The Neuroprotective Effects of Heat Shock Protein 27 Overexpression in Transgenic Animals against Kainate-induced Seizures and Hippocampal Cell Death*

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The 27-kDa heat shock protein (HSP27) has a potent ability to increase cell survival in response to a wide range of cellular challenges. In order to investigate the mode of action of HSP27 in vivo, we have developed transgenic lines, which express human HSP27 at high levels throughout the brain, spinal cord, and other tissues. In view of the particular property of HSP27 compared with other HSPs to protect neurons against apoptosis, we have tested these transgenic lines in a well-established in vivo model of neurotoxicity produced by kainic acid, where apoptotic cell death occurs. Our results demonstrate for the first time the marked protective effects of HSP27 overexpression in vivo, which significantly reduces kainate-induced seizure severity and mortality rate (>50%) in two independent lines and markedly reduces neuronal cell death in the CA3 region of hippocampus. This reduced seizure severity in HSP27 transgenic animals was associated with a marked attenuation of caspase 3 induction and apoptotic features. These studies clearly demonstrate that HSP27 has a major neuroprotective effect in the central nervous system in keeping with its properties demonstrated in culture and highlight an early stage in the cell death pathway that is affected by HSP27.

The heat shock proteins (HSPs)1 are a family of proteins originally identified as being up-regulated in response to elevated temperature, but now a wide range of cellular stresses such as hypoxia, ischemia, glutamate, and heavy metals have been shown to induce HSPs (1–6). HSPs consist of a family of highly conserved proteins grouped according to their molecular size: the high molecular mass proteins (110, 90, 70–72, and 55–60 kDa) and the small HSPs, which include HSP27, ubiquitin, αA- and αB-crystallin, and related species. Although highly conserved across species, variation in protein size occurs; for example, the 27-kDa human HSP27 has a corresponding isoform of 25 kDa in rodents referred to as HSP25. HSPs are both constitutively expressed and induced in response to stressful stimuli (e.g. HSP27 and HSP70). Rapid induction of HSP expression is mediated by specific heat shock factors (heat shock factors 1–4), which regulate transcription (7).

The HSPs play a key role in cellular defense systems, acting as protein chaperones facilitating protein folding and the removal of aberrant proteins. These properties have been shown to contribute to the enhanced cellular survival produced following preconditioning stimuli in which a subthreshold stimulus is used to raise endogenous heat shock protein levels prior to the main stimulus. Primary neuronal cultures are protected by prior exposure to mild heat or ischemic stress before subsequent more severe heat or ischemic stress or exposure to glutamate (8–10). In cardiac tissue, a mild heat shock also protects against a subsequent thermal or ischemic stress (11).

The effects of heat shock can be mimicked by overexpression of HSFs alone. Both the ND7 immortalized neuronal cell line, which is derived from dorsal root ganglia neurons, and primary cultures of dorsal root ganglion neurons can be protected against subsequent ischemic or thermal stress by overexpression of HSP70 (12–15) or HSP90 (90-kDa HSP) (13, 15). In cardiac cells or primary cultures of cardiomyocytes, overexpression of HSP70 protects against subsequent thermal or ischemic stress (16–18), but HSP90 only protects against thermal stress and does not protect against ischemic stress.

Marked differences are seen for each HSP in their tissue and cellular specificity and their response to different insults. For example, seizure activity is associated with a rapid HSP70 induction in hippocampal neuronal populations, whereas HSP27 induction is primarily in glial cells and of longer duration (19). Similarly, whereas prior heat shock protects ND7 neuronal cells and primary neurons against apoptosis due to withdrawal of serum or neural growth factor (19, 20), overexpression of HSP70 or HSP90 is not protective. Overexpression of HSP27 in ND7 neuronal cell lines does, however, protect not only against subsequent exposure to heat shock and simulated ischemia but most importantly to stimulation of apoptosis by serum withdrawal in the presence of all-trans-retinoic acid (21). Similarly, in primary dorsal root ganglion cells, infection with herpes simplex virus vectors expressing HSP27 or HSP70 protects against heat shock and simulated ischemia, whereas only expression of HSP27 protects against apoptosis induced by neural growth factor withdrawal (21). HSP27 also reduces the extent of apoptosis in monocytic cells exposed to DNA-damaging agents (22) and fibrosarcoma cells exposed to Fas-induced apoptosis.
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Although both HSPs were protective against heat shock and apoptosis in neuronal cells, which was not evident with HSP70, that transfection with HSP27 conferred protection against withdrawal or treatment with ceramide (25).

