Reduction of Caspase-8 and -9 Cleavage Is Associated With Increased c-FLIP and Increased Binding of Apaf-1 and Hsp70 After Neonatal Hypoxic/Ischemic Injury in Mice Overexpressing Hsp70

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Background and Purpose—Caspase-8 and caspase-9 are essential proteases of the extrinsic and intrinsic apoptotic pathways, respectively. We investigated whether neuroprotection associated with overexpression of heat-shock protein 70 (Hsp70), a natural cellular antiapoptotic protein, is mediated by caspase-8 and caspase-9 signaling in the neonatal mouse brain after hypoxia/ischemia (H/I) injury.

Methods—Postnatal day 7 transgenic mice overexpressing rat Hsp70 (Hsp70 Tg) and their wild-type (Wt) littermates underwent unilateral common carotid artery ligation followed by 30 minutes of exposure to 8% O₂. The expression of apoptotic proteins was quantified by Western blot analysis, and the specific interaction between Hsp70 and apoptotic protease activating factor 1 (Apaf-1) was determined by coimmunoprecipitation.

Results—Hsp70 overexpression reduced cytosolic translocation of cytochrome c without affecting the levels of Apaf-1 and pro–caspase-9 24 hours after H/I. The expression of these apoptotic proteins in the naïve neonatal brains was also not affected by Hsp70 overexpression. Reduced caspase-9 cleavage occurred in Hsp70 Tg mice compared with Wt littermates 24 hours after H/I and correlated with increased binding of Hsp70 and Apaf-1. Increased cellular Fas-associated death domain–like interleukin-1β–converting enzyme inhibitory protein (FLIP) expression and decreased caspase-8 cleavage were also observed in Hsp70 Tg compared with Wt mice 24 hours after H/I.

Conclusions—Our results suggest that the extrinsic and intrinsic apoptotic pathways mediate the neuroprotective effects of Hsp70 overexpression in neonatal H/I, specifically by upregulating FLIP and sequestering Apaf-1, leading to reduced cleavage of caspase-8 and caspase-9. (Stroke. 2006;37:507-512.)

Key Word: apoptosis • mitochondria • stress proteins

Brain injury caused by hypoxia/ischemia (H/I) in the prenatal or perinatal period affects central nervous system development and leads to neurological morbidity later in life.1 Clinical and experimental studies have revealed that outcomes and mortality after acute brain injury are age dependent, with more severe responses in infants than in adults.2 Such differences in response to injury may be explained in part by differential susceptibility to apoptosis.3 Apoptosis not only plays an essential role in normal brain development4 but is also thought to be one of the contributors to secondary neuronal loss attributable to cerebral ischemia, including neonatal H/I.5

In mammalian systems, the 2 major pathways involved in the initiation of apoptosis, namely the “extrinsic” death receptor pathway and the “intrinsic” mitochondrial pathway, converge on a family of caspases.6 In the intrinsic pathway of apoptosis, which results from alterations at the level of the mitochondria and activation of the apoptosome, release of mitochondrial cytochrome c into the cytosol initiates caspase cascade activation.6,7 After being released into the cytosol, cytochrome c binds to apoptotic protease activating factor 1 (Apaf-1) in the presence of ATP/dATP, promoting the oligomerization of Apaf-1. Concurrently or subsequently, this complex recruits pro–caspase-9, forming a complex called the apoptosome.7 Assembly of the apoptosome allows pro–caspase-9 to be autoactivated, and this is followed by the recruitment and activation of pro–caspase-3. Cleaved caspase-9 remains bound to the apoptosome, which recruits and activates...
executioner caspases such as caspase-3 and caspase-7.7 Caspase-3 cleaves the inhibitor of caspase-activated deoxyribonuclease (DNase) and activates DNase, leading to DNA fragmentation.8 Alternatively, the extrinsic pathway is driven by activation of plasma membrane death receptors and activation of caspase-8. Both pathways converge on caspase-3, and cross-talk between pathways has been described.

