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Heat Shock Protein 70 Protects the Neonatal Brains Against Ischemic Stress

Yasuhiko Matsumori, Takamasa Kayama, Yasuaki Kokubo, Daisuke Tsuchiya

Department of Neurosurgery, Yamagata University School of Medicine, Yamagata, Japan

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ABSTRACT

Apoptosis is implicated in neonatal brain injury among various forms of cell death. Here we show that over-expression of heat shock protein (Hsp) 70, an anti-apoptotic protein, protects the neonatal brain from hypoxic/ischemic (H/I) injury and the pathways involved in the protection. In our study, postnatal day 7 (P7) transgenic mice over-expressing rat Hsp70 (Tg) and their wild type littermates (Wt) underwent unilateral common carotid artery ligation followed by 30 min exposure to 8% O_2. Significant neuroprotection was observed in Tg versus Wt mice on P12, correlating with high level of constitutive but not inducible Hsp70 in the Tg. Western blot analysis showed that translocation of cytochrome c, but not apoptosis-inducing factor (AIF), from mitochondria into cytosol was significantly reduced in Tg 24 hrs after H/I compared to Wt mice. Reduced caspase-9 cleavage was also observed in Hsp70 Tg mice compared to Wt littermates 24 hrs after H/I. Co-immunoprecipitation detected more Hsp70 bound to Apaf-1 and AIF in Tg than Wt mice 24 hrs after H/I, inversely correlating with the amount of nuclear but not cytosolic AIF translocation.

In conclusion, Hsp70 could suppress the activation of caspase-9 by reducing cytochrome c release via mitochondria and directly interacting with Apaf-1 in the apoptotic pathway during neonatal H/I injury. Also interaction between Hsp70 and AIF might have reduced downstream events leading to cell death, including the reduction of nuclear AIF translocation in the neonatal brains of Hsp70 Tg mice after H/I. We hope that those findings may help identify new potential targets for anti-ischemic therapy.

Key word : Heat shock protein 70, hypoxic/ischemia, apoptosis

Address for Correspondence : Takamasa Kayama, Department of Neurosurgery, Yamagata University School of Medicine, 2-2-2 Iida-Nishi, Yamagata 990-9585, Japan

INTRODUCTION

Brain damage by anoxia and/or reduced cerebral blood flow in the prenatal and perinatal period affects central nervous system development and leads to neurological morbidity, including epilepsy, cerebral palsy, and mental retardation later in life [40),43)]. Clinical and experimental studies revealed that outocomes and mortality after acute brain injury are age dependent, with more severe responses in infants than in adults [2),6),18),23)]. Such differences in responses to injury may be explained, in part, by differential susceptibility to apoptosis [5),13),28)].

Apoptosis is thought to be one of the contributors to secondary neuronal loss due to cerebral ischemia, including neonatal hypoxic/ischemic (H/I) injury, and many other acute and chronic neurodegenerative processes [4),7),11),21),24),30),43)], even though neuronal apoptosis plays an essential role during normal development in many brain regions [15),16),24),25)].

One of the well characterized morphological features of apoptosis is caused by the activation of caspases. In the intrinsic pathway of apoptosis resulting from alterations at the level of the mitochondria and activation of the apoptosome, mitochondrial cytochrome c release into cytosol initiates caspase cascade activation [19),20),38)] (Fig.1). After being released into cytosol, cytochrome c binds to apoptotic protease activating factor-1 (Apaf-1) in the presence of ATP/dATP, promoting the oligomerization of Apaf-1 itself. Concurrently or subsequently, this complex recruits procaspase-9, forming the complex called apoptosome [3),19)]. The apoptosome assembly allows procaspase-9 to be autoactivated, and this is followed by the