Through our previous studies, we were thus able to show that transfection with HSP27 conferred protection against apoptosis in neuronal cells, which was not evident with HSP70, although both HSPs were protective against heat shock and hypoxia. This finding prompted us to develop transgenic mouse lines overexpressing the human HSP27 (hHSP27) gene in order to determine whether evidence could be obtained in vivo for protection against apoptotic and ischemic insults in CNS. No such transgenic mouse has been previously reported for HSP27, although transgenic mice overexpressing HSP70 have been generated. Studies of cerebral ischemia using HSP70 transgenic lines have not yielded consistent results; whereas some authors have shown reduced cerebral infarcts (26), others do not show reduced infarct size following permanent cerebral focal ischemia (27). This may be due to background strain differences and/or differences in expression levels, since different promoters were used in these studies (28).

In order to carry out further elucidation of the neuroprotective properties of HSP27 and to determine whether in vivo protection can be demonstrated by HSP27, we generated transgenic mouse lines in which wild-type hHSP27 was expressed at high levels in cardiac and CNS tissue. Three independent lines were generated in order to confirm that protective effects could be replicated and to ensure that the effect was due to the HSP itself rather than due to the particular site of integration. These independent transgenic lines were then tested in the CNS using the in vivo administration of kainic acid to induce seizures and hippocampal cell death. All transgenic lines showed abundant hHSP27 expression throughout the brain, with particularly high expression levels in all hippocampal subfields. The kainic acid model is a well established experimental model of temporal lobe epilepsy in which systemic kainic acid results in sustained seizures followed by a selective pattern of neuronal damage in vulnerable limbic structures, most notably the CA3 region of hippocampus (29, 30). Although the mode of cell death includes both apoptotic and necrotic components, it provides a useful model to induce apoptotic cell death in vivo (31–34).

The results obtained show that HSP27 plays an important role in CNS neuroprotection, as predicted from earlier cell culture studies, and in addition provide a preliminary insight into the site of action of HSP27 in vivo in the cell death cascade through its effects on caspase 3 induction.

EXPERIMENTAL PROCEDURES

Establishment of Transgenic Mouse Lines Expressing Human HSP27—Transgenic mice were created using a transgene containing human HSP27 cDNA with a chicken β-actin promoter and cytomegalovirus enhancer (pCAGGS) (35). In order to track expression of the transgene, a hemagglutinin (HA) tag was placed contiguous with the human HSP27 cDNA sequence. The linearized transgene was microinjected into pronuclei of fertilized eggs from C57BL10 × CBA/Ca mice, which were subsequently transferred to pseudopregnant recipients. This procedure was carried out in the Imperial College Gene Targeting Unit. At 3–4 weeks after birth, a small tail biopsy was taken from pups, and genomic DNA screened for the integration of the transgene using PCR. Founders revealed by this screening were used to establish independent transgenic lines by breeding to wild-type F1 hybrid (C57BL10 × CBA/Ca) mice. Following successful transmission, a range of tissues were the human HSP27 probes and the hemagglutinin tag probe yielded identical patterns of expression. For detection of human HSP27 mRNA, two probes (A and B) were used, which were based on the human HSP27 sequence (37). Probe A is a 34-mer oligonucleotide complementary to nucleotides 540–572, and probe B is a 33-mer oligonucleotide complementary to nucleotides 593–625. Another human HSP27 probe and the hemagglutinin tag probe yielded identical patterns of expression. For mouse HSP25 mRNA, three probes (1, 2, and 3) were used, which are based on the mouse HSP25 sequence (38). Probe 1 is a 34-mer oligonucleotide complementary to nucleotides 358–391, probe 2 is a 33-mer oligonucleotide complementary to nucleotides 497–529, and probe 3 is complementary to nucleotides 550–582. All three HSP25 probes yielded identical distributions of expression. For mouse glial fibrillary acidic protein (GFAP) mRNA, a 45-mer oligonucleotide complementary to nucleotides 1119–1163 of the mouse GFAP gene (39) was used. For detection of caspase 3 (mouse CPP32) mRNA, three probes (A, B, and C) were used, which were based on the mouse CPP32 gene sequence (40). Probe A is a 32-mer oligonucleotide complementary to nucleotides 577–608, probe B is a 31-mer oligonucleotide complementary to nucleotides 115–145, and probe C is a 32-mer oligonucleotide complementary to nucleotides 147–176. These probes gave similar distribution patterns. The data presented in this paper have been obtained using probe A. Oligonucleotide probes were 3′-end-labeled using terminal deoxynucleotidyl transferase (Promega) and [35S]dATP (Amersham Biosciences).

