70-kDa Heat Shock Protein Downregulates Dynamin in Experimental Stroke
A New Therapeutic Target?

Jong Youl Kim, PhD; Nuri Kim, BS; Zhen Zheng, MD; Jong Eun Lee, PhD; Midori A. Yenari, MD

Background and Purpose—The 70-kDa heat shock protein (Hsp70) protects brain cells in models of cerebral ischemia. Proteomic screening of mice subjected to middle cerebral artery occlusion identified dynamin as a major downregulated protein in Hsp70-overexpressing mice (Hsp70 transgenic mice). Dynamin-1 is expressed in neurons and participates in neurotransmission, but also transports the death receptor Fas to the cell surface, where it can be bound by its ligand and lead to apoptosis.

Methods—Mice were subjected to distal middle cerebral artery occlusion. Neuro-2a cells were subjected to oxygen glucose deprivation. Hsp70 transgenic and Hsp70-deficient (Hsp70 knockout) mice were compared with wild-type mice for histological and behavioral outcomes. Some mice and neuro-2a cell cultures were given dynasore, a dynamin inhibitor.

Results—Hsp70 transgenic mice had better outcomes, whereas Hsp70 knockout mice had worse outcomes compared with wild-type mice. This correlated with decreased and increased dynamin expression, respectively. Dynamin colocalized to neurons and Fas, with higher Fas levels and increased caspase-8 expression. Hsp70 induction in neuro-2a cells was protected from oxygen glucose deprivation, while downregulating dynamin and Fas expression. Further, dynamin inhibition was found to be neuroprotective.

Conclusions—Dynamin may facilitate Fas-mediated apoptotic death in the brain, and Hsp70 may protect by preventing this trafficking. Dynamin should be explored as a new therapeutic target for neuroprotection. (Stroke. 2016;47:2103-2111.
DOI: 10.1161/STROKEAHA.116.012763.)

Key Words: apoptosis ■ dynamin ■ Fas ■ neuroprotection ■ stroke

The 70-kDa inducible heat shock protein (Hsp70) is known to protect the brain from stressful stimuli, including stroke. Hsp70 also interferes with cell death pathways, such as apoptosis. Precise protective mechanisms are many, and protection by Hsp70 might lead to the identification of new therapeutic targets. To this end, we applied a proteome approach to mice overexpressing Hsp70 (Hsp70 transgenic [Hsp70Tg]) subjected to experimental stroke and compared protein expression patterns to that of similarly injured wild-type (WT) mice. The result of this analysis showed marked reduction of dynamin in the brains of Hsp70Tg mice (Figure I and Table in the online-only Data Supplement).

Dynamin is a member of a family of guanine triphosphatase (GTPase) proteins largely known for their endocytic functions. Dynamin-1, the subject of this study, is exclusive to the brain. Recently, dynamin has been shown to translocate Fas protein from the Golgi apparatus to the cell surface, where it can be bound by its ligand, FasL, and subsequently trigger one of several extrinsic apoptosis pathways leading to caspase-dependent cell death.

Ischemic stroke leads to cell death via several pathways, including apoptosis. The intrinsic apoptotic pathway occurs within mitochondria, whereas the extrinsic pathway is receptor-mediated. Extrinsic apoptosis involves the engagement of death receptors located on the plasma membrane. Receptor ligation causes activation of caspase-8, leading to activation of effector caspase-3. After focal cerebral ischemia, the interaction between the Fas receptor and its ligand (FasL) initiates intracellular signaling cascades that ultimately terminate in caspase-dependent cell death after ischemic stroke.

Dynamin has not been studied extensively and has not been studied in brain ischemia. In the central nervous system, dynamin has been associated with endocytic processes and synaptic transmission. Prior related work has shown that a similar protein in the same family, dynamin-related protein-1 (DRP-1), may be important in brain ischemia because of its role in mitochondrial fission, and DRP-1 inhibition may be.

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neuroprotective. However, to our knowledge, dynamin-1 has not been studied in brain ischemia, nor has its potential connection to Hsp70 been explored.

Methods

All experimental procedures in animals were approved by the local Institutional Animal Care and Use Committee (IACUC) and were in accordance with National Institutes of Health guidelines.