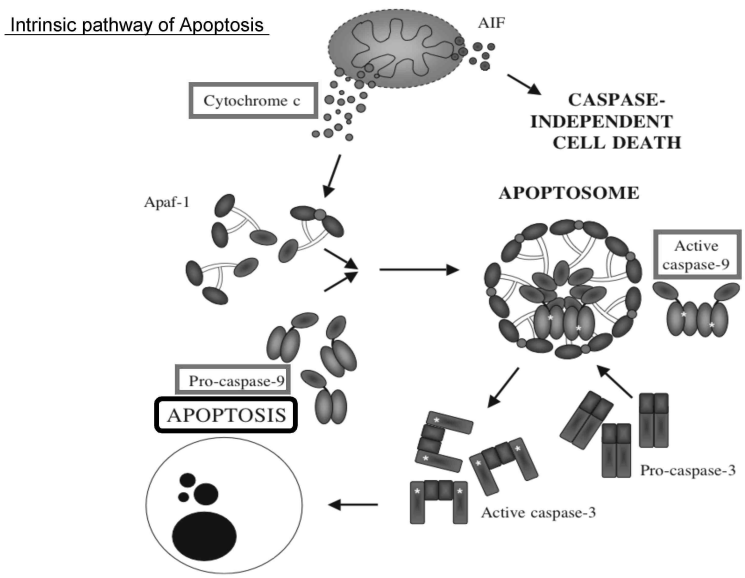

Fig. 1.
Intrinsic pathway of apoptosis. Cytochrome c released from mitochondria promotes assembly of the apoptosome. Binding of cytochrome c to Apaf-1 promotes oligomerization of the latter and recruitment of caspase-9 into a multimeric Apaf-1-caspase-9 complex that results in csapase-9, -3 activation. Active caspase-3 induces the apoptosis.

recruitment and activation of procaspase-3. Mature caspase-9 remains bound to the apoptosome which recruits and activates executioner caspases such as caspase-3 and-7 [1]. Caspase-3 cleaves the inhibitor of caspase-activated deoxyribonuclease and activates DNase, leading to DNA fragmentation[37]. A caspase-independent apoptotic pathway has also been identified in experimental models of stroke[8),9),27),29),45)], including the activation of apoptosis-inducing factor (AIF). Like cytochrome c, AIF is normally confined to the mitochondrial intermembrane and translocates into the nucleus following the induction of apoptosis. Nuclear import of AIF triggers caspase-independent nuclear changes, including large-scale (~50kd) DNA fragmentation and peripheral chromatin condensation.

Heat shock protein (Hsp) 70 joins Bcl-2 family members and the IAP to form natural cellular inhibitors of caspases. Hsp70 has been shown to provide neuroprotection from cerebral ischemia both in animal models of stroke[12),31),39),44)] and in cell culture models[14),17),22),26),34)]. Hsp70 is also known to act as a molecular chaperone protein that antagonizes apoptosis[10)]. Recent studies have revealed anti-apoptotic effect of Hsp70 in vitro, inhibiting the chromatin condensation in a caspase-independent apoptotic pathway by binding to apoptosis-inducing factor (AIF)[32)], and preventing the formation of the apoptosome by blocking the activation of caspase-9 due to binding Apaf-1[3),35)].

Despite recent advances, the anti-apoptotic mechanism of Hsp70 in vivo is still not completely understood, particularly in the neonatal CNS. This review will show our experimental results using Hsp70 overexpression transgenic neonatal mice with H/I injury model and focus on our recently identified cell-protective antiapoptotic functions of Hsp70.

Overexpression of Hsp70 reduced brain injury and apoptotic pathway after H/I injury on neonatal mice

Hsp70 overexpression transgenic neonatal mice were used with unilateral common carotid artery occlusion following exposure to 8% oxgen for 30min on postnatal day 7 (P7), which called Rice-Vannucci model[33)].

Hsp70 reduces H/I induced brain injury

Brain injury was determined in H&E stained sections by using a 0-24 brain damage scoring system described previously[36)] on 5 days after H/I (Fig. 2A). The brain damage score was significantly lower in Hsp70 Tg as compared with Wt mice at both 5 d and 14 d after H/I insult ($p<0.001$); The median brain damage scores on P12 and P21 were 7 (n=39) and 8 (n=15) in Hsp70 Tg versus 21 (n=32) and 22 (n=18) in Wt mice, respectively (Fig. 2B).

Hsp70 overexpression reduces cytosolic cytochrome c without affecting Apaf-1 and procaspase-9 expression

To investigate if overexpression of Hsp70 has an effect on the formation of apoptosome after H/I in neonatal mice brain, differential fractionation and western blots were performed on lysates of the cytosol and the mithochondria from 34 of Hsp70 Tg and 30 of Wt mice at 6, 12, 24 and 48 hr after H/I compared with naïve mice.

