Induction of HSP70 in rat brain following subarachnoid hemorrhage produced by endovascular perforation

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Current experimental research on subarachnoid hemorrhage (SAH) has been limited by the lack of a small-animal model that physiologically resembles SAH and consistently demonstrates acute and delayed cellular injury. Recently, a model for inducing SAH by endovascular perforation of the internal carotid artery has been developed in the rat. This model physiologically resembles SAH. However, little histological data detailing cellular injury after SAH are available in this or other models. Using immunocytochemistry, the authors investigated the induction of the 70-kD heat shock protein, HSP70, a sensitive marker for cellular stress or injury in the brain, 1 and 5 days following endovascular SAH. The authors also used the conventional histological techniques of cresyl violet and hematoxylin and eosin staining to investigate cellular damage 1 and 5 days after the endovascular SAH.

One day following the SAH, HSP70 was induced in all six animals examined in multiple anatomical regions, including the basal forebrain, thalamus, neocortex, striatum, and hippocampus. This HSP70 induction was observed in multiple vascular distributions bilaterally. Immunostaining with HSP70 occurred primarily in neurons but also was observed in glia and endothelium. Five days after the SAH, a similar but more intense pattern of HSP70 immunostaining was observed in all eight animals examined. Specifically, HSP70 immunoreactivity was observed in at least one region of the hippocampus more often at 5 days (six of eight animals) than at 1 day (one of six animals, p < 0.05, one-tailed Fisher’s exact test). No HSP70 immunostaining was observed in control animals at 1 day or at 5 days. Conventional histology demonstrated foci of ischemic neuronal damage and cellular necrosis; however, HSP70 immunocytochemistry detailed cellular injury far better than conventional histology in all animals tested at both 1 day and 5 days.

Our results demonstrate that HSP70 is induced in multiple regions and cell types 1 day and 5 days following endovascular SAH. Because ischemia is a known inducer of stress genes, the authors propose that acute and delayed ischemia are the processes responsible for the induction of HSP70 that was observed at 1 day and 5 days, respectively. Investigation of HSP70 induction following endovascular SAH may also serve as the basis for a new, inexpensive animal model to assess potential therapeutic interventions.

KEY WORDS • heat shock protein • ischemia • stroke • subarachnoid hemorrhage • vasospasm • rat
Induction of the 70-kd heat shock protein, HSP70, in the brain is an indicator of cellular injury. Focal and global ischemia, heat stroke, status epilepticus, and neural toxins induce HSP70 in neurons that are outside the regions of infarction but in areas where neurons are known to be damaged. Following focal ischemia, HSP70 induction is a more sensitive indicator of cellular injury than conventional histological staining with hematoxylin and eosin. Recently in our laboratory, HSP70 induction in neurons and glia has been demonstrated following injection of lysed blood into the cisterna magna of rats. We have observed that lysed blood (but not whole-blood) injection into the cisterna magna of rats produces HSP70 induction in focal regions of the forebrain and diffuse regions of the cerebellum. Therefore, HSP70 appears to be a viable marker for monitoring cellular injury following SAH in rats. Using HSP70 induction as a marker, we investigated the onset of acute and delayed cellular injury in the rat using modifications of a new, physiological model of SAH. We also compared the sensitivity of HSP70 immunocytochemistry with the conventional histological techniques of cresyl violet and hematoxylin and eosin staining. The goal of this study was to establish HSP70 induction as a sensitive, reliable marker for determining the acute and delayed cellular injuries that follow SAH and to establish the basis for a new model for testing the efficacy of future therapeutic interventions.

Materials and Methods

Experimental Model of SAH

Experimental SAH was produced in 20 female Sprague–Dawley rats, weighing 270 to 320 g, using a method developed independently in our laboratory and recently also reported by Bederson, et al. The rats were given access to food and water ad libitum before and after the experiment. Anesthesia was induced in a chamber using a mixture of 3.5% isoflurane, 66.5% N2O, and 30% O2. Rats were then intubated using an 18-gauge angiocatheter. Using a rodent ventilator, anesthesia was maintained by administration of a mixture of 1.5% isoflurane, 68.5% N2O, and 30% O2. The ventilator was adjusted to maintain pH, PO2, and PCO2 within normal ranges. The rats were then placed prone on a heating pad to maintain a body temperature of 37°C and the wound was prepared in sterile fashion and draped.

