Distribution of the 72-kd Heat-Shock Protein as a Function of Transient Focal Cerebral Ischemia in Rats

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Background and Purpose: The significance and physiological implications of the expression of the 72-kd heat-shock protein in ischemic tissue are unknown. To enhance our understanding of the relation between ischemic cell damage and 72-kd heat-shock protein expression, we evaluated the cellular expression and the anatomic distribution of 72-kd heat-shock protein in conjunction with the morphological analysis of rat brain, as a function of the duration of a single arterial occlusion.

Methods: Adult Wistar rats were subjected to graded transient middle cerebral artery occlusion (for a duration of 10, 20, 30, 60, 90, and 120 minutes and sham; n=4 per group). Forty-eight hours after reopening the artery, brain tissue sections were analyzed to determine the extent of neuronal damage (hematoxylin and eosin staining), the extent of astrocytic reactivity (immunohistochemistry, using anti-glial fibrillary acidic protein), and the distribution of 72-kd heat-shock protein (immunohistochemistry, using a monoclonal antibody to 72-kd heat-shock protein).

Results: We found that 72-kd heat-shock protein was sequentially expressed in morphologically intact neurons, microglia, and endothelial cells with increasing duration of ischemia; 72-kd heat-shock protein immunoreactivity was not detected in astrocytes. The duration of ischemia required to evoke a 72-kd heat-shock protein response in neurons was dependent on the anatomic site and followed a pattern of increasing neuronal sensitivity to ischemic cell damage with duration of ischemia: 72-kd heat-shock protein and neuronal damage were sequentially detected in the caudate putamen, globus pallidus, cerebral cortex, amygdala, and hippocampus with increasing duration of ischemia. With ischemia of long duration (≥90 minutes), neurons expressing 72-kd heat-shock protein were localized to a zone peripheral to the severely damaged ischemic core.

Conclusions: These studies suggest that 1) the expression of 72-kd heat-shock protein in neurons precedes the development of ischemic cellular alterations detectable by conventional hematoxylin and eosin light microscopy methods; 2) there is a hierarchy of cell types and anatomic sites that express 72-kd heat-shock protein, and this hierarchy reflects cellular and anatomic vulnerability to ischemic cell damage; and 3) 72-kd heat-shock protein induction in neurons bordering a necrotic ischemic core may be the morphological equivalent of the ischemic penumbra. (Stroke 1992;23:1292–1298)

Key Words • cerebral arteries • cerebral ischemia • heat-shock proteins • rats

Ischemic cell injury induces the expression of a family of stress proteins.1 The nonconstitutive 72-kd heat-shock protein (HSP-72) has been a focus of research in brain tissues injured by ischemia,2–11 largely because of the availability of the HSP-72 monoclonal antibody. Expression of HSP-72 has been reported in neurons, astrocytes, and endothelial cells exposed to ischemic insults.9 The anatomic distribution of the HSP-72 immunoreactive cells in brain varies according to the ischemic model employed. Forebrain ischemia in the rat5–6 and gerbil7,8,12 induces HSP-72 expression primarily in the hippocampus and the cerebral cortex, whereas focal ischemia in the rat localizes HSP-72 expression to the territory normally supplied by the occluded artery.5,9 In necrotic tissue, HSP-72 is absent in neurons, but the protein has been reported in glia and in the microvasculature. Expression of HSP-72 may, therefore, mirror cellular and anatomic sensitivity to ischemic damage.

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The significance and physiological implications of the expression of HSP-72 in ischemic tissue are unknown. Some data suggest that the induction of HSP-72 may protect tissue against subsequent insult7,8,12; other data indicate that only those cells that express HSP-72 undergo delayed neuronal necrosis.2,3

The objective of the present study was to evaluate the cellular expression and define the anatomic distribution of HSP-72 in conjunction with morphological analysis of
the tissue as a function of the duration of a single arterial occlusion, the model most closely resembling human ischemic stroke. Arterial occlusion of increasing duration causes a progressive increase in the distribution and intensity of ischemic cell damage. Thus, by matching the cellular distribution of HSP-72 with increasing durations of focal cerebral ischemia, we may enhance our understanding of the relation that exists between ischemic cell damage and HSP-72 expression.