Cytochrome c translocation from mitochondria to cytosol occurred as early as 6 hr after H/I in both Hsp70 Tg and Wt mice (Fig. 3A). More cytosolic cytochrome c was detected in Wt mice in a time-dependent manner after H/I,

whereas substantially smaller increase in cytosolic cytochrome c translocation was observed in Hsp70 Tg mice at 12, 24 and 48 hr after H/I. In Wt mice, significantly more cytochrome c expression in cytosol was seen 24 and 48 hr after H/I compared with control ($p<0.05$). Significant difference in the level of cytosolic cytochrome c was also seen at 24 hr after H/I between Hsp70 Tg and Wt mice ($p<0.05$).

Expression of Apaf-1 did not change at any given time after H/I during our investigation in the brain extracts of either Hsp70 or Wt mice (Fig. 3B). These results suggest that the expression of Apaf-1 is constant in neonatal mice brains during normal condition as well as after H/I.

There was a trend that the level of procaspase-9 expression was decreasing after H/I in a time dependent manner, but the change was not significant (Fig. 3C). There was also no significant difference in the level of caspase-9 between Hsp70 Tg and Wt mice at any given time points.

Reduction of caspase-9 cleavage in Hsp70 Tg after H/I

To determine how the changes in the protein components of apoptosome affected further down stream apoptotic events, we investigated the cleavage status of caspase-9 using western blot analysis from 6 to 48 hr after H/I. In the western blots, robust expression of cleaved caspase-9 was seen in Wt mice after H/I (Fig. 4A&B). The expression of cleaved caspase-9 peaked at 24 hr after H/I and then decreased again at 48 hr in Wt mice. There was a significantly difference in the amount of cleaved caspase-9 at 24 hr after H/I compared with control in the Wt mice ($p<0.05$), in contrast to the gentle increase seen in Hsp70 Tg. Significantly less cleaved caspase-9 was observed at 24 hr after H/I between Hsp70 Tg and Wt mice ($p<0.05$).

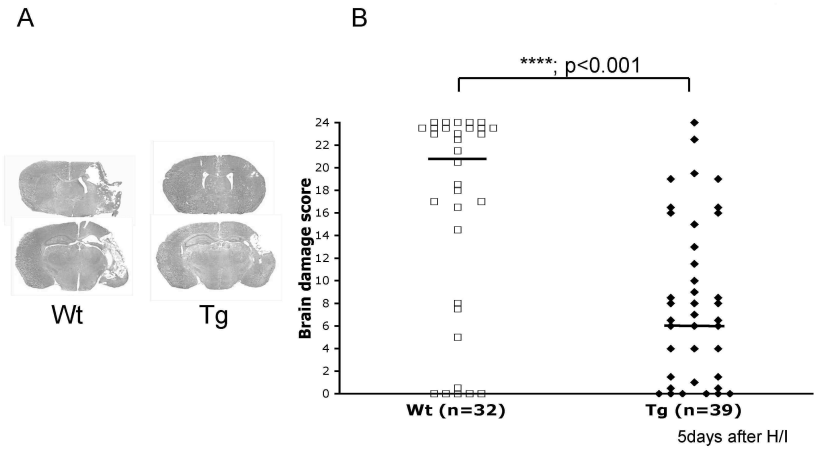

Fig. 2.
(A) Representative photographs of coronal sections, with hematoxylin and eosin staining, after hypoxic/ischemic (H/I) injury in Hsp70 Tg mice and Wt mice. (B) Reduced brain damage in Hsp70 Tg mice as compared with Wt mice 5 days after H/I injury. Significantly less brain damage was detected in Hsp70 Tg than in Wt mice after H/I insult (****; $p<0.001$).

Increased binding of hsp70 and Apaf-1

Co-immunoprecipitation was performed to investigate the physical interaction between Hsp70 and Apaf-1 after H/I insult by using cytosolic lysates from the injured and uninjured hemispheres of both Hsp70 Tg and Wt mice (n=6 each). Co-immunoprecipitation using an antibody against Apaf-1 brought down significantly more Hsp70 protein from the injured hemispheres of Hsp70Tg than Wt mice at 24 hr after H/I ($p<0.05$) (Fig. 5). There was also more Hsp70 bound to Apaf-1 in the injured than uninjured hemispheres in both the Tg and Wt mice (data not shown).