Animal Models

Male Hsp70Tg and Hsp70 knockout (Hsp70Ko) mice weighing 25 to 30 g were produced from breeder mice originally generated by the Dillmann (UCSD) and Pandita (Southwestern University) laboratories. Hemizygotic (Hsp70Tg) and homozygotic (Hsp70Ko) mice were compared with WT littermates.

Distal middle cerebral artery occlusion (dMCAO) was performed as described previously. Briefly, mice were anesthetized with isoflurane (5% for induction, 2% for maintenance via facemask) in a mixture of medical air/oxygen (3:1). The middle cerebral artery was permanently occluded by short coagulation proximal to the olfactory branch.

At the end of the experiments, mice were euthanized and transcardially perfused with normal saline and fixed in 4% paraformaldehyde (PFA) plus 20% sucrose, then frozen.

Infarct Size

Infarct size was determined from cryosections (50 μm) of brains of animals survived 14 days and stained with cresyl violet. Infarct volume was determined as previously described.

Behavior Tests

Neurological assessments were performed as previously described. All studies were recorded using a video camera, and scoring was performed by 2 different investigators.

Bederson Score

The Bederson score was modified for use in mice, as previously reported (grade 0 = no observable neurological deficit; grade 1 = unable to extend the contralateral forelimb; grade 2 = flexion of the contralateral forelimb; grade 3 = mild circling to the contralateral side; grade 4 = severe circling; and grade 5 = falling to the contralateral side).

Ladder Test

Mice were coaxed onto a 40-rung ladder. The number of forelimb faults were counted. Fewer foot falls indicated improved sensorimotor function.

Adhesive Removal (Sticky Tape) Test

50-mm² (4 mm diameter) adhesive were attached to the palm of each forepaw, and mice were observed for 2 minutes and scored for identifying the presence of and removal of the adhesive. Shorter removal times indicated improved neurological recovery.

Elevated Body Swing Test

To measure motor deficits, mice were suspended vertically by the tail with their heads elevated 3 inches above the test bench. A lateral swing was counted each time the animal moved its head >10 degrees away from the vertical axis.

In Vitro Models

Neuro-2a (N2a) cells were purchased from the American-Type Culture Collection. Cultures were grown and maintained in DMEM (Celgro) supplemented with 10% fetal bovine serum defined (Hyclone). Under humidified 5% CO₂, 95% air atmosphere and at 37°C, cells were plated in 25-cm² cell culture flasks (Corning). Media was changed 3 days after seeding and split twice a week. For experiments, cells were plated on 12-well dishes (1×10⁶ cells/well).

Cells were exposed to oxygen glucose deprivation (OGD) by placing in an anoxia chamber (O₂ tension <0.001%; Coy Laboratories) for 2 hours at 37°C. Media was removed and replaced with balanced salt solution lacking serum or glucose or oxygen. Control cultures were incubated at 37°C with balanced salt solution containing 5.5 mmol/L glucose. After OGD, glucose was added to each well to a final concentration of 5.5 mmol/L, and plates were returned to normoxia.

Cell death and viability were assessed by morphological assessment by light microscopy, vital staining (trypan blue; Sigma), and the tetrazolium dye MTT 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Sigma) assay.

17′-N-Allylamo-17-demethoxygeldanamycin and Dynasore Administration

After control or OGD exposure, N2a cell cultures were treated with 60 μM 17′-N-allylamo-17-demethoxygeldanamycin (Sigma) to induce Hsp70 or 30 μM dynasore (Dyna; Sigma), a dynamin inhibitor. Each compound was solubilized in 0.1% dimethyl sulfoxide (Sigma) and dimethylformamide (Sigma) and diluted in PBS.

Intracerebroventricular injections took place 30 minutes after dMCAO or sham surgeries. Anesthetized mice were placed in a stereotaxic frame and a total volume 2 μL of Dyna (0.3 mg/mL dose) or vehicle (0.1% dimethylformamide in PBS) was injected into the right lateral cerebral ventricle (stereotaxic coordinates: 1 mm caudal to bregma, 1.3 mm lateral to sagittal suture, and 2 mm in depth) at a speed of 0.5 μL/ min via a burr hole. The needle was left in place for 5 minutes to allow drug diffusion into tissue before it was removed and the burr hole filled with bone wax.

