Potential Role for Heat Shock Protein 72 in Antagonizing Cerebral Vasospasm After Rat Subarachnoid Hemorrhage

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Background—Cerebral vasospasm can be defined as delayed-onset narrowing of the cerebral arteries that can occur after a spontaneous aneurysmal subarachnoid hemorrhage (SAH). Despite a large number of experimental and clinical investigations, the exact pathophysiology of vasospasm remains unknown. Using a fluorescence differential-display system, we have identified the gene encoding heat shock protein 72 (HSP72) as being highly upregulated by cerebral vasospasm. We therefore elucidated the role of the HSP72 gene in cerebral vasospasm in a rat experimental SAH model.

Methods and Results—By angiography, cerebral vasospasm was detected from day 1, with maximal narrowing detected on day 2. Intracisternal injection of antisense HSP72 oligodeoxynucleotide led to specific inhibition of HSP72 gene expression and significantly aggravated cerebral vasospasm on days 2 and 3 of the angiographic studies. Oral administration of geranylgeranylacetone (GGA), an antiulcer drug, enhanced HSP72 induction and reduced cerebral vasospasm.

Conclusions—These results suggest HSP72 plays a novel role in antagonizing delayed cerebral vasospasm after SAH and that GGA provides protective effects against delayed cerebral vasospasm, at least partly via induction of HSP72. (Circulation. 2004;110:1839-1846.)

Key Words: subarachnoid hemorrhage ▪ vasospasm ▪ brain ▪ heat shock protein

The incidence of subarachnoid hemorrhage (SAH) is estimated to be 6 to 8 per 100,000 person-years.1 Delayed cerebral vasospasm often develops 3 to 9 days after aneurysmal SAH. In 27% to 38% of patients with SAH, a delayed, neurological ischemic deficit occurs. Despite successful surgical treatment for intracranial aneurysm and postoperative management, 26% to 38% of such patients suffer sequelae or die of symptomatic severe vasospasm.2,3 Although extensive research has been carried out into the mechanisms underlying delayed cerebral vasospasm and many advanced therapies have been attempted, we still have no successful treatment for this condition.3

Cerebral vasospasm has a distinct feature of a gradual resolution over 2 to 4 weeks.2,4 Recent research into the mechanisms underlying delayed cerebral vasospasm have focused on endogenous spasmogen, but the mechanisms of resolution have received little attention.2,5 We have therefore attempted to establish novel strategies aimed at therapeutic targets of cerebral vasospasm from the viewpoint of improvement of recovery outcomes.

In the present study, we adopted a rat experimental model for vasospasm6 and used fluorescence differential display (FDD)7 to identify differentially expressed genes and to evaluate the functional significance of such genes in vasospasm of the basilar artery. We identified that heat shock protein 72 (HSP72) was a gene highly sensitive to upregulation by cerebral vasospasm after SAH and that overexpression of HSP72 is a possible mechanism involved in the prevention and treatment of pathological conditions such as delayed cerebral vasospasm.

Methods

All protocols were evaluated and approved by the Animal Ethics Review Committee of Mie University School of Medicine. Animals were cared for in accordance with the Guidelines for Animal Experiments at Mie University School of Medicine.

Experimental Animals

A total of 364 male Sprague-Dawley rats (age 11 to 12 weeks, 340 to 380 g; Japan SLC Inc, Hamamatsu, Japan) were used. Animals were anesthetized by injection of chloral hydrate (400 mg/kg IP) and allowed to breathe spontaneously. A 24-gauge catheter was then placed in the right radial artery, and autologous arterial blood was withdrawn. Rats were positioned in a head holder with head flexion at 30°. The atlanto-occipital membrane was exposed through a midline occipital incision and punctured with a 27-gauge needle into the cisterna magna. A 0.1-mL volume of cerebrospinal fluid was withdrawn with this needle, after which autologous blood or physiological saline was slowly injected over a 5-minute period. After surgical closure of the wound, rats were left head down for 10 minutes and then allowed to awaken.