Using a sterile technique, a midline neck incision was made. The ECA and all of its branches were identified, dissected, cauterized, and divided. The free stump of the ECA was reflected inferiorly, and an aneurysm clip was placed across its base. A No. 3-0 nylon monofilament suture was then advanced through the ECA, into the cervical ICA, and then distally into the intracranial ICA. Resistance was encountered after insertion 22 mm beyond the common carotid artery bifurcation. The suture was advanced 11 mm further to a total of 33 mm. This maneuver punctured the ICA distal to the bifurcation of the ACA and MCA as observed in pilot studies (involving four animals) during which a craniectomy was performed and the brain was retracted during endovascular suture advancement. Brain removal and inspection following pilot studies, in which the length of suture insertion was varied, indicated that a 33-mm suture insertion ensured adequate arterial rupture. The suture was then withdrawn through the ICA into the ECA, allowing perfusion and producing SAH.

Following suture withdrawal, the ECA was ligated. The suture occluded the lumen of the ICA for approximately 1 minute. Sham-operated control rats (six animals) underwent a similar procedure; however, in that procedure the suture was advanced only to 22 mm for 1 minute and then withdrawn without ICA perfusion. After suture removal, the neck incision was closed. The rats were allowed to recover from anesthesia and were then extubated. After recovery from anesthesia, the rats were examined neurologically within 30 minutes of completion of surgery for response to an ear-pinch stimulus. The ear-pinch stimulus was performed to assess response to pain following a potentially diffuse neurological injury and to determine if absence of pain response correlates with the extent of HSP70 induction.

Conventional Histology and Immunocytochemistry for HSP70

The rats were killed at 1 day (nine animals) or 5 days (11 animals) following SAH. The animals were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and perfused through the left ventricle with 100 ml of 0.9% saline followed by 400 ml of 4% paraformaldehyde in 0.1 mol/L, pH 7.4, phosphate-buffered solution (PBS). The brains were postfixed for 1 to 3 hours after which 50-μm-thick coronal sections were cut on a vibratome and placed in PBS. The sections were washed in PBS and divided into four wells. A mouse monoclonal anti–human HSP70 antibody, C92, was used in the first well at a dilution of 1:4000. The C92 antibody recognizes the protein product of the full-length rat hsp70 complementary DNA. The C92 monoclonal antibody to HSP70 detects a single band on Western blots of heat-shocked retina, and two bands on Western blots of ischemic gerbil brain and acid-treated and heat-shocked astrocytes.

The second well was incubated without primary antibody for use as a control. After incubation in primary antibody overnight at 4°C, sections were washed three times in PBS. They were then incubated for 2 hours in sheep anti–mouse immunoglobulin (Ig) antibody (1:200). After another washing, the sections were incubated in the avidin-biotin-horseradish peroxidase complex followed by addition of 0.015% dianminobenzidine and 0.001% H2O2. Sections were dehydrated and placed on a coverslip using DePex.

Sections in the third and fourth wells were mounted on subbed slides for cresyl violet or hematoxylin and eosin staining. These sections were dehydrated, delipidized in chloroform followed by ethyl ether, rehydrated, and stained with either 1% cresyl violet acetate or hematoxylin and eosin). The sections were then dehydrated and placed on a coverslip using DePex.

Sources of Supplies and Equipment

The rats were maintained under anesthesia by means of a rodent ventilator, model 683, from Harvard Instruments, Inc., South Natick, MA. The mouse monoclonal anti–human HSP70 antibody, C92, and the sheep anti–mouse Ig antibody were obtained from Amersham, Arlington Heights, IL. The avidin-biotin-horseradish peroxidase complex was obtained from Vector Laboratories, Burlingame, CA, and the diaminobenzidine from Sigma Chemical Co., St. Louis, MO. The DePex was provided by Biomedical Specialties, Santa Monica, CA.

Results

Surgical Outcome

Twenty rats were subjected to endovascular SAH. Four of the rats were part of a pilot study to establish the optimum site of arterial perforation. These four animals were killed immediately postoperatively. Of the remaining 16 rats subjected to SAH, two (12.5%) died postoperatively. The remaining animals were often lethargic in the first 24 hours following the SAH and demonstrated little activity other than drinking. However, most of them subsequently recovered normal activity within 48 hours of the SAH. There were no postoperative deaths in the six control rats.