AIF translocation into the cytosol

AIF translocation to the cytosol began as early as 6 hr after H/I with significant amount of AIF found in the cytosol in both genotypes. The amount of AIF in the cytosol was significantly increased at 12 and 24 hr after H/I in both Tg and Wt mice as compared to the uninjured controls (Fig. 6A). There was no significant difference in the amount of

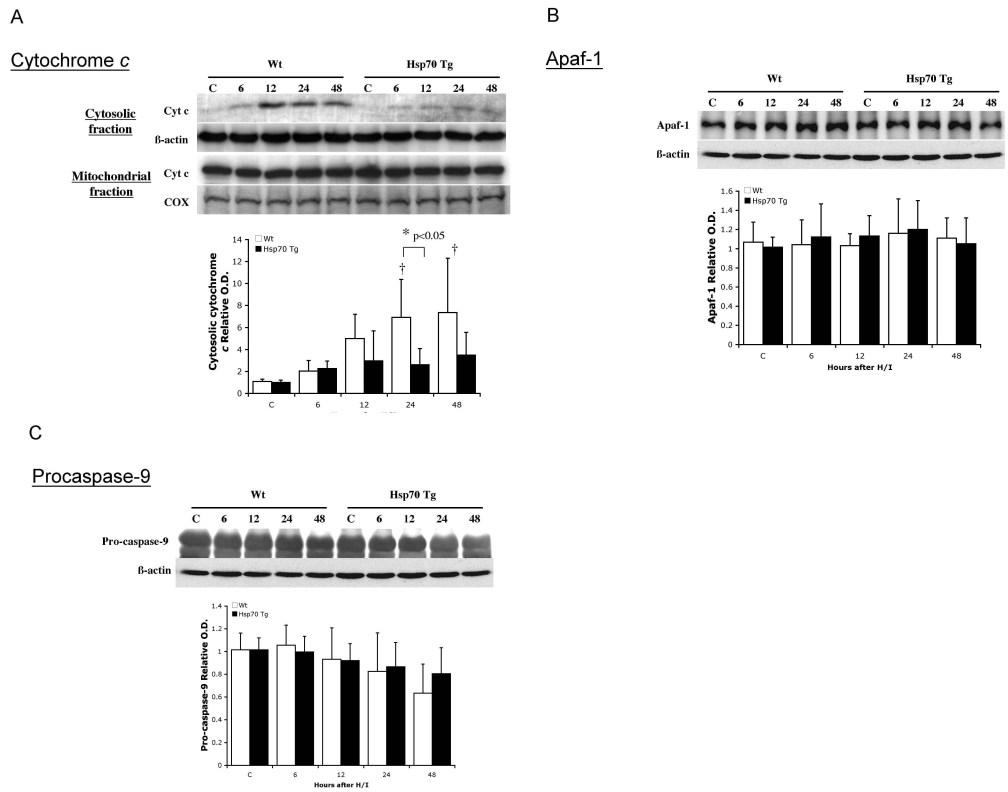

Fig. 3.
(A) Significant increase in cytochrome c translocation from mitochondria to cytosol in Wt mice 24 and 48 hr after H/I compared to control (†: $p<0.05$). Significantly reduced cytochrome c translocation to cytosol was observed in Hsp70 Tg mice as compared with Wt mice at 24 hr after H/I, (*: $p<0.05$).
(B) The expression of Apaf-1 in cytosolic fraction was not affected by H/I at any time point in both Hsp70 Tg and Wt mice. (C) The expression of procaspase-9 in cytosolic fraction was gradually decreased in a time-dependent manner, but not significantly, after H/I in both Hsp70 Tg and Wt mice. β-action was used as an internal control for cytosolic protein concentration.

cytosolic AIF between the 2 genotypes at any time point investigated. These results suggest that over-expression of Hsp70 does not affect AIF translocation from mitochondria into cytosol.