Cryostat sections were processed, and in situ hybridization was carried out as previously described (19). In brief, slides were fixed in 4% paraformaldehyde in PBS (4 °C) for 10 min, rinsed in PBS for 2 × 5 min, and then treated with 0.25% acetic anhydride in 0.1 M triethanolamine, 0.9% NaCl for 10 min. Following dehydration in increasing concentrations of ethanol, the sections were delipidated in chloroform for 5 min, rinsed in methanol, and allowed to air-dry. Sections were mounted overnight at 42 °C with 1–2 × 10⁶ cpm of labeled probe in 100 μl of hybridization buffer (5× Denhardt’s solution (0.02% bovine serum albumin, 0.02% Ficoll, and 0.02% polyvinylpyrrolidone), 4× standard saline citrate (SSC), 50% formamide, 10% dextran sulfate, 200 μg/ml polyadenylic acid, 200 μg/ml sheared single-stranded salmon sperm DNA, 0.1% sodium phosphate buffer, and 20 mM dithiothreitol). On the following day, sections were stringently washed (1× SSC at room temperature for 30 min, 1× SSC at 55 °C for 30 min, 0.5× SSC at 55 °C for 30 min, 0.1× SSC at 55 °C for 30 min, and 0.1× SSC at room temperature), dehydrated in an ascending series of ethanol, and air-dried. When dry, sections were exposed to x-ray film (BioMax MR, Eastman Kodak Co.) for 14 days and developed in Dupont grain development emulsion (Ilford K5) and exposed for 1–6 weeks at 4 °C, developed, and counterstained with toluidine blue for light microscopic analyses.

Semi-quantitative Analysis of Caspase 3 mRNA—The semi-quantitative analysis of signal intensity in the CA3 region of the hippocampus from all three lines generated. Histological comparisons of brain and cardiac tissue from the different mouse lines revealed no observable morphological differences when compared with corresponding tissue from their respective wild-type littermates, although no detailed (quantitative) studies were carried out as a part of this study.
was carried out by assigning a score in the range of 0–3 (0 = no signal, 1 = low signal, 2 = moderate signal, and 3 = maximum signal intensity obtained). All sections were hybridized, exposed to autoradiographic film, and developed together in the same experiment. Scoring of sections was carried out by three observers without knowledge of the treatment. There was good agreement of scores, and where differences occurred, a mean of scores was taken. Group scores were analyzed using the Mann-Whitney U test (InStat version 2.01).

**Analysis of Neuronal Cell Density in Hippocampus**—Serial coronal sections of the brain were cut at 10 μm, stained with hematoxylin-eosin, dehydrated through a series of alcohols, cleared in xylene, and coverslipped. Histological analysis of cell morphology and cell counts in the hippocampus were confined to approximately 1.94 to 2.46 mm posterior to bregma (41). The Nikon Eclipse E800 microscope used for this study was equipped with a motor-driven stage to traverse the x and y axes. To define the boundaries of the hippocampus, slides were viewed with a ×10 objective. The entire hippocampus was then captured by a JVC 3-CCD color video camera, and the image was tiled onto a color monitor screen. A geometrical area of interest (0.036 mm²) was placed in the CA1 and CA3 regions of the hippocampus. This procedure was standardized in order to sample the same areas of these regions. Digital images were analyzed by the Image-Pro Plus 4.1 program (Data Cell). Morphological criteria of the normal pyramidal cells were established in the control mice based on size (length) and cellular staining. To prevent counting glial cells and degenerating neurons, all counting was thresholded at a minimal cell length of 0.009 pixels. The mean neuron count was obtained from three sections per animal. Cell counts were averaged, and neuronal cell density was expressed in terms of morphologically normal nuclei per square field of 0.036 mm². Differences in neuronal cell densities between all of the treatment groups were assessed using the Kruskal-Wallis nonparametric analysis of variance test followed by Mann-Whitney U test (InStat version 2.01).

