Effects of *Hsp70.1* Gene Knockout on the Mitochondrial Apoptotic Pathway After Focal Cerebral Ischemia

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**Background and Purpose**—Murine heat-shock protein 70 (HSP70) protein, which is produced from 2 genes, *hsp70.1* and *hsp70.3*, is known to protect the brain against ischemic injury. However, little information is available on the antiapoptotic mechanism of HSP70.1 protein after cerebral ischemia. To evaluate the role of HSP70.1 protein in ischemia, we analyzed the mitochondrial apoptotic pathway using *hsp70.1* knockout (KO) mice and their wild-type (WT) mice.

**Methods**—*hsp70.1* KO and WT mice underwent focal ischemia for 120 minutes. DNA fragmentation was evaluated by TUNEL staining. Cytochrome *c* release and the activation of caspase-3 were analyzed by Western blotting and immunohistochemistry.

**Results**—*hsp70.1* mRNA was not detected in *hsp70.1* KO mice after ischemia, and HSP70 protein expression was markedly suppressed versus WT mice. KO mice showed a significantly greater infarction volume and DNA fragmentation in the cortex than WT mice at 24 hours after ischemia. At 8 hours, cytochrome *c* release into the cytoplasm was markedly higher in KO mice than in WT mice. Caspase-3 activation was also significantly enhanced in KO mice versus WT mice, as evidenced by higher levels of activated caspase-3 and cleaved gelsolin.

**Conclusions**—These findings suggest that the deletion of the *hsp70.1* gene increases cytochrome *c* release into the cytoplasm and subsequent caspase-3 activation, thereby exacerbating apoptosis after focal cerebral ischemia. *(Stroke. 2004;35:2195-2199.)*

**Key Words:** apoptosis ■ cerebral ischemia, focal ■ heat-shock proteins ■ neuroprotection

The 70-kDa heat-shock protein (HSP70) family is a group of chaperones that assist in the folding, transport, and assembly of proteins in the cell. The mouse has 2 inducible HSP70s (*HSP70.1* and *HSP70.3*), whose genes, *hsp70.1* and *hsp70.3*, are separated by only 7 kb on chromosome 17 and show 99% homology. Although both genes initiate transcription and produce HSP70 protein, they are considerably different in the 3′ untranslated region. *HSP70* induction protects cells from lethal insults other than heat shock. The in vivo protection by HSP70 has been reported in models of myocardial or cerebral ischemia. Although these forms of protection are known to be associated with chaperone function, several reports in which a cell-free system was used have recently indicated that cytoprotection might be, at least in part, the result of an antiapoptotic mechanism. However, the biochemical cascades underlying this protective role of HSP70 are still uncertain, and the individual functions of *hsp70.1* and *hsp70.3* genes are not understood.

We previously demonstrated that a marked reduction in the expression of HSP70 induces a larger infarction volume after focal ischemia. As the next experiment, using *hsp70.1* knockout (KO) mice and their wild-type (WT) littermates, we investigated how HSP70.1 protein influences apoptotic cell death after focal cerebral ischemia and the involvement of elements of the mitochondrial pathway, such as the release of cytochrome *c*, and caspase activation.

**Materials and Methods**

**Animals**

Protocols used for the care and use of animals throughout this study were approved by the animal care committee at Seoul National University. KO mice bearing a null allele and WT controls bred on C57BL/6 background were genotyped. As described previously, *hsp70.1* KO mice were generated from 129/Sv embryonic stem cells. To avoid genetic background effects, each KO mouse strain was backcrossed to the C57BL/6 strain for 9 generations to obtain congenic animals. C57BL/6 WT mice were used as control animals. Male mice (Macrogen, Seoul, Korea) weighing 25 to 30 g were used throughout.

**Focal Ischemia Model**

Focal cerebral ischemia was induced by middle cerebral artery (MCA) occlusion in *hsp70.1* KO and WT littermates, as described
Elsewhere, MCA was blocked by threading 5-0 monofilament nylon surgical sutures. MCA occlusion was documented as a decrease in laser-Doppler signals to <20% of the control values (PeriFlux System 5000, Perimed). The monofilament was removed 120 minutes after occlusion, thereby restoring blood flow.