Heat-shock protein 70 (Hsp70) joins the Bcl-2 family and inhibitors of apoptosis to form natural cellular inhibitors of caspases. Hsp70 has been shown to provide neuroprotection from cerebral ischemia in animal and cell-culture models of stroke.8 Hsp70 is also known to act as a molecular chaperone protein that antagonizes apoptosis by binding to apoptosis-inducing factor (AIF)9 or preventing the formation of the apoptosome by binding to Apaf-1 and blocking the activation of caspase-9.10,11 Despite recent advances, the antiapoptotic mechanism of Hsp70 in vivo is still not completely understood, particularly in the neonatal central nervous system. Previously, we described the specific interaction between Hsp70 and AIF after neonatal H/I insult in a caspase-independent fashion.12 In this study, we determine the neuroprotective role of Hsp70 in mediating caspase-8 and caspase-9 activation and interaction with the extrinsic and intrinsic pathway in mice overexpressing Hsp70 in a model of neonatal H/I injury.

Materials and Methods

Animals and Animal Care
Mice were housed and cared for according to the guidelines issued by the National Institutes of Health and the local institutional animal care and use committee. Transgenic mice overexpressing rat Hsp70 (Hsp70 Tg) and transgenic negative wild-type (Wt) littermates were obtained from Dr W.H. Dillmann (University of California, San Diego), and genotyped as described previously.12,13 There were no apparent phenotypic differences between Hsp70 Tg and Wt mice.

Neonatal H/I Model
The Rice-Vannucci neonatal adaptation of the Levine procedure was used to induce H/I injury.14 Postnatal day 7 Hsp70 Tg and Wt mice weighing 4 to 5 g were subjected to right common carotid artery occlusion and then exposed to 8% humidified O2 and balanced N2 for 3 minutes as described previously.12,13 These results suggest that the expression of Apaf-1 is constant in the neonatal mouse brain after H/I injury. The level of caspase-9 expression decreased after H/I in a time-dependent manner in Hsp70 Tg and Wt mice, but the change was not statistically significant (Figure 1B). There was no significant difference in the level of cytosolic cytochrome c was detected in Wt mice at 24 and 48 hours after H/I compared with control mice (P=0.89). However, substantially less cytochrome c translocation from the mitochondria to the cytosol was observed in Hsp70 Tg mice 24 hours after H/I compared with Wt mice (P<0.05; Figure 1C). There was no significant difference in the level of pro–caspase-9 between Hsp70 Tg and Wt mice at any time point (P=0.05). A significant difference in the level of cytosolic cytochrome c was also seen between Hsp70 Tg and Wt mice 24 hours after H/I. Robust COX signals were detected in the mitochondrial fractions (Figure 1C) from Hsp70 and Wt mice but not from the cytosolic fractions (data not shown), indicating proper subcellular fractionation without cross-contamination. No significant differences in cytosolic cytochrome c, Apaf-1, and pro–caspase-9 expression were observed in the naïve controls between Hsp70 Tg and Wt mice (Figure 1A through 1C), suggesting that germline Hsp70 overexpression has no effect on baseline expression of apoptosome components attributable to a compensatory effect.

Immunohistochemistry
Five days after H/I, pups were deeply anesthetized and transcardially perfused with 4% paraformaldehyde as described previously.12 Serial 30-mm coronal sections were cut with a cryostat. Immunohistochemistry was performed to evaluate the spatial expression pattern of cleaved caspase-9 after H/I by using rabbit anticleaved caspase-9 (1:10000; Cell Signaling Technology Inc.). Biotinylated goat anti-rabbit Ig (1:200; Amersham Life Science) were used as secondary antibodies and visualized as described previously.12

Subcellular Fractionation and Western Blot Analysis
To investigate the differential changes in apoptosome assembly after H/I, subcellular fractionation and Western blotting were performed on lysates from 34 Hsp70 Tg and 30 Wt mice subjected to H/I injury and compared with results from 6 naïve mice. Protein samples for Western blot analysis were prepared from injured hemispheres 6, 12, 24, and 48 hours after H/I.12 Cytosolic, mitochondrial, and membrane fractions were obtained by serial centrifugation.12,13 Protein concentrations were determined by using the Bradford protein assay (Bio-Rad). Ten micrograms of protein were loaded in each lane and blotted with primary mouse anti-Hsp70 (1:2000; Stressgen); rabbit anti–Fas-associated death domain–like interleukin-1β–converting enzyme inhibitory protein (FLIP; 1:100), and rabbit anti–Fas death receptor (FDR; 1:2000; Santa Cruz Biotechnology); rabbit anti–caspase-9 and rabbit anticleaved caspase-9 (1:10000; Cell Signaling Technology Inc.); and rat anti–Apaf-1 (1:10000; Chemicon). β-Actin (1:10000; Sigma) and mouse anti-cytochrome oxidase subunit IV (COX IV; 0.2 mg/mL; Molecular Probes) were used as internal controls for the cytosolic and mitochondrial fraction, respectively. Alternatively, equal loading was validated by quantification of Coomassie-stained gels by using optical densitometry. The signals were scanned and optical density quantified as described previously.12