**Western Blot Analysis**—Tissues were snap-frozen in liquid nitrogen, and protein was extracted directly by homogenization in SDS sample buffer containing 10% SDS and a mixture of protease inhibitors. When quantification of protein concentration was required, the samples were homogenized in lysis buffer plus protease inhibitors to give a final protein concentration of 100 mg/ml based on tissue wet weight. Protein concentration was determined using the detergent-compatible protein assay (Bio-Rad). For protein gels, 100 μg of total protein was then further solubilized in 20 μl of 10% SDS sample buffer for each gel track. Proteins were separated on 10–20% acrylamide gradient Tris-glycine gels (Bio-Rad) and transferred onto polyvinylidene difluoride membranes (Amersham Biosciences) for 1 h at 100 V. Membranes were then air-dried. The efficiency of protein transfer was assessed by staining the membrane with 1% Amido Black in 10% methanol plus 10% acetic acid, destaining, and washing in Tris-buffered saline, pH 8, 0.05% Tween 20 (TBST). Non-specific binding was blocked using a 5% solution of dried milk powder (Marvel) in TBST. Western blot analysis was carried out using an antibody directed against the HA tag (Bio-Rad). For protein gels, 100 μg of total protein was then further solubilized in 20 μl of 10% SDS sample buffer for each gel track. Proteins were separated on 10–20% acrylamide gradient Tris-glycine gels (Bio-Rad) and transferred onto polyvinylidene difluoride membranes (Amersham Biosciences) for 1 h at 100 V. Membranes were then air-dried. The efficiency of protein transfer was assessed by staining the membrane with 1% Amido Black in 10% methanol plus 10% acetic acid, destaining, and washing in Tris-buffered saline, pH 8, 0.05% Tween 20 (TBST). 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probe for the HA tag sequence, which was contiguous with the HSP27 transgene. All three probes produced identical RNase A-sensitive patterns of expression in brain sections from unstressed hHSP27(18) mice, whereas no signal was detected in sections from unstressed and kainate-stressed wild-type mice (HSP27-probe B, data shown in Fig. 3, A–D; HSP27-probe A and HA tag probe, data not shown). Probe B was used for further characterization of CNS expression of the HSP27 transgene.

To validate further the specificity of these probes for the HSP27 transgene, three additional probes (probes 1–3) directed to the endogenous inducible mouse HSP25 gene (38) were synthesized. All three probes (1, 2, and 3) yielded identical hybridization signals in brain sections from kainate-stressed hHSP27(18) and kainate-stressed wild-type mice that greatly contrasted with the hybridization pattern for the HSP27 transgene (probe 1 data shown in Fig. 3, G and H; probe 2 and 3 data not shown). Furthermore, the HSP25 mRNA signal was absent in mouse brain sections from unstressed hHSP27(18) mice (Fig. 3E), unstressed wild type mice (Fig. 3F), and RNase A-treated sections (data not shown), thus confirming the species specificity of the probes. Therefore, the transgenic mice clearly contain greatly elevated levels of HSP27 mRNA, which is present in the absence of stress.

In kainate-treated mice, HSP25 mRNA expression was induced intensely in thalamus, moderately in hippocampus and at low levels in the cerebral cortex (Fig. 3, G and H) being predominantly localized to glial cells (data not shown). Induction of HSP25 appeared to be similar in both control and transgenic animals, indicating no suppression of endogenous HSP25 induction by the transgene.

A molecular marker of brain injury in response to kainate, GFAP induction, was used to substantiate the behavioral response to kainate. GFAP mRNA expression was up-regulated following kainate administration to a similar extent in both wild-type and hHSP27(18) mice (Fig. 3, K and L) compared with PBS-treated control mice (Fig. 3, I and J). High expression of GFAP mRNA was seen in hippocampus, entorhinal cortex, and septal nuclei, whereas moderate to low levels were observed in corpus callosum and thalamus (Fig. 3, K and L).

