Targeted Disruption of Hsp110/105 Gene Protects Against Ischemic Stress

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Background and Purpose—Hsp110/105 belongs to the HSP110 heat shock protein family, which is a subgroup of the HSP70 family. In mammals, Hsp110/105 is constitutively expressed but exhibits particularly high levels in the brain. It has recently been shown that both Hsp110/105 and Hsp70 are elevated after cerebral ischemia. To study the physiological role of this protein in vivo, we generated hsp110/105 knockout (KO) mice and investigate the effect of reduced Hsp110/105 levels on focal cerebral ischemia.

Methods—hsp110/105 KO and wild-type mice were subjected to 30 minutes of transient middle cerebral artery occlusion followed by reperfusion for 24 hours. The infarct volume and neurological scores were measured and compared. The Hsp70 chaperone activity of thermally denatured firefly luciferase was measured in hsp110/105 KO embryonic fibroblasts.

Results—The infarct volume and neurological deficit scores were significantly (P<0.05) reduced in hsp110/105 KO mice compared with wild-type controls. In addition, hsp110/105 KO embryonic fibroblasts exhibited a dose-dependent suppression of Hsp70 chaperone activity by the presence of Hsp110/105.

Conclusions—These results demonstrate that hsp110/105 KO mice are resistant to ischemic injury and that the protective effects of hsp110/105 deficiency in cerebral ischemia may partly be mediated by an increase in the chaperone activity of Hsp70. (Stroke. 2008;39:2853-2859.)

Key Words: cerebral ischemia □ focal □ Hsp70/Hsc70 chaperone activity □ Hsp110/105 □ knockout □ mice

Heat shock proteins (HSPs) are classified into several families on the basis of their apparent molecular weights: HSP110, HSP90, HSP70, HSP60, HSP40, and small HSPs (25 to 28 kDa). In the brain, several studies have shown that HSPs are involved in protecting neurons from a variety of brain injuries, including ischemia, traumatic injury, and neurodegenerative diseases. The mechanism of this protective action has largely been attributed to their chaperone functions that prevent protein aggregation and assist in the refolding of denatured polypeptides.

We have previously cloned 2 members of the Hsp110/105 protein family in mice: Hsp105α and Hsp105β. Hsp105α is a 105-kDa stress protein, which is expressed constitutively and induced by a variety of stressors, whereas Hsp105β is an alternatively spliced form of Hsp105α that is specifically induced by heat shock at 42°C. Hsp105α and Hsp105β exist as complexes that are associated with Hsp70 and its cognate protein Hsc70 (Hsp70/Hsc70) in mammalian cells. The mammalian HSP110 family is known to have diverged from the HSP70 family and consists of 3 members: Hsp105/105, Apg-1/Osp94, and Apg-2/Irp94. Hsp110/105 is present in all of the cerebral neuronal regions, including those in the cerebral cortex and the hippocampus. Using a murine model of cerebral ischemia, it has been reported that not only Hsp110/105, but also Apg-1 and Apg-2/Irp94 are induced in neurons of the cerebral cortex and hippocampus either at mRNA and/or protein levels.

Hsp110/105 has been reported to suppress the chaperone activity of Hsp70/Hsc70 by inhibiting its ATPase. This is in contrast to the role of Hsp40 (Hdj-1), which has been shown to enhance the function of Hsp70/Hsc70 by stimulating nucleotide hydrolysis. The reported inhibitory function of Hsp110/105 against Hsp70 ATPase activity is controversial because a conflicting study describes that Sse1, a yeast ortholog of mammalian Hsp110/105, synergistically stimulates the yeast Hsp70 Ssa1 ATPase activity with Hsp40.
Recently, yeast Sse1 and mammalian Hsp110/105 have been reported to act as potent nucleotide exchange factors for their respective Hsp70s by accelerating the dissociation of nucleotide from Hsp70/ADP complexes.\textsuperscript{19–21} Interestingly, the effect of Hsp110/105 on the refolding cycles of denatured substrates by Hsp70 in vitro and in vivo is also controversial.\textsuperscript{17,19–21} In contrast to Hsp70, very little is known about the functions of Hsp110/105.