Increased binding of Hsp70 and AIF

Co-immunoprecipitation was performed to investigate the physical interaction between Hsp70 and AIF after H/I insult by using whole cell lysates from the injured and uninjured hemispheres of both Hsp70 Tg (n=5) and Wt (n=4) mice. Immunoprecipitation using an antibody against AIF brought down significantly more Hsp70 protein from the injured hemispheres of Tg than Wt mice at 24 hr after H/I ($p<0.05$) (Fig. 6B). There was also more Hsp70 bound to AIF in the injured than uninjured hemispheres in both the Tg and Wt mice (data not shown).

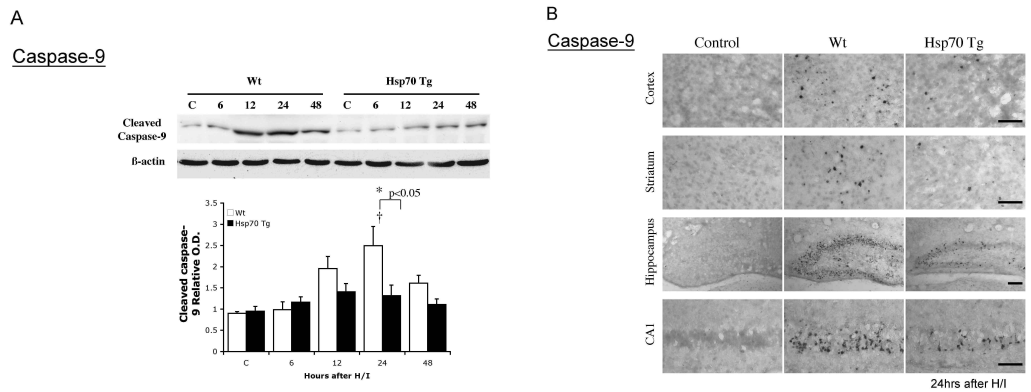

Fig. 4.
(A) In Wt mice, significantly increased caspase-9 cleavage was observed at 24 hr after H/I compared with control († ; $p<0.05$). Significantly educed cleavage of caspase-9 was seen in Hsp70 Tg as compared with Wt mice 24 hr after H/I (*; $p<0.05$). (B) Significantly reduced numbers of caspase-9-positive cells in cortex, striatum, hippocampus and CA1 region of Wt mice compared with Hsp70 Tg mice 24 hr after hypoxic/ischemic injury. Scale bars are 50 μm.

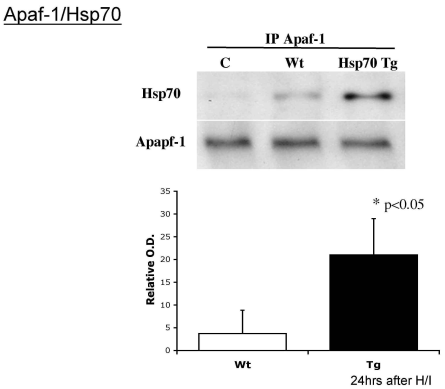

Fig. 5.
Relative OD of Hsp70/Apaf-1 showing significantly more Hsp70/Apaf-1 was detected in Hsp70 Tg as compared with Wt mice (*; $p<0.05$). OD, optical density.

Reduction of AIF nuclear translocation in Hsp70 Tg mice

To determine whether interaction between AIF and Hsp70 affected further down stream apoptotic events, lysates from cytosolic and nuclear fractions of 5 Tg and 11 Wt mice were used in Western blotting. Significantly less AIF was detected in the nuclear extracts from the injured hemispheres of Tg than Wt mice ($P<0.05$) (Fig. 6C).