Expression of hHSP27 mRNA was extensive throughout cerebral cortex, CA1 and CA3 of hippocampus, dentate gyrus, striatum, and some thalamic regions (e.g. ventro-posterior lateral and medial thalamic nuclei). Moderate levels of expression were seen in lateral and medial habenula nucleus, dorsal lateral geniculate, and lateral septal nucleus. Little expression was seen in white matter (Fig. 4). There was some variation in the level of expression between lines; for example, in line 18, expression was highly abundant in cerebral cortex, hippocampus, striatum, and cerebellum, whereas for lines 59 and 64, a moderate but more widespread expression was seen throughout the brain.

Cellular Labeling of hHSP27 mRNA—Analyses of emulsion-dipped sections at the light microscopic level revealed a predominantly neuronal expression for hHSP27 mRNA in the dentate granule cells, CA1, CA3 (Fig. 5, B and D), and hilar cells of the hippocampus, striatum (Fig. 6B), and cerebral cortex (Fig. 6D) in all three lines. Intense labeling was seen in Purkinje cells of the cerebellum (data not shown), and moderate glial labeling was observed in the corpus callosum (Fig. 6F) of all mice expressing the hHSP27 transgene. Small cells with the characteristic appearance of glial cells, which were positive for hHSP27, were also seen in hippocampal regions (data not shown).
Behavioral Effects of Kainate Administered Intraperitoneally—For the present study, we have concentrated on a single dose of kainate (25 mg/kg, intraperitoneally) that produces a consistent and maximal effect in our hands. Higher doses increase the mortality rate, and lower doses (20 mg/kg or lower) are less consistent, with fewer animals developing seizures (Fig. 7, inset). Animals were observed for 4 h following kainate administration to allow an evaluation of short term effects of epileptogenesis and seizure activity, and tissue was examined at 24 h and 7 days to characterize gene expression and to investigate morphological effects of cell death, respectively. Wild-type animals injected with kainate exhibited behavioral abnormalities as early as 15 min after administration of kainate, notably behavioral arrest and staring spells. This was followed by facial clonus and masticatory seizures within 30–60 min. Seizure behavior then progressed to forelimb clonus, rearing, and loss of balance within 1–2 h after injection with kainate. Most animals developed status epilepticus, but this varied in duration. In some animals, brief episodes (1–2 min) of generalized tonic-clonic seizures were observed. This behavioral profile in response to kainate injection is consistent with previous studies in mice (42–46).

The time course of response to kainate injection (intraperitoneally) was markedly different in transgenic animals; hHSP27(18) mice overall exhibited much milder seizures throughout most of the 4-h observation period following kainate administration (Fig. 7). Further analysis showed that mean seizure scores were significantly reduced in kainate-treated hHSP27(18) mice at 60 min ($p < 0.01$), 90 min ($p < 0.05$), 120 min ($p < 0.005$), 150 min ($p < 0.005$), and 180 min ($p < 0.05$) compared with wild-type animals (Mann-Whitney U test) (Fig. 7). Seizure scores for a second transgenic line, hHSP27(64) mice, were analyzed in two separate experiments and were also shown to be significantly lower than wild-type mice following kainate injection ($p < 0.05$), and there appeared to be a delay in time to maximum seizure in hHSP27(64) mice, but this property did not reach statistical significance (data not shown). No significant behavioral changes were observed in vehicle-treated animals.

hHSP27 overexpression also had a significant effect on reducing mortality in the two independent lines tested. The mortality rate for hHSP27(18) mice was 18.4%, and the rate for hHSP27(64) mice was 16% after kainate treatment, which was significantly reduced compared with wild-type littermates (38.5%, $p < 0.05$; 40%, $p < 0.05$, respectively, by Fisher’s exact test).

Cell Morphology following Kainate Administration—Typical morphological changes were seen at the light microscopic level following kainate administration (intraperitoneally), which included pyramidal cell loss in CA3 of the hippocampus with the appearance of vacuoles, atrophic changes, cell shrinkage, and chromatin condensation. These effects were seen in kainate-treated wild-type animals but were much reduced in the kainate-treated transgenic line (Fig. 8). The CA3 region showed signs of neuronal degeneration, with some of the neurons exhibiting eosinophilic cytoplasm and basophilic chromatin clumping (Fig. 9, C and E). These features are characteristic of the light microscopy appearance of apoptotic neurons, although no attempt was made to confirm this type of cell death. There were also signs of perineuronal vacuolation, evident in sections from all kainate-treated mice (Fig. 9, D–F).