Coimmunoprecipitation, Membrane Fractionation, and Immunoblotting

For immunoblots, ipsilateral hemispheres or N2a cells were homogenized and solubilized in radioimmunoprecipitation assay (RIPA) lysis buffer (Sigma) with protease inhibitor mixture (ROCHE). Coimmunoprecipitation was performed as previously described by our laboratory. Brain lysates were incubated with 2.5 mg of mouse anti-Hsp70 (Stressgen) or an IgG isotype control (2.5 mg normal mouse IgG; Santa Cruz), and the protein A/G PLUS-Agarose was collected. To detect Fas in the cell membrane, a Subcellular Protein Fractionation Kit (Thermo) was used according to the manufacturer’s instructions to separate and isolate membrane from tissue samples. Twenty micrograms protein samples were subjected to 10% SDS-polyacrylamide gel electrophoresis, then transferred to polyvinylidene fluoride membranes (Millipore), and probed for the protein of interest by incubation with mouse anti-Hsp70 (1:1000; Stressgen) or dynamin-1 (1:1000; Santa Cruz) and rabbit anti-caspase-8 (1:1000; Santa Cruz) or Fas (1:1000; Santa Cruz) antibodies, followed by a horseradish peroxidase-conjugated secondary antibody. Blots were visualized using the enhanced chemoluminescence (ECL) system (Amersham) according to the manufacturer’s directions and imaged using LAS-4000 (Fuji).

Flow Cytometry

After OGD, 1×10⁶ N2a cells were washed once in PBS and blocked with flow cytometry staining buffer (eBioscience) for 10 minutes. Samples were stained with mouse anti-Fas (1:1000; Santa Cruz) followed by Alexa Fluor 488-conjugated goat anti-mouse IgG (1:1000; Invitrogen). Mouse IgG-PE (Santa Cruz) was used as an isotype control. N2a cells were sorted on FACS Calibur (BD Biosciences), and data were analyzed using CellQuest (BD Biosciences).

Immunohistochemistry

Three days after dMCAO, brain sections (10 μm thick) were incubated with primary antibodies against mouse anti-dynamin-1 (1:1000; Santa Cruz) and rabbit anti-caspase-8 (1:500; Santa Cruz) or Fas (1:200; Santa Cruz), followed by secondary biotinylated antibodies and 3,3′-diaminobenzidine (Vector Laboratories) and counterstained with cresyl violet.
Brain sections were colabeled with antibodies against dynamin-1 plus cell markers for neurons (MAP-2), astrocytes (GFAP), microglia (CD11b), or Fas plus dynamin-1 to determine which cell populations expressed dynamin-1, followed by a fluorescent secondary antibody.

TUNEL Staining
Terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL, ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit; Millipore) was used according to the manufacturer’s instructions to assess DNA fragmentation in neuron and described previously. Number of TUNEL-positive cells in the ipsilateral hemisphere were normalized and the numbers of cells counted from brains of sham animals.

Statistical Analysis
All studies were randomized and assessments performed by investigators blinded to experimental conditions. All data were analyzed with standard statistical methods (t test; Systat Software, Inc). Data were presented as the mean±SE. Differences between the 2 groups were compared using an unpaired t test, and multiple comparisons were performed using 1-way analysis of variance followed by Bonferroni’s post hoc test. P≤0.05 was considered significant.

Results
Hsp70 Protects Against Experimental Stroke
Consistent with previously published reports, Hsp70 over-expression led to improved neurological outcomes, whereas Hsp70 deficiency led to worsened outcomes compared with WT mice. Infarct size 14 days after dMCAO from WT, Hsp70Ko, and Hsp70Tg mice was compared. Infarct volumes quantified from cresyl violet–stained sections were significantly smaller in the Hsp70Tg mice compared with WT and Hsp70Ko mice (Figure 1A). Motor function was also improved among Hsp70Tg mice and worsened in Hsp70Ko mice (Figure 1B). Behavioral indices among sham controls did not reveal any baseline differences because of gene manipulation (data not shown).