To address the physiological mechanism that regulates Hsp70 chaperone activity by Hsp110/105 in vivo, we generated \textit{hsp110/105} knockout (KO) mice and examined the effect of Hsp110/105 on transient focal ischemia.

**Materials and Methods**

**Gene Targeting and Generation of \textit{hsp110/105}-Deficient Mice**

Genomic clones containing the murine \textit{hsp110/105} locus were isolated from the 129/SvJ \(\lambda\)-phage genomic library by Southern blotting using probes corresponding to the murine \textit{hsp110/105} cDNA. The genomic organization of the locus was determined by subcloning portions of these genomic inserts into pBluescript II SK (+) (STRATAGENE). One of the genomic clones containing the first to seventh exons of \textit{hsp110/105} was used to construct the targeting vector. The neomycin resistance gene was flanked by the 2.4-kb SalI/Xhol 5' genomic fragment and the 5.0-kb SalI/NcoI 3' fragment. The HSV-TK negative selection cassette was intro-
duced at the 3’ end of the genomic fragment. E14.1 embryonic stem cells were electroporated with the linearized targeting vector and selected with G418 and ganciclovir. Homologous recombination in doubly resistant clones was screened by Southern blotting and polymerase chain reaction analysis. Heterozygous embryonic stem clones were microinjected into C57BL/6 blastocysts to generate chimeric mice. Chimeric male mice were mated with C57BL/6 female mice to generate heterozygous mice. Germline transmission was confirmed by Southern blotting using the probe indicated in Figure 1A. hsp110/105 KO mice were backcrossed into the C57BL/6 strain over 10 generations and obtained by heterozygous intercrosses. All experimental procedures were approved and carried out in accordance with the Institutional Animal Care and Use Committee of Kyoto University.

Focal Cerebral Ischemia Models

C57BL/6 male mice (wild-type [WT]) and hsp110/105 KO mice weighing 25 to 30 g were subjected to cerebral ischemia using the standard intraluminal middle cerebral artery occlusion method.22 Briefly, each mouse was anesthetized with 1% halothane in 30% oxygen and 70% nitrous oxide using a face mask. Rectal temperature was maintained at 37.0±0.5°C with a thermostat-controlled heating pad. After a midline skin incision, the left external carotid artery and its branches were isolated and ligated. A 8–0 nylon monofilament coated with silicon was introduced into the left internal carotid artery through the common carotid artery to occlude the origin of middle cerebral artery. After 30 minutes of occlusion, the monofilament was withdrawn and the mouse was placed in a thermally controlled incubator (32.0°C) for 2 hours before being returned to its cage. In randomly selected animals, the left femoral artery was cannulated to facilitate blood pressure monitoring (iWorx) and sampling. Blood samples were taken before, during (15 minutes after occlusion), and after the first 15 minutes of reperfusion; samples were analyzed for blood gases (Pao2 and Paco2) and pH using a blood gas analyzer (FUSO). Regional cerebral blood flow at the middle cerebral artery territory was monitored by a laser Doppler flowmetry (OMEGA-WAVE). Only mice that satisfied the criteria that the regional cerebral blood flow in the middle cerebral artery territory was reduced to <25% of the baseline during ischemia and that it recovered to >55% of the baseline during reperfusion were used in subsequent experiments. According to these criteria, 10 of 15 of WT and 11 of 15 of hsp110/105 KO mice were selected, among which one mouse in each group died during reperfusion. At 24 hours after reperfusion onset, neurological deficit scores were evaluated as described23: 0, no neurological deficit; 1, flection of torso and contralateral forepaw when lifted by the tail; 2, turning to the contralateral side; 3, circling to the contralateral side; and 4, no spontaneous walking with depressed level of consciousness.

Measurement of Infarct Volume

Each brain was sectioned (20 μm thick) at 600-μm intervals at coronal levels (+2.0, +1.4, +0.8, +0.2, −0.4, −1.0, −1.6 mm from the bregma) on a cryostat. Sections were stained with 0.1% cresyl violet to identify viable tissue. The infarct area was quantified by using image analysis software (ImageJ, Version 1.36). Infarct volumes were calculated by the integration of infarcted areas on each brain slice.