Materials and Methods
Animal Model
Twenty-eight male Wistar rats weighing 260–300 g were studied. Middle cerebral artery (MCA) occlusion was induced for 10, 20, 30, 60, 90, and 120 minutes using a method of intraluminal vascular occlusion. Rats were fasted overnight preceding surgery but allowed free access to water. Halothane was used to induce (3.5% in a mixture of 70% N2O and 30% O2) and maintain (1%) anesthesia. A face mask was used to maintain anesthesia. The rectal temperature of the animal was maintained at 37°C with a heating pad. The femoral artery was cannulated for serial measurements of pH, PO2, PCO2, and plasma glucose concentration. The right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were isolated via a ventral midline incision. The ECA distal end was ligated with 5-0 silk suture at the branch of occipital artery, and the ECA origin was tied loosely with 5-0 silk suture. Two microvascular clips were placed across the CCA and ICA. A 5-cm length of 4-0 nylon monofilament with its tip rounded by heating near a flame was introduced into the ECA lumen through a puncture between the two silk sutures at the ECA. The silk suture around the origin of the ECA was tightened around the intraluminal nylon suture to prevent bleeding, and the two clips on the CCA and ICA were removed. Approximately 18.0–19.0 mm of the nylon suture, the length determined by body weight, was advanced into the ICA to block the origin of the MCA and to enter the anterior cerebral artery (ACA). The skin incision was closed. Restoration of MCA blood flow was accomplished by withdrawing the intraluminal suture until the tip had cleared the ACA–ICA lumen and was in the ECA stump.

Electroencephalogram and electrocardiogram were recorded before and during ischemia. Induction of ischemia was evident by slowing of the electroencephalogram. The electrocardiogram did not exhibit obvious change. All rats with right MCA occlusion exhibited focal neurological deficits characterized by left hemiparesis with failure to extend the left forepaw. An additional four rats served as sham-operated controls; they had a 15-mm-long nylon monofilament inserted into the ICA (one rat for each period of 10, 20, 30, or 60 minutes). This length of nylon monofilament is too short to occlude the MCA. None of these rats exhibited a slowing of the electroencephalogram or left-sided neurological deficits.

HSP-72 Immunohistochemistry and Histopathology
Since we wanted to investigate a fully mature lesion, we killed the rats 48 hours after recirculation (authors’ unpublished observations). The immunohistochemical technique used to detect HSP-72 was essentially as described in detail by Vass et al. All rats were given an overdose of pentobarbital and fixed by transcardial perfusion with heparinized sodium phosphate buffer (pH 7.4), followed by 4% paraformaldehyde in the buffer. The brain was removed and placed in the same fixative overnight. Eight coronal sections of 3-mm thickness were obtained using a rodent brain matrix. Vibratome coronal sections (50 μm) were obtained. Blocking of nonspecific background staining was accomplished with normal sheep whole serum. A mouse monoclonal antibody to induced HSP-72 (C92, RPNI 1197; Amersham, Cleveland, Ohio) was used in 1:200 dilution and incubated for 10 hours. Endogenous peroxidase was blocked with H2O2 and methanol. Biotinylated sheep anti-mouse immunoglobulin G was incubated for 1 hour each. Peroxidase was detected with diaminobenzidine. Sections were gelatin-mounted on slides for light microscopic evaluation. Control sections were prepared on each rat by performing immunohistochemistry, except that primary antibody was deleted. The remaining coronal slices from these rat brains were embedded in paraffin. Sections (6 μm) adjacent to those cut on the vibratome were stained with hematoxylin and eosin for neuronal evaluation, anti-glial fibrillary acidic protein (GFAP) for astrocytic evaluation, factor VIII–related antigen for endothelial cell evaluation, and with lysozyme and muramidase for microglial evaluation.

Results
All systemic pH, PO2, PCO2, and plasma glucose data were within normal limits. Sham-operated rats did not exhibit cerebral cell damage or HSP-72 synthesis. The contralateral hemisphere, cerebellum, and brain stem of rats with MCA occlusion also did not exhibit obvious brain damage or HSP-72 induction. HSP-72 was detected bilaterally in the choroid plexus for all durations of ischemia.

10-Minute Ischemia
No morphological evidence of ischemic cell injury in the MCA territory was noted in the cerebrum, except for very few nuclear pyknotic and eosinophilic neurons (red neurons) found in the margin of the caudate putamen close to subcortical white matter.

Many HSP-72–positive neurons were present in the caudate putamen and some in the globus pallidus (Figure 1a). HSP-72 was induced only in morphologically intact neurons. HSP-72 was present in the soma and processes of small to medium-sized neurons but not in large neurons. No HSP-72–positive astrocytes were detected.