After surgery, the rats were examined for neurological deficits within 30 minutes of the SAH using an ear pinch as a stimulus. In the six control subjects, three demonstrated bilateral reaction to the ear pinch. The three remaining control rats were without reaction to ear pinch on...
the side ipsilateral to the suture insertion. In the 14 SAH rats that were subjected to and recovered from anesthesia, eight had no observed reaction to ear pinch bilaterally, four demonstrated bilateral reaction to the ear-pinch stimulus, and two had a reaction to ear pinch only on the side contralateral to the hemorrhage. Of the eight animals that demonstrated no reaction to pain bilaterally, diffuse HSP70 induction was observed in seven.

The distribution of subarachnoid blood around the brain was also examined. None of the control animals exhibited blood in the subarachnoid spaces around the brain (Fig. 1 left). In the SAH group, 13 of the 14 animals had clotted blood at the base of the brain adjacent to the area of arterial perforation (Fig. 1 right). Two animals demonstrated dissemination of blood across the basal cistern to surround the contralateral ICA. In three animals, blood was also observed on the dorsal aspect of the neocortex. One animal had intraventricular extension of the hemorrhage.

**Induction of HSP70 1 Day After SAH**

Induction of HSP70 was not observed in the sham-operated control animals at 1 day after surgery (Fig. 2A). There also was no immunochemical or conventional histological evidence of brain injury caused by suture penetration. However, 1 day following the SAH, a heterogeneous pattern of HSP70 induction developed, which ranged from a single focus of neuronal HSP70 induction in two animals (Fig. 2B) to massive endothelial HSP70 induction in the MCA distribution in two others (data not shown). The remaining two animals demonstrated striatal, neocortical, and basal forebrain induction of HSP70 (Fig. 2C and D). Induction of HSP70 in the striatum, neocortex, and basal forebrain occurred primarily in neurons (Fig. 2D), but occasional HSP70 immunoreactivity was also observed in glia. In two animals, HSP70 immunoreactivity was observed primarily in the endothelium throughout the neocortex and striatum (data not shown). In these animals, only a rim of neuronal HSP70 immunostaining surrounding the region of endothelial induction was observed. Neocortical induction of HSP70 in the ipsilateral hemisphere was observed most commonly in the MCA distribution (Fig. 2B and C). Induction of HSP70, however, also occurred within the ACA territory. In two of six animals HSP70 induction was observed in the contralateral MCA distribution, in addition to the HSP70 immunostaining observed on the ipsilateral side.

**Induction of HSP70 5 Days After SAH**

Induction of HSP70 was not observed in the sham-operated control animals at 5 days (Fig. 3A). Five days after SAH was induced, a more intense, but also heterogeneous, pattern of HSP70 immunostaining developed. One animal subjected to SAH demonstrated a small amount of periventricular, neuronal HSP70 immunoreactivity (data not shown), and another showed focal HSP70 induction in the striatum and basal cortex (Fig. 3B). In the remaining six animals, HSP70 induction occurred throughout the forebrain in multiple anatomical regions, including the hippocampus, neocortex, thalamus, striatum, basal forebrain, and septum (Figs. 3B–D). Induction of HSP70 was observed in multiple vascular distributions (in the ACA, MCA, and posterior cerebral artery (PCA); Fig. 3B–D). Induction in these regions was primarily neuronal (Fig. 3D); however, HSP70 induction was also observed in glia and endothelium (data not shown).

The HSP70 immunoreactivity observed did not correspond to any known pattern of afferent innervation or specific anatomical neuronal distribution. However, in the neocortex HSP70 induction occurred diffusely and sometimes focally in neurons surrounding penetrating blood vessels (Fig. 3D). The HSP70 immunoreactivity was often observed in the ipsilateral PCA distribution including the thalamus, the CA1 to CA4 regions of the hippocampus, and the dentate gyrus. In addition to the ipsilateral hemisphere, HSP70 immunoreactivity occurred in the hemisphere contralateral to the hemorrhage in four of eight animals in which it was observed in the dorsal cortex, basal forebrain, and hippocampus.