Statistical Analyses
Data were expressed as mean±SD and evaluated by ANOVA, followed by Tukey-Kramer post hoc tests when appropriate. P values <0.05 were considered statistically significant. Statistical analyses were performed with StatView (Version 5.0.1; SAS Institute Inc.).
Hsp70 Overexpression Reduces Caspase-9 Cleavage After H/I

The expression of cleaved caspase-9 peaked at 24 hours after H/I and then decreased at 48 hours in Wt mice. There was significantly more cleaved caspase-9 in Wt mice 24 hours after H/I compared with naïve control Wt mice \((P<0.05)\) in contrast to the mild increase seen in Hsp70 Tg mice exposed to H/I compared with naïve control Hsp70 Tg mice (Figure 2A and 2B). Significantly less cleaved caspase-9 was detected 24 hours after H/I in Hsp70 Tg than Wt mice \((P<0.05)\). Corroborating results also demonstrated a significant reduction in the number of cells immunoreactive to antibodies against cleaved caspase-9 in the Hsp70 Tg mice compared with Wt mice at 24 hours after H/I (Figure 2C).

More Hsp70 Coimmunoprecipitated With Apaf-1 I in Hsp70 Tg Mice After H/I Compared With Wt Mice

Coimmunoprecipitation was performed to investigate the physical interaction between Hsp70 and Apaf-1 after H/I insult. Coimmunoprecipitation using an antibody against Apaf-1 brought down significantly more Hsp70 protein from lysates of the injured hemispheres of Hsp70 Tg mice than Wt mice 24 hours after H/I \((P<0.05;\) Figure 3).

Marked Inhibition of FDR Signaling in Hsp70 Tg Mice

Wt and Hsp70 Tg mice expressed substantial amounts of FDR protein at baseline and after H/I, and there were no differences in the expression of the FDR protein at any time point measured (Figure 4A). In contrast, there were marked differences in the expression of the short (S; 28 kDa) and long (L; 55 kDa) isoforms of FLIP. A minimal amount of FLIP-L was expressed at baseline followed by a mild induction of both isoforms 6 hours after H/I in Wt and Hsp70 Tg mice. Expression of FLIP-L dissipated by 12 hours after H/I in Wt and Hsp70 Tg mice, but recovered with robust expression in the brains of Hsp70 Tg mice 24 hours after H/I. No FLIP-L was detectable in Wt animals at 24 hours after H/I (Figure 4B). A similar pattern was seen in the expression of FLIP-S.
The robust expression of FLIP at 24 hours after H/I was associated with a decrease in the expression of 2 of the cleaved forms of caspase-8 in Hsp70 Tg mice (18/20 kDa: Wt 0.55/H11006 0.14, Tg 0.3/H11006 0.04; 33/kDa: Wt 3.2/H11006 2.7, Tg 1.0/H11006 0.016; optical density; Figure 4B).

**Figure 2.** Reduced caspase-9 cleavage in Hsp70 Tg mice after hypoxic and ischemic insult. A, Representative Western blots showing the expression of cleaved caspase-9 in the cytosolic fraction after 6, 12, 24, and 48 hours after H/I in Hsp70 Tg and Wt mice. B, In Wt mice, significantly increased caspase-9 cleavage was observed 24 hours after H/I compared with the controls (P<0.05). Significantly reduced cleavage of caspase-9 was seen in Hsp70 Tg mice compared with Wt mice 24 hours after H/I (P<0.05). β-Actin was used as an internal control for cytosolic protein concentration. C, Representative photomicrographs showing reduced immunoreactivity to antibodies against cleaved caspase-9 at 24 hours after H/I in the Hsp70 Tg mice compared with Wt mice in regions indicated.