20-Minute Ischemia
As in 10-minute ischemia, most neurons were morphologically intact throughout the brain except for those located in the caudate putamen. Most neurons in the caudate putamen (>50%) were red neurons and ghost neurons, and the remaining neurons exhibited acute morphological change, characterized by nuclear shrinkage, and cytoplasmic scalloping. The neuropil was pale and slightly spongy. The large neurons of the caudate putamen appeared normal.

The area of HSP-72–positive staining was enlarged (Figure 1b) compared with staining found after 10 minutes of ischemia. HSP-72 was detected in neurons and glia. Many HSP-72–positive neurons were present.
FIGURE 1. Coronal 50-μm vibratome sections show topographic profile of 72-kd heat-shock protein (HSP-72)-positive neurons 48 hours after graded transient middle cerebral artery occlusion, as described in text. Panel a: 10-minute ischemia; HSP-72 induction is localized to caudate putamen and globus pallidus. Panel b: 20-minute ischemia; extensive HSP-72 induction in globus pallidus, olfactory tubercle, and piriform, insular, and parietal cortices. Panel c: 30-minute ischemia; anatomic distribution of HSP-72 at this time encompassed a larger area compared with other durations of ischemia. Panel d: 60-minute ischemia; less prominent distribution of HSP-72 than that found after 30-minute ischemia. Panel e: 90-minute ischemia; HSP-72-positive cells are present in peripheral ring but absent from necrotic core. Figure for 120-minute ischemia is not shown because 120-minute ischemia reveals neuronal HSP-72 distribution and intensity similar to that found after 90 minutes of ischemia. Panels a–e, x5.4.

in the small and medium-sized neurons of the globus pallidus, olfactory tubercle and adjacent structures, and preoptic areas. HSP-72 was induced in nuclei, cytoplasm, and processes of neurons in all six cortical layers, with the most intense staining found in the middle cortical laminae of layers II–V of the piriform, insular, and parietal cortices (Figure 2a). HSP-72 induction was observed in scattered microglia in the caudate putamen. HSP-72-positive neurons were no longer present in the caudate putamen (Figure 1b). The processes of the HSP-72-positive glia were thicker proximal to the cell body and thinner at the more distal branches (Figure 2b). These processes were freestanding and did not form vascular end feet. No GFAP-positive cells were present in the HSP-72-positive glial cell area, suggesting that these cells are not astrocytes. However, HSP-72-positive glia were detected in the region in which the rod-shaped nuclei and cytoplasm of glia were stained by lysozyme and muramidase, suggesting that these HSP-72-positive cells are microglia.
30-Minute Ischemia

Morphologically intact neurons were present in most brain areas, with red or ghost neurons localized to the caudate putamen (>50%). A few large neurons, exhibiting only mild ischemic change, with a normal nucleus and scalloping at the cytoplasmic border or nuclear shrinkage and smeared nuclear–cytoplasmic border, were found in the caudate putamen. Very few (1%) scattered red neurons and other mildly ischemic neurons were found in the parietal cortex.

HSP-72 induction was more extensive and prominent than in the specimens with shorter durations of ischemia (Figure 1c). Positive neuronal staining for HSP-72 immunoreactivity spread from the piriform, insular, and parietal cortices to the limbic, frontal, and perirhinal cortices. HSP-72 induction was now also present in neurons of amygdala. HSP-72 was present in scattered microglia in the caudate putamen.

60-Minute Ischemia

Morphologically intact neurons were present in most areas of the occluded MCA territory. A distinct zone of neuronal damage was localized to the caudate putamen, in which most neurons (>80%) were red neurons or ghost neurons. Most (>90%) large neurons in the caudate putamen were intact, except for a few (<10%) large neurons that showed mild ischemic damage, or moderate ischemic damage with shrunken nuclei and no nuclear–cytoplasmic detail (dark neurons). Very few (1%) scattered mildly ischemic neurons (dark or red neurons) were found in the cortices.
HSP-72-positive neurons were less prominent in the piriform, insular, parietal, limbic, frontal, and perirhinal cortices (Figure 1d). However, dense HSP-72 staining was now widespread in neurons within the occipital and temporal cortices. No HSP-72-positive neurons were found in the caudate. HSP-72-positive microglia were found in the caudate putamen, globus pallidus, olfactory tubercle and adjacent structures, and preoptic area.