Western Blot Analysis

Proteins were extracted from the cerebral cortex. Aliquots of the lysate containing 20 μg protein were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and were subsequently transferred to a polyvinylidene difluoride membrane. The primary antibodies used were mouse anti-Hsp110/105 (1:500; Transduction Laboratories), mouse anti-Hsp90 (1:1000; StressGen), rabbit anti-Hsp70/Hsc70 (1:1000; a gift from Dr K. Ohtsuka), and mouse anti-Actin (1:1000; Chemikon). The secondary antibodies were either alkaline phosphatase-conjugated antimouse IgG (BIOSOURCE) or antirabbit IgG (BIOSOURCE). The immunoreaction was detected using 5-bromo-4-chloro-3-indolylphosphate and nitro blue tetrazolium Buffer Substrate Tablets (Sigma).

Establishment of hsp110/105 Knockout Embryonic Fibroblasts and Luciferase Refolding Assay

Primary embryonic fibroblasts (MEFs) were prepared from day 13.5 hsp110/105 KO mice embryos. The tissue was dissociated into individual cells with trypsin. The reactivation of thermally denatured luciferase was estimated as described previously.24 Briefly, the cells were transfected with firefly luciferase (Promega), human hsp70 (pCMV-luc, a gift from Dr K. Ohtsuka), mouse hsp110/105WT, and mutant hsp110/105K69A, which lacks ATP-binding activity using Lipofectamine and Plus Reagent (Invitrogen). Twenty-four hours after transfection, the cells were preincubated in a medium containing cycloheximide (20 μg/mL) for 30 minutes at 37°C to inhibit HSPs and luciferase synthesis, and subsequently luciferase was inactivated by heat shock for 15 minutes at 45°C. After the specified recovery periods at 37°C allowing the reactivation of luciferase, luciferase activity was determined with the luciferase reporter assay system (Promega).

Table. Physiological Parameters Before, During, and After Middle Cerebral Artery Occlusion*

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Occlusion</th>
<th>Reperfusion</th>
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<tr>
<td>WT</td>
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<td></td>
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<tr>
<td>pH</td>
<td>7.28±0.03</td>
<td>7.24±0.03</td>
<td>7.25±0.04</td>
</tr>
<tr>
<td>Pao2, mm Hg</td>
<td>135±7.70</td>
<td>124±3.97</td>
<td>127±2.04</td>
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<tr>
<td>Paco2, mm Hg</td>
<td>37.3±1.49</td>
<td>43.3±5.46</td>
<td>43.5±2.42</td>
</tr>
<tr>
<td>Glucose, mg/dL</td>
<td>163±17.1</td>
<td>157±28.2</td>
<td>147±23.9</td>
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<tr>
<td>MABP, mm Hg</td>
<td>95.8±4.56</td>
<td>93.8±5.80</td>
<td>90.6±0.38</td>
</tr>
<tr>
<td>hsp110/105 KO</td>
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<tr>
<td>pH</td>
<td>7.30±0.03</td>
<td>7.20±0.03</td>
<td>7.16±0.04</td>
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<tr>
<td>Pao2, mm Hg</td>
<td>134±12.1</td>
<td>149±14.8</td>
<td>120±0.39</td>
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<tr>
<td>Paco2, mm Hg</td>
<td>33.0±2.26</td>
<td>35.0±1.84</td>
<td>37.8±0.81</td>
</tr>
<tr>
<td>Glucose, mg/dL</td>
<td>161±17.2</td>
<td>164±7.33</td>
<td>158±9.25</td>
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<tr>
<td>MABP, mm Hg</td>
<td>96.6±5.98</td>
<td>92.4±4.25</td>
<td>84.5±2.88</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SEM (n=4 mice for each group). MABP indicates mean arterial blood pressure.

Statistical Analysis

The data are expressed as mean±SEM. Comparison among multiple groups was performed using an analysis of variance (Dunnett’s test), whereas comparisons between 2 groups were achieved using the Student’s t test. Differences with P<0.05 were considered statistically significant.