Quantitative analysis of neuronal loss at 7 days following kainate injection was carried out by automated cell counting of hematoxylin-eosin stained sections in four zones within the CA1 and CA3 regions of the hippocampus in four animal groups: PBS-treated ($n = 3$) and KA-treated ($n = 6$) wild-type animals and PBS-treated ($n = 7$) and KA-treated ($n = 6$)
transgenic animals (hHSP27(18)). Quantitative analysis of the neuronal cell density showed a significant loss of 33.1% (p < 0.05) in CA3 (zone III) in kainate-treated wild-type mice, but this was not significant in other regions analyzed (Fig. 10). There was no significant cell loss in CA1, in agreement with previous reports of kainate neurotoxicity in mice (47, 48). In kainate-treated transgenic animals, cell loss was much attenuated and was not significantly different from the respective vehicle-treated control group.

**Induction of Caspase 3 mRNA in Wild-type and Transgenic Animals**—In order to obtain insight into the mechanism of action of HSP27 in vivo, the levels of caspase 3 mRNA were analyzed, which is known to be an early marker of the initiation of the apoptotic cell death cascade. This response is seen within a few hours of kainate administration, and two representative times of 4 and 8 h after kainate injection. In parallel, the endogenous mouse HSP25 mRNA, which is also rapidly induced by kainate was investigated at 4 and 8 h after kainate injection. In wild-type animals, a prominent induction of caspase 3 was seen in CA3, CA1, and dentate gyrus at 4 h following kainate injection (Fig. 11, B, C, and D), which increased further in CA3 and CA1 at 8 h but decreased in dentate gyrus (Fig. 11, E–G). This effect was markedly attenuated in transgenic animals (hHSP27(18)) at both time points, being absent in most animals at 4 h after kainate injection (4 of 6; e.g. Fig. 11B) or present at low to moderate levels in some (2 of 6; e.g. Fig. 11, C, and D). Similarly, at 8 h most transgenic animals showed little or no induction of caspase 3 (7 of 9; e.g. Fig. 11, E, and F) or moderate induction (2 of 9; e.g. Fig. 11G).

Little induction of mHSP25 mRNA was seen at 4 h after kainate injection, in contrast to the levels of caspase 3 mRNA at 4 h following kainate injection, in contrast to the levels of caspase 3 mRNA.
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![Image](https://via.placeholder.com/150)

**FIG. 9.** High magnification of hematoxylin-eosin-stained sections of the hippocampal CA3 pyramidal cell layer 7 days following vehicle (PBS) or kainic acid (25 mg/kg) administration (KA) to wild-type (Wt) and hHSP27(18) transgenic animals. High magnification sections (>400) of the CA3 subfields show the extent of degeneration in KA-treated wild type mice (C and E). Hematoxylin-eosin staining clearly shows neurons with eosinophilic cytoplasm and large, round, basophilic clumps (an arrowhead points to three in C and four in E). There are also signs of perineuronal vacuolation in the KA-treated tissue (an arrowhead points to three in D, two in E, and three in F). Glial cells in D are indicated by asterisks. Scale bar, 20 μm.

mRNA and protein was abundant within neurons of the cerebral cortex, hippocampus, striatum, cerebellum, and thalamic nuclei. In addition, glial expression was also evident. All three lines demonstrated a similar and robust pattern of expression of human HSP27, which was distinguishable from the expression of endogenous mouse HSP25 at the mRNA level by the use of selective oligonucleotide probes and both at the protein and mRNA level by use of an antibody or oligonucleotide probe specific to the HA tag sequence, which is contiguous with the hHSP27 cDNA sequence.

Our previous studies in cell culture indicated that HSP27 possessed a unique property among heat shock proteins tested to date of being able to protect neuronal cells in culture against apoptotic cell death (21). It was therefore of particular interest to use an *in vivo* model in which apoptotic cell death is known to occur as evidenced from morphological and molecular markers (42). Hence, we used the intraperitoneal administration of kainate to test the neuroprotective properties of hHSP27 overexpression in the transgenic lines generated. This model is well established in several laboratories and leads to a characteristic generation of seizures with induction of caspase 3 in the CA3 and CA1 regions of hippocampus (19, 49, 50), followed by the appearance of apoptotic changes in these regions and cell death (33, 43, 51). We therefore compared the behavioral responses to kainate injection with responses to vehicle injection in groups of two transgenic lines (lines 18 and 64). Morphological effects have been compared in parallel in line 18. A marked effect of neuroprotection was seen in both lines as evidenced from enhanced survival and significantly reduced seizure severity sustained over the major period of seizures (60–180 min).