**90-Minute Ischemia**

Two distinct zones of neuronal damage were apparent. Total neuronal and astrocytic necrosis was found in the caudate putamen, lateral globus pallidus, and the piriform, insular, and parietal cortices. In a marginal zone, peripheral and adjacent to the ischemic center, a few either dark or red neurons were scattered among the mostly morphologically intact neurons in limbic and frontal cortices and medial globus pallidus.

A sharp demarcation of HSP-72-positive neurons was present; morphologically intact HSP-72-positive neurons were present in the peripheral zone and absent from the necrotic core (Figure 1e). In the necrotic core, HSP-72 was present in cells with bipolar processes in the cortices (Figure 2c) and in endothelial cells (detected by factor VIII staining) with open and round lumen and in some collapsed endothelial cells with oval and narrow lumen (Figure 2d) in the caudate putamen and globus pallidus. Neuronal induction of HSP-72 in the periphery of the neuropil was distinctly outlined as an irregular ring in the cortex and in the medial globus pallidus. The irregular ring, approximately 2 mm in height and 1.25–2.5 mm in width, included neurons in the limbic, frontal, temporal, and occipital cortices.

In the cortices, in the region between the HSP-72-positive neurons in the periphery and the necrotic neurons in the core of the lesion, HSP-72 induction was found in scattered microglia (Figure 3). In the globus pallidus peripheral to the necrotic center, HSP-72 was detected in neurons, microglia, cells with bipolar processes, and endothelial cells. These HSP-72-positive cells formed a band similar to that found in the cortex. HSP-72-positive cells (neurons, microglia, bipolar cells, endothelial cells) were also observed in the olfactory tubercle and adjacent structures and in the amygdala. These cells formed an irregular pan-shaped border area, together with HSP-72-positive neurons in the globus pallidus and cortices.

**120-Minute Ischemia**

As in 90-minute ischemia, two distinct zones of tissue damage were detected. A zone of neuronal and astrocytic necrosis overlapped the necrotic region, extending to include parts of the limbic, frontal, temporal, and occipital cortices. The neuropil was pale and spongy. A marginal zone bordering the necrotic region exhibited few dark or red neurons (<1%). This region extended to the cingulate and orbital cortices and the lateral hypothalamus.

HSP-72 induction was similar to that found in 90-minute MCA occlusion, except that the region devoid of neuronal HSP-72 was larger compared with that found in 90-minute MCA occlusion. HSP-72-positive neurons, microglia, bipolar cells, and endothelial cells were also found in the amygdala. An irregular boundary of HSP-72-positive neurons, 2 mm in height and 1.25–2.5 mm in width, was detected adjacent to the region of neuronal necrosis, in the cortical layers. The HSP-72-positive neuronal boundary to the necrotic core enlarged to encompass the cingulate and orbital cortices and the lateral hypothalamus. HSP-72-positive neurons were also observed in hippocampal CA1-CA4 regions and formed an irregular pan-shaped border together with HSP-72-positive neurons in the globus pallidus and cortices.

A diagrammatic summary of the progressive changes in neuronal damage (indicated by hematoxylin and eosin staining) and neuronal HSP-72 expression with increasing durations of ischemia is presented in Figure 4.

**Discussion**

A progressively larger lesion and more extensive ischemic cell damage occurred with increasing duration of ischemia (10–120 minutes). Ten minutes of MCA occlusion followed by 48 hours of reperfusion caused no cerebrovascular neuronal necrosis, except for a very few neurons located in the caudate putamen. A longer duration of ischemia resulted in the appearance of increasingly larger numbers of red and ghost neurons. The areas where neuronal ischemic injury developed exhibited a predictable progression initially involving the caudate putamen and followed by the globus pallidus, cerebral cortex, and then the amygdala. The caudate putamen was always the most severely affected area in each rat. This difference in damage between caudate putamen and cortex may reflect the diversity in terminal arteries and collateral circulations.
HSP-72 was expressed only by endothelial cells. The absence of HSP-72–positive astrocytes is in contrast to the report of HSP-72–positive astrocytes found after transient focal ischemia in rats by other investigators. However, the cells identified as astrocytes may be microglia. In our preparations, the distribution and location of the HSP-72–positive glia were consistent with microglia, as identified by lysozyme and muramidase staining. The absence of processes reaching the capillaries does not support the astrocytic identity of these cells. Hypertrophic astrocytes, identified by GFAP in our study, were detected in regions of HSP-72–positive neurons, but no HSP-72–positive astrocytes were detected. HSP-72–positive astrocytes are prominently induced by brain hyperthermia. The shape of HSP-72–positive astrocytes after hyperthermia is distinctly different from that of the microglia.