Hsp70 Overexpression Decreases and Interacts With Dynamin-1
Using our in vitro model, we induced Hsp70 in N2a cells using 17-N-allylamino-17-demethoxygeldanamycin as we previously reported. 17-N-Allylamino-17-demethoxygeldanamycin treatment led to decreased cell death after OGD (Figure IIA and IIB in the online-only Data Supplement) and 2-fold higher Hsp70 induction and 2.3-fold lower dynamin-1 expression relative to controls (Figure IIC in the online-only Data Supplement).

Consistent with observations from the proteomic analysis, Hsp70Tg mice showed reduced dynamin-1 levels compared with knockout and WT mice after brain ischemia, whereas Hsp70Ko mice showed the highest dynamin-1 levels (Figure 2A and 2B). This was seen especially within cells of the ischemic border zone. Double labeling showed that dynamin-1 was present in neurons but not astrocytes or microglia under noninjury conditions. In ischemic brains, dynamin-1,
Fas, and Hsp70 were expressed in neurons (Figure III in the online-only Data Supplement).

Coimmunoprecipitation studies showed that Hsp70 was associated with dynamin-1 (Figure 2C). The highest Hsp70–dynamin-1 associations were seen in the transgenic mice and the least in the knockout mice. Colabeling for dynamin-1 and Fas showed that both colocalized to neuron-like cells and were the highest in Hsp70Ko brains and lowest in the transgenic brains (Figure 2D). Further, several dynamin-positive cells were found to be TUNEL-positive (Figure 2D).

Hsp70 Attenuates Fas-Mediated Apoptotic Death
To explore a link between Hsp70- and Fas-mediated cell death, dMCAO-exposed brains from WT, Hsp70Ko, and Hsp70Tg mice were stained for Fas. Fas-positive cells were found to be increased by brain ischemia, and brains of Hsp70Ko mice expressed more Fas compared with those of either WT or Hsp70Tg mice. Because Fas must be expressed on the cell’s surface to trigger cell death, immunoblots of membrane fractions showed that membrane-bound Fas was significantly decreased in the brain of Hsp70Tg mice, whereas Hsp70Ko mice had 4-fold higher Fas expression (Figure 3A). Further, N2a cells exposed to OGD showed increased Fas-positive cells, and treatment with 17-N-allylamino-17-demethoxygeldanamycin decreased these numbers by ≈42% (Figure 3B). The percentage of Fas+ cells were calculated as follows. R2 demarcated the fluorescein isothiocyanate (FITC) signal threshold for stained cells, excluding baseline FITC levels in unstained controls. R3 excludes FITC levels from nonspecific staining in control samples (secondary antibody only control). R3 thereby

**Figure 2.** A, Dynamin-1 is increased more markedly in 70-kDa heat shock protein knockout (Hsp70Ko) mice 3 days after insult compared with wild-type (WT) mice, whereas expression is reduced in Hsp transgenic (Hsp70Tg) mice brains (n=3/group, scale bar=50 μm). B, Immunoblots show dynamin-1 from WT, Hsp70Ko, and Hsp70Tg mouse brains 1 day after distal middle cerebral artery occlusion (dMCAO). Relative intensities of protein normalized to β-actin show that dynamin-1 was increased in Hsp70Ko compared with WT and Hsp70Tg mice (n=3/group; *P<0.01, **P<0.05). C, One day post ischemia, brain lysates were immunoprecipitated using anti-Hsp70. Immunoblots (IB) of the immunoprecipitate (IP) showed significant levels of dynamin-1, indicating that Hsp70 associates with dynamin-1 (n=3/group; *P<0.01). Relative intensities of the immunoblots showed that Hsp70 highly associated with dynamin-1 in Hsp70Tg. D, Three days after dMCAO, dynamin-1 (red) and Fas (green) colocalized (Merge, yellow). Fas and dynamin-1 were reduced in Hsp70Tg brains and increased in Hsp70Ko brains compared with WT (n=3/group, scale bar=10 μm). In ischemic brain, dynamin-1 also colocalized with TUNEL-positive cells in WT mice.
conservatively estimates the population of Fas-expressing cells within a given sample.