This review has outlined some of our recent data

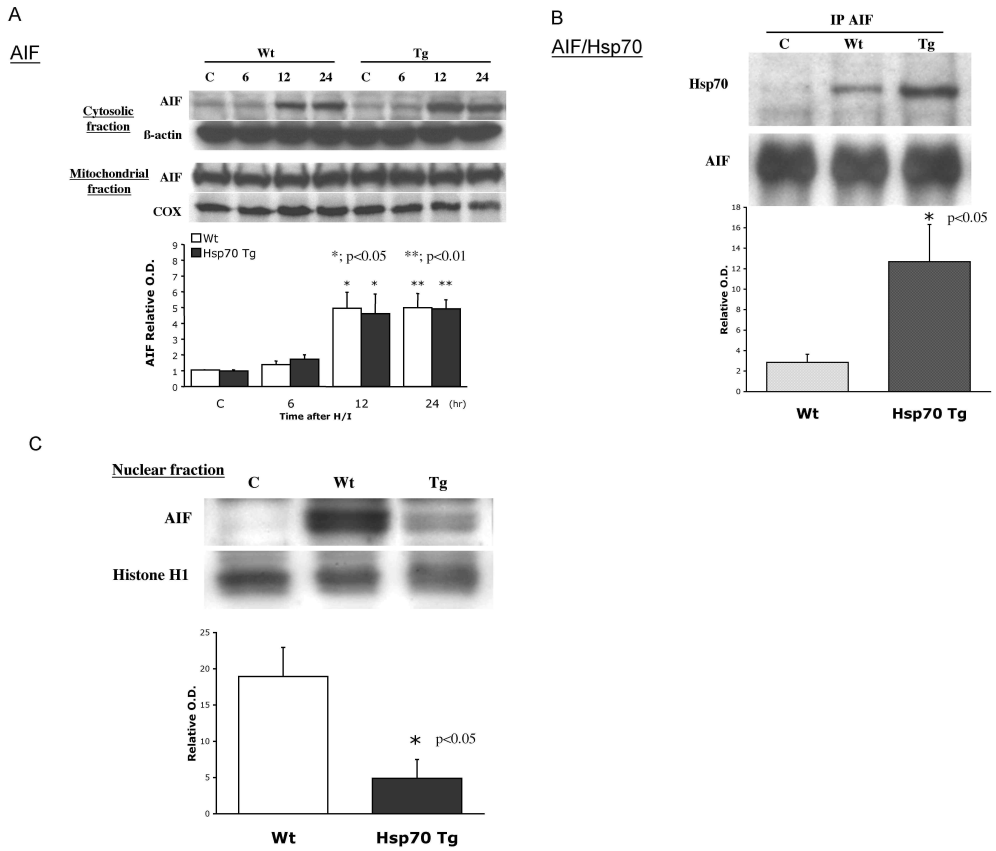

Fig. 6.
(A) Significant translocation into the cytosol was observed 12 and 24 hr after H/I in both Hsp70 Tg and Wt mice as compared to control (*: $p<0.05$, **$p<0.01$). However, the amount of cytosolic AIF did not differ significantly between Hsp70 Tg and Wt mice at these three time points. (B) Increased binding of apoptosis inducing factor (AIF) with Hsp70 in Hsp70 Tg mice at 24 hr after H/I injury. Representative Western blots probed with Hsp70 and AIF as indicated from immunoprecipitated lysates with an antibody against AIF in injured brains of Hsp70 Tg and Wt mice at 24 hr after H/I. Little or no Hsp70 was detected in the immuno pull-down from brain lysates of naive Wt control mice (C). Significantly more Hsp70/AIF was detected in Hsp70 Tg as compared with Wt mice (*: $p<0.05$). AIF signals did not differ among control, Hsp70 Tg and Wt mice. (C) Reduced AIF nuclear translocation in the Hsp70 Tg mice at 24 hr after H/I insult. Western blots detected significantly less AIF translocation into nuclear fraction in Hsp70 Tg as compared with Wt mice (*: $p<0.05$) at 24 hr after H/I. Little or no AIF was detected in the nuclear fraction of brain lysates from naive Wt control mice (C). Histone H1 was used as an internal control for nuclear protein concentration.

elucidating specific ways in which Hsp70 could protect neonatal brain against H/I injury. Hsp70 is one of antiapoptotic proteins, which could reduce translocation of apopotosiselated proteins and reflect one of several different protein-protein interactions. Overexpression of Hsp70 has been shown to protect both in animal and cell models of cerebral ischemia so far. How it exerts these protective effects remains to be elucidated. Because brain damage induces a complex array of gene expression related to glutamate exicitotoxicity, oxidative stress, and apoptotic cascade, multiple neuroprotective effects of Hsp70 can be postulated. Additional studies using molecular techniques are needed to clarify this issue. We hope that understanding those underlying mechanisms may identify specific cell-destructive changes which contribute to brain cell loss in ischemia and help identify new potential targets for anti-ischemic therapy.

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