Infarction Volume
After cardiac perfusion-fixation with 4% paraformaldehyde, brains were cut into 30-μm-thick coronal sections on a freezing microtome (CM 3050S, Leica). A total of 7 brain sections were processed for Nissl staining. Infarction volume was measured with an image analysis program (Image-Pro Plus, Media Cybernetics). The percentage of infarction was determined by comparing the volume of Nissl staining. Infarction volume was measured with an image analysis program (Image-Pro Plus, Media Cybernetics). The percentage of infarction was determined by comparing the volume of Nissl staining. Infarction volume was measured with an image analysis program (Image-Pro Plus, Media Cybernetics). The percentage of infarction was determined by comparing the volume of Nissl staining.

Immunohistochemistry
Cryosections were incubated overnight at 4°C in mouse monoclonal HSP70 (sc-24; 1:500; Santa Cruz Biotechnology, Santa Cruz, Calif) or goat polyclonal cytochrome c (sc-8385; 1:1000; Santa Cruz Biotechnology, Santa Cruz, Calif) antibody. Slides were then incubated in biotinylated anti-mouse/anti-rabbit/anti-goat immunoglobulin G (K0690; Dako, Glostrup, Denmark). After they were washed, the sections were treated with 3,3′-diaminobenzidine tetrahydrochloride (DAB; Aldrich, St. Louis, Mo) as the chromogen. Sections were counterstained with methyl green.

Northern Blots
As described previously, total RNA was prepared by homogenizing the brain tissues in an acid guanidinium thiocyanate solution. Ten micrograms of RNA was loaded and separated by electrophoresis on denaturing agarose gels and subsequently transferred to a nylon membrane (Amersham). Membranes were hybridized with the use of hsp70.1- or hsp70.3-specific probes labeled with α-32P dCTP (Amersham) with a random-priming DNA labeling kit (Amersham).

Western Blots
Protein extraction of the subcellular fraction was performed as described previously, with modification. The homogenates were first centrifuged at 750g for 10 minutes and then at 8000g for 20 minutes; the 8000g pellets were used as the mitochondrial fraction. The supernatant was further centrifuged at 100 000g for 20 minutes, and the final supernatant was collected as the cytoplasmic fraction. Western blots were performed using antibody for cytochrome c (sc-8385; 1:500; goat polyclonal; Santa Cruz Biotechnology, Santa Cruz, Calif), cytochrome oxidase subunit IV (COX-IV) (A21348; 1 μg/mL; mouse monoclonal; Molecular Probes, Eugene, Ore), active caspase-3 (551150; 1:200; rabbit monoclonal; Pharmingen, San Diego, Calif), gelsolin (G37820; 1:1500; mouse monoclonal; Transduction Laboratories, San Diego, Calif), or HSP70 (sc-24; 1:500; mouse monoclonal; Santa Cruz Biotechnology, Santa Cruz, Calif).

In Situ Labeling of DNA Fragmentation
TUNEL was performed with the use of a commercially available kit (Oncogene) as described previously. The sections were incubated in a TdT-labeling reaction mixture for 90 minutes, colored with DAB solution, and counterstained with methyl green. According to morphological criteria, TUNEL-positive nuclei with chromatin condensation and fragmented nuclei (>2 apoptotic bodies) were considered probable apoptotic cells, and TUNEL-positive cells with diffuse light labeling of nucleus and cytoplasm were considered probable necrotic cells.

Statistical Analysis
Results are presented as mean±SD. Statistical comparisons were made with the Student t test or the Mann-Whitney U test. Mean differences were considered significant when P<0.05.

Figure 1. HSP70 mRNA and protein expression. Northern blots confirmed the absence of hsp70.1 mRNA in hsp70.1 KO mice. The expression of hsp70.3 mRNA was increased in both groups (A); hsp70.3 produces 2 transcripts, whereas hsp70.1 produces only 1 transcript. This is caused by different nucleotide composition and length in 3′ untranslated region. Western blots of HSP70 and β-actin showed strong HSP70 expression in WT mice and a very weak signal in KO mice (B). β-Actin was used as an internal control. HSP70 immunostaining with methyl green counterstaining was seen (C to F). HSP70 immunoreactivity was much lower in KO mice (C and E) than in WT mice (D and F).