The effect of HSP27 overexpression on cell death was examined at 7 days after kainate injection, where maximal cell death occurs in the CA3 region of the hippocampus. The severity of cell death usually ranges from 30 to 50% in the mouse (*e.g.* a 25% reduction in cell density following a dose of 34 mg/kg...
kainate). This level of cell loss between kainate and vehicle-treated animals was seen in the control littermates, being 33% (p < 0.05). In contrast, whereas cell density was similar in vehicle-treated transgensics compared with controls, no significant cell loss was detected in transgenic animals after kainate administration.

In order to control for strain differences in susceptibility to kainate, breeding at all generations was always with an F1 hybrid C57BL/CA/Ca to maintain a hybrid genetic background. This strategy was adopted to avoid the marked differences that have been reported between inbred strains in behavioral tests involving kainate (e.g. Refs. 48 and 52). All tests were carried out between littermates that either carried the transgene (+ve) or lacked it (−ve), thus avoiding problems arising from the use of inbred strains. Thus, the reduced seizure susceptibility seen here in two independent transgenic lines is likely to have resulted from the overexpression of the human transgene.

We also show for the first time in this study the gross anatomical labeling pattern of induced endogenous mouse HSP25 mRNA in mice following kainic acid administration. Mouse HSP25 mRNA was predominantly localized to glial cells with modest labeling of neurons, particularly in the pyramidal cell layers of the hippocampus (data not shown), which reproduces the finding in rats (19). The distribution of mouse HSP25 mRNA was similar to that for GFAP mRNA as assessed on adjacent sections by in situ hybridization, although the up-regulation of GFAP mRNA was more profound and widespread, in agreement with similar studies in rats (19). Up-regulation of GFAP has been described as a marker for neurotoxicity and aberrant neuronal activity (53, 54). The comparable pattern of expression and induction levels of mouse HSP25 mRNA and GFAP mRNA in both transgenic and wild-type mice in response to kainic acid would suggest that mice of both genotypes were subjected to a similar level of noxious stress.

The effect of overexpression of hHSP27 clearly showed a significant and rapid effect in the behavioral response to kainate injection, indicating an immediate suppression of the spread of the seizure activity. This may be due to an interaction of HSP27 with components of the excitatory signaling cascade involved in seizure propagation, which have not as yet been elucidated. HSP27 is known to be involved in apoptotic signaling cascades mediated by both caspase-dependent and Daxx components of Fas-mediated pathways (55), and these sites of action are likely to contribute to the longer term effects of HSP27 to reduce overall cell death.

**Mechanism of Neuroprotection Mediated by hHSP27**—In this study, we investigated whether one of the early markers of the cytochrome c-caspase-dependent pathway of apoptosis, caspase 3 induction, was affected in hHSP27 transgenic animals. This response is well known to be associated with kainate-induced cell death. We found a marked attenuation of caspase 3 mRNA induction in transgenic animals at both time points examined, which characterizes the initiation of this cascade and precedes other activation events such as the induction of endogenous mouse HSP25, which is delayed in onset compared with caspase 3 (Fig. 11). This is in keeping with the subsequent attenuated cell death seen in transgenic animals. These preliminary results therefore indicate that in vivo hHSP27 can moderate an early reaction in caspase 3-dependent apoptosis at a stage preceding caspase 3 induction, which in turn has a distinct time course compared with the delayed induction of the endogenous mouse HSP25 mRNA and its translation. The attenuated induction of mHSP25 may reflect a reduced caspase-dependent activation of the heat shock response but could also result from a direct down-regulation of heat shock factor activation of the transcription of heat shock proteins in the absence of denatured proteins.