The existence of a “penumbra” or ring around the focal ischemic center in cortices, consisting of possibly reversibly injured neurons, has been demonstrated via hemodynamic measurements in the rat. A zone of HSP-72–positive neurons sharply demarcated the focal ischemic center from viable neurons, and this zone of morphologically intact HSP-72–positive neurons may therefore correspond to the penumbral area around the focal ischemic center.

HSP-72 expression 48 hours after MCA occlusion provides a sensitive indicator of progressive changes after ischemia with increasing duration of ischemia. This study also suggests that HSP-72 expression may be a sensitive and early marker of ischemic cell damage, with expression of HSP-72 preceding changes detectable by light microscopy. Neurons that do not express HSP-72 are either necrotic or possibly not stressed. Neurons that express HSP-72 are morphologically intact and possibly viable. Thus, the presence of HSP-72 in brain cells may have potential applications for therapeutic intervention.

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References

The report in this issue by Li and colleagues describes the magnitude and extent of the "heat shock" or stress protein response which develops in brain during focal ischemia. Using a model of reversible MCA occlusion in rats, Li detected HSP-72-like immunoreactivity (HSP-72-LI) in vulnerable brain regions within the distribution of the MCA territory and in its immediate surround. The appearance of the immunoreactive gene product anticipated the appearance of cell injury (by H&E staining) and cell death by many minutes. Most importantly, HSP-72-LI became expressed within the peri-infarct zone, indicating that brief pretreatment with hyperthermia or ischemia which conferred cytoprotection, cell populations expressing these proteins, and mechanisms by which this cytoprotection is achieved. Nevertheless, the data make the point quite clearly that pathophysiological events such as ischemia activate specific genetic programs which make certain cells relatively resistant to subsequent injury. Consistent with this overall formulation, recent reports indicate that brief pretreatment with hyperthermia or ischemia attenuates ischemic brain damage in vivo. The challenge now is to determine just how this neuroprotection is achieved so that rational strategies can be developed to detect and manipulate gene expression in brain cells vulnerable to ischemia and other forms of brain injury.

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Editorial Comment

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Two papers published in Neuron recently by Rordorf, Koroshetz, and Bonventre (Boston) and Lowenstein, Chan, and Miles (San Francisco) provide strong and confirmatory evidence in vitro that the induction of stress proteins is indeed cytoprotective to neurons. Newly synthesized stress proteins mediate ameliorative effects against glutamate-induced excitotoxicity in cultures of rat cortical and cerebellar granule cells. Heating cells (e.g., cortical neurons to 42.5°C for 20 min) caused new expression of specific families of heat-shock proteins and at the same time suppressed the synthesis of all other proteins as detected by the incorporation of 35S-methionine into protein. The heat-shock response caused a shift to the right in the dose-toxicity relation between glutamate and cell death. The protective interval lasted 3-24 hours but was less than 48 hours, presumably well beyond the half-life of the relevant proteins. The cytoprotective effect depended upon new protein synthesis inasmuch as the preventive effects of prior heating were blocked by the addition of cycloheximide or actinomycin D (an inhibitor of protein and RNA synthesis, respectively) prior to heat-shock exposure. Not surprisingly, the stress protein response did not confer absolute protection inasmuch as the protection against glutamate toxicity was greatest under conditions in which mild degrees of cell injury occurred. Nevertheless, the stress protein response decreased excitotoxic damage caused by all doses of glutamate except those causing maximal injury. Cell survival within the ischemic core versus penumbral zone of the intact animal must be considered in this context.

Questions remain as to the precise stimulus for gene activation, heat-shock proteins or pattern of proteins which confer cytoprotection, cell populations expressing these proteins, and mechanisms by which this cytoprotection is achieved. Nevertheless, the data make the point quite clearly that pathophysiological events such as ischemia activate specific genetic programs which make certain cells relatively resistant to subsequent injury. Consistent with this overall formulation, recent reports indicate that brief pretreatment with hyperthermia or ischemia attenuates ischemic brain damage in vivo. The challenge now is to determine just how this neuroprotection is achieved so that rational strategies can be developed to detect and manipulate gene expression in brain cells vulnerable to ischemia and other forms of brain injury.

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