**Discussion**

Our findings show that the neuroprotection from H/I injury associated with Hsp70 overexpression in the neonatal mouse brain is related to the reduction in mitochondrial cytochrome c release and caspase-9 cleavage attributable to an increased binding of Hsp70 to Apaf-1. Hsp70 overexpression also reduced cleaved caspase-8, possibly by increasing the expression of FLIP. To our knowledge, this is the first report to show that intrinsic and extrinsic apoptotic pathways are involved in mediating the neuroprotective effects against H/I injury of Hsp70 overexpression in the neonatal mouse brain.

The neuroprotective effect of Hsp70 overexpression after neonatal H/I injury is probably related to high levels of constitutive Hsp70 protein before insult in the Hsp70 Tg mice because in a previous study of ours, a robust induction of Hsp70 observed in Wt mice 6 hours after H/I did not effectively protect the brain from H/I injury. The neuroprotection observed in Hsp70 mice was not attributed to variance in cerebral blood flow or body temperature because there was no significant difference in cortical cerebral blood flow and rectal temperature between the Hsp70 Tg and Wt mice at any given time during the investigation.

Among the major apoptotic pathways identified, Apaf-1 and caspase-9 play an essential role during development.4,16
The majority of Apaf-1 or caspase-9 knockout mice die perinatally and have a markedly enlarged brain or cerebellum, craniofacial malformations, persistence of the interdigital webs, and alterations of the eye, all caused by reduced apoptosis during brain development.17–19 Constitutive amounts of cleaved caspase-9 detected in the brains of postnatal day 7 neonatal mice not subjected to H/I suggest an ongoing process of apoptosis in normal development (Figure 2). In the present study, we observed more Hsp70 bound to Apaf-1 in Hsp70 Tg mice compared with Wt mice 24 hours after H/I (Figure 3). Apaf-1 is known to bind to pro–caspase-9 via the caspase recruitment domain of Apaf-1.20 A recent study revealed that Hsp70 inhibits oligomerization of Apaf-1 and association of Apaf-1 with pro–caspase-9 by competing for the caspase recruitment domain binding domain.10 Considering these results, the direct binding of Hsp70 to Apaf-1 may be one of the mechanisms that reduce caspase-9 cleavage, which leads to reduced apoptosis. Hsp70 can interfere with apoptosis at several other points in the death cascade in lieu of blocking Apaf-1 formation, including antagonizing AIF12 and increasing Bcl-2 expression.21–23

The fact that Fas is upregulated after H/I in rats24 and neonatal mice lacking FDR are resistant to H/I brain injury15 suggests the involvement of the extrinsic pathway of apoptosis in neonatal H/I injury. Although we did not observe changes in FDR expression after H/I in either Hsp70 Tg or Wt mice, we detected a biphasic response of c-FLIP (Figure 4), a Fas-mediated signal transduction protein, similar to the finding reported after traumatic brain injury.25 c-FLIP was increased initially then decreased to control level at 12 hours, increasing robustly again at 24 hours after H/I in the Hsp70 Tg mice. FLIP acts as an endogenous cytoplasmic decoy26 for caspase-8, competitively inhibiting its binding to Fas-associated protein death domain and its subsequent autocatalytic cleavage to active forms. Decreased expression of the fully active 20/18 kDa caspase-8 24 hours after H/I in Hsp70 Tg mice compared with Wt mice suggests a dominant-negative effect of FLIP in the present model (Figure 4). Reduction of activated caspase-8 might lead to reduced Bid cleavage and subsequently reduced mitochondria-dependent apoptosis.

Summary
In conclusion, our results suggest that the extrinsic and intrinsic apoptotic pathways mediate the neuroprotective effects of Hsp70 overexpression in neonatal H/I. Specifically, there is marked expression of the caspase-8 decoy protein FLIP in mice overexpressing Hsp70, and this correlates with decreased expression of the active cleaved form of caspase-8. In addition to the reduction of cytochrome c release, Apaf-1 is sequestered and downstream caspase-9 cleavage decreased in Hsp70 overexpressors after neonatal H/I.

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