**Conventional Histology Versus HSP70 Immunocytochemistry**

In neocortical neurons demonstrating HSP70 immunoreactivity 1 day after the SAH, histological examination using cresyl violet and hematoxylin and eosin staining on adjacent sections did not reveal any evidence of necrotic cell death (data not shown). A similar result was observed in the striatum, basal forebrain, and septum. Focal regions of HSP70 induction in neurons from these regions did not demonstrate evidence of neuronal damage on adjacent sections stained with cresyl violet or with hematoxylin and eosin. In one animal HSP70 immunostaining of the hippocampus 1 day after SAH demonstrated HSP70 immunoreactivity in neurons from the CA1, CA3, and CA4 regions of the contralateral hippocampus (Fig. 4A and C). Histological examination using cresyl violet on an adjacent section in this animal demonstrated no evidence of ischemic neuronal damage in the CA1 region of the hippocampus (Fig. 4B and D). Five days after the SAH, HSP70 immunoreactivity was
commonly observed in the CA1 to CA4 regions of the hippocampus as well as in the dentate gyrus (Fig. 5A and C). In two animals, HSP70 induction occurred in the CA2 to CA4 regions of the hippocampus but sparsely in the CA1 region (Fig. 5A, arrow). Cresyl violet staining on an adjacent section confirmed cellular necrosis in the CA1 region in which no HSP70 induction was observed (Fig. 5B, arrow). Normal cresyl violet and hematoxylin and eosin staining were observed, however, in those CA2 to CA4 regions that did demonstrate induction of HSP70 (Fig. 5D).

**Discussion**

**Acute Cerebral Ischemia and HSP70 Induction After SAH**

One day after endovascular SAH, HSP70 was induced in several regions of the brain encompassing multiple vascular distributions. The most common region involved...
was the MCA distribution ipsilateral to the hemorrhage site, although other vascular territories also demonstrated HSP70 immunostaining. Because both global and focal ischemia are known to induce HSP70 in the rat brain,7,13,22 we proposed that acute ischemia following SAH was the process responsible for inducing this protein. Intracranial pressure measurements were not performed in these experiments and are reserved for future studies. However, intracranial pressure changes have been well described in this model.4 Following endovascular SAH in rats, intracranial hypertension develops rapidly, and significant reductions in CBF are also observed during this same period.4,26 However, as intracranial hypertension resolves, CBF remains diminished in these same animals.4,26 Thus, following endovascular SAH, cerebral perfusion pressures can be reduced acutely. This reduction may produce acute ischemia. In dogs, SAH has also been shown to produce perfusion defects throughout the brain in the acute phase.2

The preponderance of HSP70 immunostaining found in the MCA distribution ipsilateral to the hemorrhage 1 day after SAH suggested a focal process as the cause of the HSP70 induction observed. Because ischemia is the proposed mechanism that produces HSP70 induction, acute vasospasm in the ipsilateral MCA, near its origin, may have developed subsequent to the SAH. After endovascular advancement of the nylon suture, arterial perforation was observed near the ACA–MCA bifurcation.4 Proximal ACA rupture may yield large amounts of clotted blood around the proximal MCA and may predispose the vessel to acute vasospasm. An acute phase of angiographic vasospasm has been described after injection of blood into the cisterna magna of rats.6

Another mechanism for MCA ischemia could have been a mechanical intimal injury to the ICA caused by the nylon suture. However, HSP70 immunostaining was observed in multiple vascular territories bilaterally, and multifocal ipsilateral lesions typical of embolization were not observed. Furthermore, the pattern of HSP70 immunostaining after endovascular SAH differed from that observed after prolonged endovascular ICA occlusion alone.13

**Delayed Cerebral Ischemia, Vasospasm, and HSP70 Induction After SAH**

Induction of HSP70 5 days after SAH was more extensive than that observed 1 day after SAH. Five days after SAH occurred, HSP70 induction was observed in several
separate anatomical regions, including the hippocampus, thalamus, striatum, neocortex, septum, and basal forebrain. The HSP70 immunostaining was often observed in two or three vascular distributions; bilateral HSP70 immunoreactivity occurred in four of eight animals. We propose that the increase in HSP70 expression observed in our study 5 days following SAH was a manifestation of delayed cerebral ischemia due to vasospasm. Cerebral blood flow was not measured in this preliminary study, and future CBF experiments would be of interest. However, delayed cerebral ischemia following SAH has been demonstrated experimentally in a similar animal model. Following basilar arterial perforation in rats, CBF was reduced and remained diminished for several days. Angiographic studies of rat cerebral vasculature has shown a delayed phase of vasospasm approximately 48 hours after injection of blood into the cisterna magna.