Bars=50 μm.

Figure 2. Increased infarction size in hsp70.1 KO mice. Representative slices of Nissl-stained coronal brain sections 24 hours after ischemia of WT and KO mice (A) are shown. The infarction volume was significantly greater in KO mice (n=5) than in WT (n=5) mice (B). Data are mean±SD; *P<0.05.
of positively stained cells were greater in WT than in KO mice (Figure 1C to 1F). Western blots also showed that increased HSP70 expression was observed in both KO (n=4) and WT mice (n=4) 24 hours after ischemia, but its expression was markedly higher in WT mice (Figure 2B).

Nissl staining at 24 hours after ischemia (Figure 2A) showed that infarction volume was significantly greater in KO mice than in WT mice. The infarction percentages in KO mice (n=5) and in WT mice (n=5) were 39.5±2.3% and 32.2±3.5%, respectively (P<0.05; Figure 2B).

Cytochrome c protein expression was determined immunohistochemically and by Western blotting 8 hours after ischemia in both groups (n=5). Cytoplasmic immunoreactivity was observed in the ischemic core and more prominently in the adjacent cortex (Figure 3). The number of cytochrome c-immunoreactive cells in the cortex was significantly higher in hsp70.1 KO mice (132.2±9.5 cells/×200 field) than in WT mice (74.6±26.8 cells/×200 field; P<0.05). In the striatum, no obvious differences were seen between the 2 groups (KO, 108.5±21.3 cells/×200 field; WT, 92.9±32.8 cells/×200 field). Morphologically, cytochrome c immunoreactivity was detected not only in neurons but also in astrocytes, oligodendrocytes, and microglia. Cytochrome c-positive endothelial cells were also frequently observed. Cytochrome c immunobLOTS showed that levels of mitochondrial cytochrome c were similar in both groups, whereas cytoplasmic levels were significantly higher in KO mice (Figure 4A; P<0.05). The relative optical densities of cytoplasmic cytochrome c were 8.2±1.6 in KO mice (n=4) and 1.8±0.2 in WT mice (n=4) (Figure 4B).

Eight hours after ischemia, caspase-3 activation was also significantly higher in KO mice than in WT mice, as indicated by increased 17-kDa bands (Figure 5A). In addition, Western blots of gelsolin showed that 41-kDa cleavage products, characteristic of caspase-3 activation, were higher in KO mice. The relative optical density of cleaved caspase-3 was 3.7±0.1 in KO mice (n=4) and 1.2±1.3 in WT mice (n=4) (Figure 5B).

Apoptotic cells with TUNEL positivity were seen in the entire ischemic area in both groups 24 hours after ischemia (Figure 6A and 6B). Quantitative analyses (n=5 each) showed a significantly higher level of apoptotic cells in KO mice than in WT mice in the cortex (Figure 6C and 6D; Figure 6A and 6B). Significantly higher in the cortex of KO mice (A and C) than in the cortex of WT mice (B and D). Cytoplasmic cytochrome c immunoreactivity was observed in the ischemic core and more prominently in the adjacent cortex (Figure 3). The number of cytochrome c-immunoreactive cells in the cortex was significantly higher in hsp70.1 KO mice (132.2±9.5 cells/×200 field) than in WT mice (74.6±26.8 cells/×200 field; P<0.05). In the striatum, no obvious differences were seen between the 2 groups (KO, 108.5±21.3 cells/×200 field; WT, 92.9±32.8 cells/×200 field). Morphologically, cytochrome c immunoreactivity was detected not only in neurons but also in astrocytes, oligodendrocytes, and microglia. Cytochrome c-positive endothelial cells were also frequently observed. Cytochrome c immunobLOTS showed that levels of mitochondrial cytochrome c were similar in both groups, whereas cytoplasmic levels were significantly higher in KO mice (Figure 4A; P<0.05). The relative optical densities of cytoplasmic cytochrome c were 8.2±1.6 in KO mice (n=4) and 1.8±0.2 in WT mice (n=4) (Figure 4B).