Results

Targeted Disruption of the hsp110/105 Gene and Generation of hsp110/105-Deficient Mice

To investigate the physiological function of Hsp110/105, we generated hsp110/105 KO mice by gene targeting. A null mutation was introduced in 129/Sv embryonic stem cells by replacing exon 1 of the hsp110/105 gene with a neomycin resistance cassette (Figure 1A). DNA from the tails of the resulting littermates was analyzed by Southern blotting (Figure 1B) and by polymerase chain reaction (data not shown). The absence of protein expression in hsp110/105 KO mice was confirmed by Western blot analysis using mouse brain lysates (Figure 1C). The hetero- and homozygous mice are viable and fertile and show no gross developmental abnor-
malities. Because Hsp110/105 expression were reported during various stages in the development of the nervous system, we anticipated that the disruption of Hsp110/105 would cause postnatal abnormalities in brain structure. However, histological and morphological analyses revealed no differences compared with their WT littermates.

We next examined the expression of Hsp90, Hsp70, and Hsc70 in the brains of hsp110/105 KO mice because overexpression of these proteins could functionally compensate for the loss of hsp110/105 expression. The constitutive expression of Hsp90 and Hsc70 did not significantly change, and the expression of inducible Hsp70 was not observed in either WT or hsp110/105 KO mice (Figure 1C). These results suggest that the loss of Hsp110/105 expression in the brain is not compensated for by increased expression of Hsp90 or Hsp70/Hsc70 under normal physiological conditions.

Hsp110/105 Deficiency Reduces Infarct Volume and Improves Neurological Scores
To generate a focal ischemia model in C57BL/6 mice, we occluded the left middle cerebral artery for 30 minutes followed by 24 hours of reperfusion. Laser Doppler flowmetry confirmed that the mean ± SEM values of regional cerebral blood flow in the middle cerebral artery territory reduced satisfactorily both in WT and hsp110/105 KO mice (16.2 ± 1.7% in WT [n = 10] and 15.9 ± 1.3% in hsp110/105 KO [n = 11] mice, expressed as a percentage of preischemic baseline). Similar levels of regional cerebral blood flow recovery were also observed after the onset of reperfusion (71.1 ± 3.4% and 73.7 ± 4.1% in WT and hsp110/105 KO mice, respectively). There were no differences in physiological parameters such as arterial blood gases and blood glucose between WT and hsp110/105 KO mice before,
during, and after reperfusion (Table). The mice were euthanized 24 hours after reperfusion and the infarct volume was evaluated by Nissl staining of the coronal brain sections (Figure 2A). hsp110/105 KO mice exhibited a smaller infarct area than WT mice in all of the 5 coronal sections (Figure 2B). We calculated the infarct volume and found that it was significantly smaller in hsp110/105 KO mice than in WT mice (Figure 2C; 5.71 \pm 1.64 and 8.21 \pm 2.88 mm³, respectively). We evaluated the severity of the neurological deficit scores of these mice 24 hours after reperfusion by quantifying the mouse behavior using a 5-point score (see “Materials and Methods”). The degree of neurological deficits in hsp110/105 KO mice was significantly less severe than that observed in WT mice (Figure 3; 0.73 \pm 0.19 and 1.40 \pm 0.22, respectively).

**Hsp110/105 Suppresses Hsp70 Chaperone Activity**

Because the disruption of Hsp110/105 caused the suppression of infarct volume and improved the neurological scores after ischemia and reperfusion, we next examined the possible effect of Hsp110/105 on the function of Hsp70, because Hsp70 is one of the major molecular chaperones involved in the protection against ischemic stresses. To examine whether Hsp110/105 affects Hsp70 chaperone activity, we transiently expressed Hsp110/105 and examined the luciferase-refolding activity in hsp110/105 KO MEFs. When firefly luciferase was expressed in the cells, and treated at high temperature, the luciferase activity was recovered through its refolding from the denatured state with the aid of the chaperone activity of Hsp70 in a dose-dependent manner during the recovery period after heat shock (Supplemental Figure I, available online at http://stroke.ahajournals.org). Under the conditions used here, Hsp70 was not induced during 2 hours recovery after heat shock (Supplemental Figure II) and we have already confirmed that Hsp110/105 itself did not exert any chaperone activity (Supplemental Figure III). The cells were also cotransfected with hsp110/105WT or hsp110/105K69A, a substitution mutant that lacks ATP binding activity and cannot interact with Hsp70 (N. Yamagishi, K. Ishihara, Y. Saito, and T. Hatayama, unpublished data, 2004). When increasing amounts of hsp110/105WT were transfected into the cells, we observed a significant decrease in Hsp70 chaperone activity (Figure 4A). In contrast to Hsp110/105WT, Hsp110/105K69A did not affect the Hsp70 chaperone activity, indicating that the effect of Hsp110/105WT was dependent on the interaction with Hsp70 (Figure 4B).