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1839
Angiography
Vertebral basilar angiography to assess the basilar artery was performed by manual injection of contrast medium (1 mL Iomeron 400, Eisai Corp) into the right radial artery. Exposure settings were 50 mA - s, 150 kV, and 0.5 second. The focus-to-objective distance was 50 cm and the focus-to-film distance was 100 cm, resulting in a 2.0-fold linear magnification.

Measurement of Arterial Diameter
Basilar artery diameters were determined at 3 different points: just above the junction of the vertebral arteries, at the midpoint of the posterior cerebral arteries, and immediately below the bifurcation of the posterior cerebral arteries. NIH Image software was used to determine the mean values.

RT-PCR Analysis
Total RNA was isolated from the basilar artery or brain with ISOGEN reagent (Nippon Gene). RNA was then reverse-transcribed with Superscripts II reverse transcriptase (RT) (GIBCO BRL) according to the supplier’s protocol. The resulting cDNA was used as a template for polymerase chain reaction (PCR) amplification. The primers for rat HSP72 mRNA were 5'-TGGCTGACCAAGATGAG-3' and 5'-AGAGTCGATCTCCAGGC-3'. The primers for rat HSP73 mRNA were 5'-GTCCTGGGTGTGTTCTAC-3' and 5'-CCTTTTCGTGTCTTATATCTAC-3'. The primers for rat β-actin mRNA were 5'-GGGAAATCGTGCTGACAT-3' and 5'-CAGGGAGGCAATGATCCTT-3'. PCR products were run on 2% agarose gels, stained with ethidium bromide, and scanned.

Real-Time RT-PCR Analysis
Real-time RT-PCR analysis was performed with TaqMan universal PCR master mix (PE Biosystems) according to the supplier’s protocol. The primers for rat HSP72 mRNA were 5'-CCGGAGAAGGAGTAGAATCTGAAG-3' and 5'-TTGGAT- TAGAGGCTTCTTCTGACTC-3'. The probe for rat HSP72 mRNA was 5'-(carboxyfluorescein [FAM])-TGCCACCGAGCCTAAAGGTCTCGT-(6-carboxy-tetramethyl-rhodamine [TAMRA])-p3'. The primers for rat β-actin mRNA were 5'-TGCCACCGAGCCTAAAGGTCTCGT-(6-carboxy-tetramethyl-rhodamine [TAMRA])-p3'. The probe for rat β-actin mRNA was 5'-(FAM)-TGTCACGATCCACGTCCAGGTGGTTG-(TAMRA)-p3'. PCR and the resulting relative increase were monitored in real time with a 7700 sequence detector (PE Biosystems). Signals were analyzed with the PE Biosystems sequence detector program. Standard curves for HSP72 and β-actin were generated by serial dilution of cDNA obtained from the basilar arteries on day 2. The expression levels of target genes were evaluated by the ratio of the level of target mRNA to β-actin mRNA.

Intracisternal Injection of Phosphorothioate ODNs
The antisense HSP72 oligodeoxynucleotide (ODN) sequence used was 5'-TGTTCCTCGCTACCT-3', and the sense HSP72 ODN sequence was 5'-ATGGCCCAAGAACA-3'. These were diluted in saline to a concentration of 200 μmol/L. The ODN solution was injected twice into the cisterna magna: The first injection (0.5 mL) was administered 24 hours before SAH and the second injection (0.5 mL) 24 hours before SAH.

Western Blot Analysis
Equivalents of 50 μg protein extracted from the basilar arteries were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10%) and transferred to polyvinylidene difluoride membranes. Membranes were incubated with a rabbit polyclonal antibody against rat HSP72 (SPA-760, StressGen Biotechnologies Corp) at a dilution of 1:20 000. Signal development was performed with an enhanced chemiluminescence detection kit.