This site of action of HSP27 is consistent with other studies where HSP27 has been shown to suppress cytochrome c-caspase 3-dependent apoptosis, affecting the release of cytochrome c (56), binding directly to cytochrome c, and thereby inhibiting subsequent apoposome formation and preventing caspase 9 maturation (57). Recently, Benn et al. (58), using...
dissociated primary cultures of dorsal root ganglion cells to study neural growth factor withdrawal-induced apoptosis, showed that infection with herpes simplex virus vectors expressing the sense orientation of HSP27 rescued cells and that these cells were characterized by diffuse cytochrome c labeling in the cytoplasm but lacked activated caspase. This indicated that HSP27 action occurred downstream of cytochrome c release from mitochondria but prior to caspase 3 activation, which is consistent with the present findings. HSP27 appears able to act at multiple sites in this pathway, including more downstream events, through a direct interaction of HSP27 with caspase 3 processing, inhibiting cytochrome c-dependent apoptosis in 293T and L929 cells (59). The cell specificity of these actions of HSP27 remains to be elucidated in more intact systems. Events upstream of cytochrome c are also known to be affected by HSP27 and could contribute to protective effects, for example through the reduction of F-actin damage and through mobilization of members of the Bcl-2 family (56). The inhibitory effect of HSP27 is specific for cytochrome c-caspase-dependent apoptosis and does not affect the actions of apoptosis-inducing factor, which is also released from mitochondria during apoptosis. It has been demonstrated both in vitro and in vivo that large nonphosphorylated oligomers of HSP27 are the active form of the protein responsible for the caspase-dependent antiapoptotic effect of HSP27 (57).

In addition, HSP27 is known to inhibit Fas-mediated apoptosis (55), preventing c-Jun amino-terminal kinase 3 activation. Phosphorylation of HSP27 modulates activity by producing a large change in the supramolecular organization of the protein with a shift from oligomers to dimers. In Fas-mediated apoptosis, phosphorylated dimers of HSP27 interact with Daxx, a mediator of Fas-mediated apoptosis, preventing the interaction with Fas and Ask1 and blocking Daxx mediated apoptosis (55). The Daxx-dependent pathway is distinct from that mediated by FADD, which is not sensitive to HSP27. The involvement of this pathway in neuronal cell death in vivo remains to be elucidated.

It will also be important to determine the role of phosphorylation in mediating both the short and longer term effects of HSP27, which we have observed in vivo. An increase in phosphorylation is detected within minutes of a cellular stress, with the increased expression occurring after a few hours. The rapid stress-induced phosphorylation results from stimulation of the p38 mitogen-activated protein kinase cascade and leads to the phosphorylation of mammalian HSP27 at two or three serine residues by MAPKAP kinase 2. However, phosphorylation differentially affects the protective properties of HSP27. Thus, chaperone activity and the ability to block the mitochondrial pathway are inhibited by phosphorylation, whereas the ability to block Daxx-mediated apoptosis is stimulated by phosphorylation.

In contrast to the different effects of overexpression of HSP27 and HSP70 in neuronal cultures (21), a similar degree of protection has been demonstrated in cardiac cultures (25) and for cardiac tissue from transgenic animals overexpressing either HSP70 (60, 61) or HSP27 using the transgenic mice described here, indicating that both HSPs can interact with damaging oxidative cascades either through their protein chaperone properties or other mechanisms. It is already known that HSP27 increases glutathione (reduced form)-dependent chaperone activity against misfolded or oxidized proteins (62), which could contribute to protection against reactive oxygen species. Using in vivo models of cerebral ischemia, conflicting results have been obtained where protection has been reported by Rajdev et al. (26), whereas no significant difference in infarct size assessed at 24 h was found by Lee et al. (27). In the present study, a clear neuroprotective effect was seen with overexpression of HSP27 in vivo. These results provide further support for the idea that HSP27 is as important as HSP70 in the nervous system.

These studies demonstrate for the first time in vivo an important role for HSP27 in CNS tissue in response to apoptotic injury and provide support for the unique property of HSP27 among HSPs to protect neurons from apoptotic cell death. Recently, we demonstrated that intracerebral viral delivery of HSP27 reduced kainate-induced neuronal cell death in hippocampus in rats (63). Thus, HSP27 shows enormous potential as a target for neuroprotective intervention.

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The Neuroprotective Effects of Heat Shock Protein 27 Overexpression in Transgenic Animals against Kainate-induced Seizures and Hippocampal Cell Death
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