Other postischemic reactions that cause cytotoxicity or stress may play a role in the increased HSP70 induction that was observed 5 days after SAH in this study. Following temporary endovascular MCA occlusion, HSP70 expression was observed in the ischemic territory as late as 7 days after the ischemic event. Furthermore, hemoglobin and catalytically active iron released after SAH can lead to lipid peroxidation and DNA degradation, which may contribute to delayed cellular damage and subsequent HSP70 induction. Thus the HSP70 expression that we observed 5 days after SAH may have been a manifestation of neurochemical processes that occurred within 24 hours of the hemorrhage.

Heat Shock Protein 70 as a More Sensitive Indicator of Cellular Injury After SAH

It has been suggested that the hsp70 heat shock gene serves as a marker of neural injury in cells not necessarily destined to die. Experimentally denatured proteins, when injected into normal cultured cells, induce HSP70 and other heat shock genes. Injection of amino acid analogs that induce the formation of abnormal, denatured proteins also induces hsp70. Furthermore, severe hyperthermia just short of that required to kill cells induces hsp70, whereas mild hyperthermia does not. Finally, focal ischemia induces expression of HSP70 in neurons.
that are outside the regions of infarction but in areas in which surviving neurons are known histologically to be damaged.⁷,¹⁷

In our experiments, neuronal damage was observed using conventional histological examination in the hippocampus 1 day and 5 days after SAH. Three hours after endovascular SAH occurred, neuronal damage was evident in the hippocampus using hematoxylin and eosin staining in another recent study.²⁶ Of considerable interest, however, was the observation that HSP70 immunostaining on adjacent sections demonstrated a greater degree of cellular injury than that observed using conventional histology (Figs. 4 and 5). In neocortex and basal forebrain, HSP70 immunostaining also demonstrated a greater degree of cellular injury than that demonstrated by conventional histology.

In a previous study investigating focal ischemia in rats, HSP70 induction was shown to be a more sensitive marker for cellular injury than hematoxylin and eosin staining.¹³ Following focal and global ischemia in rats, HSP70 is induced in a cellular and anatomical hierarchy.¹³,²² Cell types and anatomical regions that are more sensitive to ischemia, such as neurons, induce HSP70 earlier than cell types more resistant to ischemia, such as glia and endothelium.¹³,²² If acute or delayed ischemia is sufficiently severe, HSP70 immunocytochemical analysis may demonstrate the severity and distribution of the ischemia according to the cell types and regions in which the HSP70 immunoreactivity is observed. Immunostaining with HSP70, therefore, appears to be a more sensitive method than standard means of histological examination for detecting cellular and regional ischemia and injury following SAH. The HSP70 immunostaining may also detect sublethal metabolic cell stress that does not progress to irreversible injury and necrosis.⁷,¹³

Heat Shock Protein 70 as a Marker for Cellular Injury in a New Animal Model of SAH

Experimental data on therapies to treat the injury caused by SAH and subsequent acute and delayed ischemia are limited because of the lack of a histological endpoint. Using endovascular SAH and HSP70 immunocytochemistry, early and delayed cellular injury can be delineated easily in a physiological model of SAH. However, significant variability is observed between animals in this model with respect to the sites and extent of injury following SAH. In future studies, such variable response patterns may require the use of large numbers of experimental animals. Variable patterns of injury may also limit the opportunity to perform quantitative injury studies. However, the use of a pain stimulus as a screening test may allow animals without evidence of extensive injury to be screened. Following SAH, eight rats demonstrated no response to ear pinch bilaterally. Seven of these eight animals demonstrated diffuse cellular injury in multiple regions of the brain. Therefore, the absence of reaction to a pain stimulus may serve as a simple indicator for predicting which animals will display significant cellular injury after endovascular SAH. This ability to predict cellular injury may significantly decrease problems caused by the variability within this model. Variability within this model may also be reduced by controlling the extent of the SAH. Veelken, et al.,²⁶ varied the degree of SAH in this model by altering the amount of reperfusion through the site of arterial perforation. They subsequently demonstrated significant differences in CBF in the first 3 hours after SAH, depending on the degree of hemorrhage.²⁶ Therefore, it is possible that variability in the extent of HSP70 induction may be minimized by controlling the extent of SAH. Thus HSP70 immunocytochemical analysis in the rat brain after endovascular SAH may ultimately serve as the basis of a useful new animal model for testing therapeutic interventions.

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