.05). No significant differences in α-tubulin immunoreactivity were found among the different groups, underscoring the validity of using α-tubulin as an internal control. All experiments were repeated at least 6 times.

**HSP70 mRNA Expression**

In the ischemic hemispheres of wild-type mice, both hsp70.3 and hsp70.1 mRNAs were prominently expressed, as shown by Northern blot analysis (Figure 4C). However, in the hsp70.1 knockout mice, hsp70.1 mRNA expression was absent from both hemispheres, whereas hsp70.3 mRNA was significantly elevated in the left hemisphere. Interestingly, a moderate signal for hsp70.3 mRNA was detected in the contralateral nonischemic hemisphere in the wild-type group.

**Discussion**

The present study was undertaken to investigate the effect of HSP70 reduction on cerebral ischemia. The results show that reduced HSP70 protein expression increases cellular damage during acute focal cerebral ischemia in hsp70.1 knockout mice. Moreover, this is the first study that demonstrates the neuroprotective effect of HSP70 after cerebral ischemia in the hsp70.1 knockout mouse model. Our findings are consistent with previous observations that the overexpression of HSP70 confers neuroprotection against cerebral ischemia. The findings also strongly support the hypothesis that HSP70 is a cytoprotective protein within the brain and that it is at least partially responsible for cerebral protection after transient MCA occlusion.

In the present study, we used a transient focal ischemia model consisting of 2-hour MCA occlusion and 4-hour reperfusion. We used this model because the majority of hsp70.1 knockout mice were unable to survive even 24 hours after 2-hour MCA occlusion, although the hsp70.1 gene knockout in itself was not lethal. Therefore, there is a possibility that the differences observed were not due to differences in infarct volume but to differences caused by infarct evolution. However, the mean infarct volume in the wild-type littermates even at the 24-hour stage (data not shown) was significantly smaller than that in the knockout mice after the 2-hour occlusion/4-hour reperfusion, and this finding cannot be explained by differences in lesion evolution. It has been reported that after 60 minutes of focal ischemia, HSP70 synthesis increased within 4 hours, peaked at 24 hours, and persisted for up to 7 days, which suggests that HSP70 might not be fully expressed in the brains of...
that ischemic damage and density in hsp70.1 noninfarcted areas. In the present study, it was evident mRNA has been shown to be expressed in infarcted areas and infarction after permanent MCA occlusion, although observations indicating that HSP70 protein is localized in neurons ganglia. This is partially consistent with previous observations. 

Figure 4. A. Western blots revealed lower HSP70 protein levels in hsp70.1 knockout (KO) mice than in the wild-type (WT) mice, in both the ischemic and nonischemic hemispheres. The HSP70 signal in the sham-operated control group was minimal. An α-tubulin protein was used as a control for equal loading of protein. R indicates right hemisphere; L, left hemisphere. B. The level of HSP70 protein was expressed in relative optical density, as determined from Western blots (n = 6 mice each). Compared with the mean level of hsp70 in sham-operated WT mice, relative values are expressed as mean ± SD. *P<0.05 between the WT and the KO mice, analyzed by Student t test. C. Northern blots confirmed the absence of hsp70.1 mRNA in hsp70.1 KO mice, whereas hsp70.1 mRNA was detected in the ischemic (left) hemisphere in the WT mice. Expression of hsp70.3 mRNA was increased in the ischemic hemisphere of the KO mice and in both hemispheres of the WT mice but was higher in the WT mice than in the KO mice.

either the hsp70.1/−/− mice or the wild-type littermates during our study period. Thus, our investigation is limited in not examining the long-term effects of HSP70. In addition, the anti-HSP70 antibody used in the present study recognizes both HSP70 and the HSC70. This cross-reaction might influence the baseline level of HSP70 protein in sham-operated animals. However, it is well known that HSP70 protein increases greatly after stress, and the considerable increase of protein in the ischemic hemisphere was most likely due to the expression of HSP70. Because we did not directly compare the level of HSP70 with that of HSC70 in the present study, we could not identify the fraction of HSC70 in the expressed protein. Prior studies indicate that the HSC70 abundant in normal cells exhibits a slight increase after stress compared with a robust increase in the expression of HSP70 protein. In addition, the HSC70 gene was not under the influence of targeted deletion in the present study; thus, we believe that the comparison between the 2 groups under the same conditions was valid.