Oral Administration of GGA
Geranylgeranylatedone (GGA; a gift from Eisai Co, Tokyo, Japan) as an emulsion with 5% arabic gum and 0.008% tocopherol was administered orally. To examine the time-dependent expression of HSP72 in the brain or basilar artery, rats were killed 0, 4, 12, 24, or 48 hours after a single dose of GGA (400 mg/kg). To examine the dose-dependent expression of HSP72, rats were killed 24 hours after administration of 1 of 4 different doses of GGA (0, 400, 600, or 800 mg/kg).

HSP72 induction in the basilar artery after 0.4 mL saline or SAH on treatment with GGA or its vehicle (5% arabic gum and 0.008% tocopherol emulsion) administration was evaluated by RT-PCR, Western blotting, and angiography. Rats treated with GGA were administered orally at a dose of 400 mg/kg every 12 hours. Rats with SAH and treated with vehicle or GGA were injected with 0.4 mL autologous blood as described earlier and were administered orally every 12 hours at the same dose of vehicle or GGA (400 mg/kg).

Statistical Analysis
All data are presented as mean±SEM. A Student t test was used for analysis of the statistical difference between 2 means. Comparisons among multiple groups were assessed by repeated-measures ANOVA, followed by the Dunnett test. A value of P<0.05 was considered statistically significant.

Results
RT-PCR showed a >8.0-fold upregulation of HSP72 mRNA on day 2 after SAH compared with the no-treatment group and a 1.9-fold upregulation of HSP72 mRNA on day 2 after SAH compared with the results in the saline-injection group (Figure 1A). Constitutive HSP73 mRNA after SAH was unchanged compared with levels measured after saline injection (Figure 1A). Real-time RT-PCR also showed a notable induction (1.8-fold) of HSP72 mRNA expression on day 2 after SAH (Figure 1B). HSP72 protein induction was determined by Western blotting with an antibody that specifically recognizes inducible HSP72. A significant induction (2.7-fold) of HSP72 protein was detected on day 2 after SAH compared with that seen on day 2 after saline injection (Figure 1C).

Angiographic examination of cerebral vasospasm after SAH in the basilar artery was performed. In the 0.3-mL SAH group of rats, cerebral vasospasm was detected from day 1, with the maximal vasospasm occurring on day 2. Here, the average arterial diameter decreased to 86% of that seen in baseline angiograms (Figure 1D). The vasospasm then slowly abated and was gone by day 7. In the 0.5-mL SAH group of rats, cerebral vasospasm was similarly detected from day 1, with the maximal vasospasm occurring on day 2. In this case, the average arterial diameter decreased to 76% of that in baseline angiograms (Figure 1D). Comparisons of angiographic arterial diameters between the 0.3-mL and the 0.5-mL SAH groups showed a significant difference (Figure 1D), suggesting that although the time courses of development and disappearance of cerebral vasospasm were relatively similar, the extent of vasospasm was different for the 2 treatment protocols. In the saline groups, no significant differences in basilar artery diameters were observed for either group between day 2 measurements and baseline angiograms (data not shown). No rats in either of the blood-injected groups were observed to develop neurological deficits.
after SAH; however, 15% of these rats died after administration of additional anesthesia.

To determine the functional significance of induction of the HSP72 gene in the basilar artery, we examined the effect on vasospasm of selective HSP72 inhibition by using an antisense HSP72 ODN. RT-PCR showed that successful inhibition of HSP72 mRNA upregulation was observed on day 2 after intracisternal injections of antisense HSP72 ODN (Figure 2A). Densitometric analysis demonstrated that antisense HSP72 ODN decreased the level of HSP72 mRNA to 57.6% of the control value obtained with sense HSP72 ODN. Real-time RT-PCR also showed notable inhibition (0.74-fold) of HSP72 mRNA expression on day 2 after intracisternal injections of antisense HSP72 ODN (Figure 2B). The antisense HSP72 ODN was also found to significantly reduce upregulation of the HSP72 protein (Figure 2C). Densitometric analysis demonstrated that antisense HSP72 ODN decreased the
level of HSP72 protein to 54.1% of the control value obtained with sense HSP72 ODN.