Because caspase-8 is activated downstream of Fas, but not during intrinsic (mitochondria) apoptosis, we assayed for caspase-8. Caspase-8 expression was the most decreased among ischemic Hsp70Tg brain samples compared with either WT or Hsp70Ko mice, whereas caspase-8 expression was highest among Hsp70Ko (Figure 3C). Increased dynamin-1, Fas, and caspase-8 protein also corresponded to an increased number of TUNEL-positive injured neurons, particularly in Hsp70Ko mice. Hsp70Tg mice showed significantly reduced numbers of TUNEL-positive cells by ≈20% to 25% (WT versus transgenic) and ≈35% to 40% (knockout versus transgenic), respectively (Figure 3D). These data suggest that dynamin contributes to Fas-mediated cell death in experimental stroke and that Hsp70 may protect by inhibiting dynamin expression and preventing surface expression of Fas.

Dynamin Inhibition Is Protective
To determine functional significance of dynamin, OGD-exposed N2a cells were treated with Dyna, a dynamin inhibitor which interferes with its GTPase activity. This led to improved cell viability and reduced cell death (Figure 4A and 4B) and 3.4-fold lower dynamin-1 expression relative to Veh groups (Figure 4C) and decreased numbers of Fas-positive N2a cells as demonstrated by flow cytometry (Figure 4D).

At the in vivo level, we first established that Dyna could engage target tissues in the brain. Texas red-conjugated Dyna (courtesy of Dr Nick Cairns, Combinix, Inc Mountain View, CA) was injected intracerebroventricular into uninjured animals. Fluorescent signals were observed in brain tissues showing that Dyna did in fact travel to and bind to brain cells (Figure IV in the online-only Data Supplement). Dyna administration did not seem to cause any seizures or obvious neurological impairment in uninjured mice.

Dyna treatment in dMCAO mice led to smaller strokes compared with vehicle (Figure 5A)-treated controls, with improved motor function (Figure 5B). Dynamin-1 expression was also decreased with Dyna treatment. To determine whether dynamin inhibition might prevent Fas translocation to the cell surface, immunoblots of cytoplasmic and membrane fractions showed that dMCAO led to increased expression of Fas in the membrane fraction, and Dyna treatment decreased this (Figure 5C).

To explore any synergistic actions of Hsp70 and dynamin inhibition, we administered Dyna to Hsp70Tg mice exposed to dMCAO. However, Dyna did not lead to any further lesion size reduction or improvement in neurological function (Figure V in the online-only Data Supplement).
Hsp70Tg mice exposed to stroke led to the identification of dynamin as a markedly downregulated protein. To our knowledge, this is the first report of dynamin in brain ischemia. We show that dynamin increases after stroke, along with Fas and caspase-8, and these proteins are decreased in mice overexpressing Hsp70, whereas the opposite was observed in Hsp70 deficiency. Further, we show that the proportion of membrane-bound Fas is increased after stroke, but is reduced by Hsp70Tg overexpression. This is consistent with prior work that showed that dynamin trafficks Fas to the cell’s surface and is in line with our hypothesis that Hsp70 prevents this trafficking (Figure 6). We also demonstrate for the first time that inhibition of dynamin improves outcome from experimental stroke.

Hsp70 has previously been shown to interfere with many aspects of the intrinsic apoptotic pathway by inhibiting caspase activation, preventing mitochondrial release of cytochrome c or increasing the anti-apoptotic protein Bcl-2. Less has been studied with respect to the extrinsic or receptor-mediated apoptotic pathways. Death receptors include Fas, which initiates cell death with the binding of Fas by its ligand FasL and leads to caspase-8 activation and apoptosis. Fas activation has been documented in ischemic stroke and related pathologies. Fas and its ligand have been documented in the brain after ischemia, and several studies, including some from our laboratory, have shown that interrupting this pathway is protective.

In neurological disease, dynamin-1 deficiency has been linked to defects in γ-aminobutyric acid transmission and...
epilepsy. However, intact dynamin has also been shown to have negative consequences. Dynamin has been linked to Alzheimer’s disease pathology, whereas its inhibition or deficiency led to decreased neuronal degeneration. Dynamin has been shown to traffic Fas protein from the Golgi apparatus through the Trans Golgi network to the cell surface where it can be bound by FasL and may suggest an additional role in brain cell death and degeneration. There are scant reports of dynamin in the ischemia literature. The most widely studied seems to be DRP-1 and its role in mitochondrial fission. In cardiac and renal ischemia, DRP-1 inhibition has been shown to be cytoprotective by preventing mitochondrial apoptosis. There are a few reports characterizing DRP-1 in experimental stroke, and DRP-1 inhibition decreased apoptosis.