Our results showed prominent expression of HSP70 in endothelial cells and minimal expression within the basal ganglia. This is partially consistent with previous observations indicating that HSP70 protein is localized in neurons outside the infarced area and in endothelial cells within the infarction after permanent MCA occlusion, although hsp70 mRNA has been shown to be expressed in infarcted areas and noninfarced areas. In the present study, it was evident that HSP70 protein expression was elevated more in the cerebral cortex and in the ischemic penumbra than in the basal ganglia. Several earlier reports have also concluded that the protective effect of HSP70 occurs mainly in the ischemic penumbra. In the present study, TTC staining showed that ischemic damage and density in hsp70.1 were greater in knockout mice than in wild-type mice. Overall, the infarction-reducing effect in wild-type mice, which was prominent in the cerebral cortex, may be due to HSP70 expression. In the ischemic core, the neuroprotective effects may be limited by a translational block of hsp70 mRNA caused by severe ATP loss, because HSP70 generation is an energy-consuming process, but this possibility was not confirmed in the present study.

We measured hsp70.3 expression with hsp70.1. However, the individual functions of the 2 genes in stressful conditions are unclear. It is known that their nucleotide sequences are strikingly similar and although they are considerably different in the 3’ UTR, both genes are known to initiate transcription after stress and to produce the same HSP70 proteins. Because there is no definitive evidence that the 2 genes show different responses to stress, it is believed that they might respond differently to various stimuli. Recently, it has been suggested that the 2 genes may respond separately to a single stress. Akçetin et al reported that rphp70.2 mediates a short and sensitive transient response after ischemia/reperfusion injury in rat kidney, whereas rphp70.1 mediates the major and long-lasting protective defense mechanism. However, there has been no corresponding report regarding the nervous system. Although we did not compare the neuroprotective effects of hsp70.1 and hsp70.3, it may at least be true that the expression of hsp70.1 is important in “early” neuroprotection after focal cerebral ischemia. To confirm the role of hsp70.3 in cerebral ischemia, an hsp70.3 knockout or combined hsp70.1–70.3 knockout study would be helpful.

Northern blot analyses produced another interesting result. In the contralateral hemisphere, hsp70.3 mRNA was clearly expressed in wild-type mice but not in knockout mice. There have been a few studies reporting that focal ischemia induced
expression of the HSP70 protein in the contralateral hemisphere, but the mechanism has not been established. Some of the suggested mechanisms were elevated intracranial pressure due to edema and secondary events, such as deafferentation or transynaptic activation. A recent study reported that the synthesis of HSP70 might be an index of ongoing repair or a compensatory mechanism related to neuronal remodeling, contributing to facilitate the recovery of postischemic neurological deficits. Whatever the mechanism, it was interesting that no hsp70.3 mRNA in knockout mice and no hsp70.1 mRNA in wild-type mice was found in the contralateral hemisphere. We do not consider these results to have been caused by experimental error, because repeated analyses showed the same result in other animals. We believe that this may be associated with possible differences in expression pattern or a functional interaction between hsp70.1 and hsp70.3, but this was not investigated in the present study. Further investigation of the different responses and individual functions of hsp70.1 and hsp70.3 is needed.

In summary, our results demonstrate that ischemic damage is increased without hsp70.1 and that this is probably due to the detrimental effect of hsp70.1 deficiency on HSP70 expression. However, the long-term effects of hsp70.1 expression, which were not examined in the present study, remain to be resolved. HSP70 protein is known to play a very important role in the protection of cells against various stresses, including cerebral ischemia, but the individual functions of hsp70.1 and hsp70.3 and the molecular mechanisms of HSP70 (apoptosis block or chaperone function) are not yet understood. Further investigation is required to successfully develop novel therapeutic interventions based on HSP70 protein for stroke.

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