Significant differences in arterial diameters measured by angiography were observed on days 2 and 3 after intracisternal injection of antisense HSP72 ODN or sense HSP72 ODN (Figure 2D). In this way, intracisternal injections of antisense HSP72 ODN significantly aggravated the extent of vasospasm measured angiographically on days 1 to 5 after SAH. No differences in the extent of vasospasm were observed when animals were treated with sense HSP72 ODN injection compared with SAH. Vasospasm gradually subsided until day 7 in the group injected with antisense HSP72 ODN.

In addition to the aforementioned experiments, we found that GGA induced HSP72 expression in the brain and basilar artery. The dose-dependent effects of GGA on the expression of HSP72 in the brain and basilar artery were assessed 24 hours after single oral administration of GGA (Figure 3A and 3B). HSP72 expression in both the brain and basilar artery was observed for doses in excess of 400 mg/kg and increased in a dose-dependent manner up to 600 mg/kg. The time-
dependent effects of GGA on the expression of HSP72 in the brain and basilar artery were also evaluated after a single-dose administration of GGA (400 mg/kg, Figure 3C and 3D). HSP72 expression in the brain and basilar artery was observed from 4 hours, then slowly decreased, and had disappeared by 48 hours.

Levels of HSP72 in the basilar artery were examined by RT-PCR and Western blot analysis after saline injection or SAH with oral administration of GGA or its vehicle only (Figure 4A and 4B). The overexpression of HSP72 mRNA by the end of day 2 after SAH or saline injection with just 2 orally administered GGA boluses on day 1 (Figure 4A, lanes 2 and 5, lanes 4 and 7). Furthermore, on day 2, the overexpression of HSP72 mRNA after SAH with oral GGA treatment was significantly enhanced compared with that after SAH with vehicle treatment and after saline injection with GGA treatment (Figure 4A, lanes 5 and 7, lanes 6 and 7). Overexpression of HSP72 protein by the end of day 3 after saline injection after 6 12-hourly administered oral GGA boluses was dose-dependently increased compared with that after saline injection with just 4 orally administered GGA boluses on day 2 (Figure 4B, lanes 2 and 5). Furthermore, on day 3, overexpression of HSP72 protein after SAH with oral GGA treat-

Figure 3. Oral administration of GGA induces HSP72 mRNA expression. A and B, Dose-dependent effects of GGA on expression of HSP72 mRNA in brain (A) and basilar artery (B) were assessed 24 hours after GGA administration. C and D, Time-dependent effects of GGA on expression of HSP72 mRNA in brain (C) and basilar artery (D) were evaluated after single-dose administration of GGA (400 mg/kg). Top, Representative RT-PCR. Bottom, Mean densitometric data expressed as ratio of β-actin mRNA (n=4 in each group).
ment was significantly enhanced compared with that after SAH with vehicle treatment (Figure 4B, lanes 6 and 7).

Comparisons of arterial diameters as measured by angiography on days 1, 2, and 3 after SAH with oral administration of GGA showed significant improvement compared with cases when GGA was not administered (Figure 4C).

Discussion

The mechanisms underlying cerebral vasospasm have been widely reported, with vasospasm well recognized by experimental and clinical evidence to be associated with the presence of exudate blood in the subarachnoid space. Oxyhemoglobin and deoxyhemoglobin in SAH are probably the principal pathogenetic agents. However, the mechanisms involved in the genesis of vasospasm are yet to be elucidated. The pathogenesis of delayed cerebral vasospasm is multifactorial, involving morphological changes to smooth muscle cells (proliferation and/or necrosis) and endothelial cells (endothelial cell damage and apoptosis), a variety of molecular mediators, and proinflammatory mediators.