**Discussion**

In this study, we examined the function of Hsp110/105 in vivo by generating mice lacking the hsp110/105 gene. We found that the hsp110/105 gene is not a gene that is essential for mouse development. To further examine the role of Hsp110/105 under conditions of stress in vivo, hsp110/105 KO mice were subjected to transient focal cerebral ischemia. Unexpectedly, hsp110/105 KO mice were protected from ischemic injury induced by middle cerebral artery occlusion. Behavioral observation demonstrated that neurological deficits in hsp110/105 KO mice were significantly lower than those in WT mice. These data suggest that hsp110/105 KO mice are more resistant to ischemic injury than WT mice.

The induction of several classes of HSP after cerebral ischemia has been extensively investigated in animal models. In the central nervous system, HSPs are specifically induced in response to a variety of brain injuries, including focal and global ischemia, and function to protect neurons against cell death.26,27 As described for other HSPs, it has been reported that HSP110 family members are also upregulated after cerebral ischemia.12-16 Consistent with previously reported observations,13 we observed a significant upregulation of hsp110/105 and hsp70 mRNAs 24 hours after reperfusion in the ipsilateral ischemic hemisphere of WT mice (data not shown).

Because previous studies have demonstrated that Hsp110/105 exists as complexes associated with Hsp70/Hsc70 in mammalian cells,6,7 and suppresses Hsp70/Hsc70 chaperone activity by inhibiting the ATPase activity of Hsp70/Hsc70,17 we speculate that the inhibition of Hsp110/105 on Hsp70 chaperone functions may contribute to the neuroprotective effect we observed in hsp110/105 KO mice. To confirm this, we assessed the Hsp70 chaperone activity in hsp110/105 KO MEFs and found that the luciferase refolding activity was suppressed by Hsp110/105WT. Under pathological conditions such as cerebral ischemia, unfolded or misfolded proteins exposing their hydrophobic segments accumulate in the affected neurons and these denatured proteins are prone to aggregate, which subsequently cause cytotoxicity.28,29 To avoid aggregation within the cells, abnormal proteins are either stimulated to refold by the Hsp70/Hsp40 chaperone system or are quickly degraded by the ubiquitin/proteasome system.30 We propose that Hsp110/105 functions as a negative regulator of Hsp70 both in vitro and in vivo. Although the studies presented previously17 and here favor the notion that Hsp110/105 inhibits Hsp70 chaperone activity by inhibiting the hydrolysis of ATP bound to Hsp70, Hsp110/105 and yeast homolog Sse1 have been recently shown to possess...
nucleotide exchange factor activity.19–21 Further studies will be needed to address the precise mechanism as to whether Hsp110/105 inhibits or promotes the Hsp70/Hsp40 refolding cycle.

It is also reported that Hsp70 protects neurons through antiapoptotic mechanisms.30 Hsp70 blocks the apoptosis death cascade at several different levels. The ischemic insult (30 minutes of middle cerebral artery occlusion) used in this study is mild, which is known to induce cell death limited to ischemic penumbra mainly through the induction of apoptosis. Therefore, it is also possible to expect that neuroprotective effect in hsp110/105 KO mice is caused by the enhancement of Hsp70 antiapoptotic function in the absence of Hsp110/105, although it is not examined whether Hsp110/105 affects antiapoptotic function of Hsp70.

In conclusion, we have shown that an hsp110/105 gene deficiency confers resistance against ischemic brain injury in mice. An increase in the Hsp70 chaperone activity detected in hsp110/105 KO MEFs may contribute to the neuronal protection in these hsp110/105 KO mice. These results suggest that Hsp110/105 could be a therapeutic target for stroke.

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Disclosures
None.

References


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