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expression of HSP70 can be interpreted as a molecularly defined penumbra of protein denaturation. Our results are consistent with previous findings that showed obvious HSP70 expression mainly in the cortex, although a remarkable difference was observed in the expression intensity of hsp70.1 KO mice and WT mice. When the properties of HSP70 are considered, it is evident that HSP70 can protect neurons from protein-denaturing stimuli. In the present study hsp70.1 KO mice showed 22.7% more infarction than WT mice, and these results are in accordance with previous studies. Infarct volume increase caused by hsp70.1 gene deletion was significant, but not prominent, and this can be explained as follows. First, as mentioned above, this may be related to the mechanism of HSP70.1 protein that is mainly operated in the penumbra or the peri-infarct area rather than in the ischemic core. This phenomenon is strongly associated with limitation of HSP70 action (in regard to need for ATP) and is also associated with the experimental data showing that apoptotic cell death is most frequently identified in this area. Second, we should consider types of cell death after focal ischemia; the major type of ischemic cell death is necrosis, and apoptosis is observed in only 10% to 20%. Thus, considering these data, we believe that the protective effects of HSP70.1 protein found in this study are obviously significant.

In regard to the HSP70 cytoprotective mechanism, although a few reports have indicated the inhibition of necrosis, recent studies tend to concentrate on apoptosis, which is a dominant type of cell death in the ischemic penumbra. An in vitro study using hsp70-transfected cell lines found that HSP70 overexpression protected cells from heat shock–induced cell death by inhibiting cytochrome c release; these findings were consistent with recent data from an in vivo study using hsp70 transgenic mice in a model of focal ischemia. On the other hand, some reports showed that HSP70 may protect against apoptosis by interfering with apoptosome formation or a process downstream of caspase-3 activation. Several recent studies reported an association between HSP70 and c-Jun N-terminal kinase (JNK) and upregulation of the antiapoptotic protein Bcl-2 by gene transfer of HSP72. These disparate results regarding the antiapoptotic mechanism of HSP70 may be caused by the different species and types of stress or stress intensities used, but it is more likely that HSP70 is a potent antiapoptotic protein that can influence the apoptotic cascade at multiple sites. Our results suggest that mitochondrial cytochrome c might be a target of HSP70.1 protein action, at least after focal cerebral ischemia. Although the functions of hsp70.1 and 70.3 have not been established, the deletion of hsp70.1 leads to a remarkable reduction in the expression of HSP70 protein after focal cerebral ischemia versus WT mice. These results suggest that the hsp70.1 gene may be more important functionally in neuroprotection, particularly in apoptosis inhibition, after cerebral ischemia than the hsp70.3 gene. In addition, we found that hsp70.3 mRNA expression was also lower in KO mice than in WT mice, and this result may suggest that hsp70.1 and hsp70.3 may be associated functionally in the response to ischemic stimuli. This possibility of association has not been proposed until now. However, this hypothesis should be examined by comparing results with the use of hsp70.3 or hsp70.1-70.3 combined KO mice.

Our study demonstrates that deletion of the hsp70.1 gene remarkably suppresses the expression of HSP70 protein and increases infarction volume and apoptotic cell death after transient focal ischemia. Moreover, increased apoptosis in KO mice was associated with increased release of cytochrome c into the cytoplasm and the subsequent activation of...
caspase-3. These increased apoptotic features were more prominent in the cortex, where the extent of HSP70 protein expression was obviously upregulated. However, in this study we did not examine the caspase-independent pathway via the apoptosis-inducing factor,27 the JNK pathway,22–24 or the proteins influencing apoptotic cell death, including Bcl-2 family proteins. In this context, the in vivo mechanism of HSP70 after cerebral ischemia remains to be resolved. For the clinical applications of HSP70, these molecular mechanisms must be investigated further.

Acknowledgments

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