Cerebral vasospasm is an angiographically demonstrable, variable arterial narrowing that can be clinically asymptomatic, but it can also give rise to increasing neurological deficits or even death. For this study, to investigate the mechanisms underlying the condition, we developed a rat SAH model wherein delayed cerebral vasospasm could be reproduced in the basilar artery after intracisternal autologous blood injection. In this way, cerebral vasospasm was detected by angiography from day 1, with maximal vasospasm occurring on day 2, followed by a gradual reduction and then disappearance by day 7. The degree of vasospasm depended on the volume of injected blood. This rat model is suitable for quantitative evaluation because a constant volume of blood can be injected into the cisterna magna. Using FDD, we observed a significant level of upregulation of HSP72 mRNA
and protein in the basilar artery on day 2 after SAH. These findings reveal that during cerebral vasospasm, HSP72 mRNA and protein were significantly induced in the basilar artery.

Several recent studies have demonstrated that intracisternal administration of antisense ODN has provided a powerful tool for studying the consequences of inhibiting the expression of individual genes in vivo. \textsuperscript{14–16} We introduced antisense HSP72 ODN in this SAH model and revealed a novel function for HSP72 in the cerebral artery. Angiographies showed that vascular narrowing was markedly aggravated between days 1 to 5 after treatment with antisense HSP72 ODN. However, cisternal injection of sense HSP72 ODN had no significant effect on arterial diameter. Significant enhancement of vasospasm was evident on days 2 and 3, with maximum effects seen on day 2. The peak time for vasospasm after injection of antisense HSP72 ODN was found to be the same as that after injection with sense HSP72 ODN. After treatment with antisense HSP72 ODN, HSP72 mRNA and protein levels were found to be significantly downregulated on day 2.

The results presented herein demonstrate that orally administered GGA, which is known to induce HSP72 predominantly in gastric mucosal cells, \textsuperscript{17} liver, \textsuperscript{18} and heart \textsuperscript{19} of rats, also induces HSP72 expression in rat basilar arteries and brains and provides protective effects against delayed cerebral vasospasm, at least partly via induction of HSP72. GGA was reported to specifically induce transcriptional activation of HSF1 and expression of HSP72 mRNA in cultured guinea pig gastric mucosal cells \textsuperscript{20} and to exert priming effects for enhanced HSP72 induction. \textsuperscript{20} It has been reported that protein kinase C mediates the phosphorylation of HSF1, resulting in HSP72 expression in cultured human epidermoid A431 cells. Oral administration of GGA activates protein kinase C, leading to the phosphorylation and translation of HSF1, and thus promotes the expression of HSP72 protein. \textsuperscript{21} Similar mechanisms may underlie the induction of HSP72 with GGA treatment after SAH in the basilar artery.

In the present study, we found that intracisternal administration of antisense HSP72 ODN aggravated delayed cerebral vasospasm and that enhanced HSP72 gene induction by GGA after SAH was one of the key mechanisms responsible for protecting against this. Therapeutic gene induction of HSP72 by GGA may therefore prove to be a novel approach for the prevention and treatment of pathological conditions such as delayed cerebral vasospasm.

Among several multimember families of HSPs, HSP72 is the best-known endogenous factor protecting cell and tissue injury under various pathological conditions. Overproduction of HSP72 has been reported to increase resistance to ischemia-reperfusion injury in brain, \textsuperscript{22} liver, \textsuperscript{23} and heart \textsuperscript{24} in vivo and plays a cytoprotective role against injury-induced smooth muscle cell proliferation \textsuperscript{25} and/or necrosis, as well as to protect against endothelial cell damage, \textsuperscript{26} apoptosis, \textsuperscript{27} \textsuperscript{28} and free radical damage and to reduce the production of proinflammatory mediators, \textsuperscript{29} the effects of which are observed in delayed cerebral vasospasm. These findings suggest that HSP72 may have a potential role in a range of cytoprotective mechanisms against such pathological effects in delayed cerebral vasospasm after SAH.

We therefore propose that HSP72 induction is an intrinsic protective mechanism and that it provides a potential therapeutic target for the treatment of cerebral vasospasm. Future studies will be required to confirm the protective effects of GGA via HSP72 induction.

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