The relationship of dynamin to Hsp70 is unknown, but the present data indicate an inverse relationship between Hsp70 and dynamin in the ischemic brain. Hsp70 overexpression attenuated increases in dynamin after experimental stroke, whereas its deficiency did the opposite. Transgenic mice overexpressing Hsp70 had decreased Fas, caspase-8, and membrane-associated Fas. Based on our own experiments and reports in the literature, we postulate 2 potential mechanisms for this relationship. Hsp70 may regulate dynamin at the transcriptional level. Dynamin’s promoter region contains a sequence similar to those recognized by nuclear factor kappa B (NF-kB), as does Fas. Hsp70 is known to inhibit NF-kB’s transcriptional activity and may disrupt dynamin at the transcriptional level. However, it is also possible that Hsp70 may directly inhibit dynamin-dependent Fas

Figure 5. Intracerebroventricular (ICV) injection of Dyna protects against distal middle cerebral artery occlusion (dMCAO). Fourteen days after dMCAO, cresyl violet–stained brain sections show smaller infarcts in mice treated with Dyna compared with vehicle (Veh)-treated mice. A, Infarct size among mice treated with Dyna was reduced compared with mice treated with Veh. B, Bederson, Ladder test, adhesive removal, and body swing tests show that mice given Dyna performed better than Veh-treated mice (n = 7–9/group; *P < 0.01, **P < 0.05). C, Dynamin inhibition attenuated the amount of Fas present on the cell surface in experimental stroke. Immunoblots of brain samples show decreased dynamin-1 expression at 3 days after Dyna treatment after dMCAO (n = 3/group; *P < 0.01). Immunoblots of Fas from membrane and cytosolic fractions of brain tissue 3 days after MCAO show increased membrane Fas in Veh-treated mice compared to sham, and this increase was reduced by Dyna treatment (n = 4/group; *P < 0.01).
translocation by containing dynamin in the cytosol through specific chaperone interactions.

Pharmacological inhibition of dynamin can also protect against stroke. Dynamin inhibition led to protection in our stroke models and reduced Fas expression on the cell surface. However, it should be noted that we administered dynasore intracerebroventriculally shortly after stroke onset. Although this paradigm was designed as a proof of concept study, future studies should further address the optimal timing and dosing of dynamin inhibitors.

Conclusions
We reveal a previously unknown mechanism of protection by Hsp70 in the ischemic brain and identify dynamin as a potential therapeutic target. Future studies may focus on more precise interactions between Hsp70 and dynamin and the development of dynamin inhibitors.

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Disclosures
None.

References

Figure 6. Proposed dynamin and Fas interactions during stroke. Stroke leads to increased membrane Fas expression, presumably because of its trafficking from the Golgi apparatus by dynamin. Fas ligand, also increased after stroke, binds Fas and activates caspase-8 through engagement of its adaptor molecule Fas-associated death domain (FADD), which then leads to cell death through apoptosis. 70-kDa heat shock protein (Hsp70) may reduce both Fas and dynamin expression, but also prevents Fas membrane expression.


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Supplemental Material

Methods

2-dimensional electrophoresis (2-D)

2-D analysis: Brain tissues samples collected 1 day after injury were washed twice in ice-cold PBS and lysed in sample buffer composed with 7 M urea, 2 M thiourea containing 4% CHAPS, 1% DTT and 2% pharmalyte and 1 mM benzamidene. Proteins were extracted for 1 hour at room temperature with vortexing. After centrifugation at 8,000 g for 1 hour at 4°C, insoluble material was discarded and soluble fraction was used for 2-D gel electrophoresis. Protein loading was normalized by Bradford assay. A. 2-D PAGE: IPG dry strips were equilibrated for 12-16 hours with 7 M urea, thiourea containing 2% 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS, Sigma), 1% dithiothreitol (DTT, Sigma), 1% pharmalyte in 200 μg samples. Isoelectric focusing (IEF) was performed at 20°C using a Multiphor II electrophoresis unit and EPS 3500 XL power supply (Amersham) following manufacturer’s instruction. For IEF, the voltage was linearly increased from 150 to 3,500 V during 3 hours for sample entry followed by constant 3,500 V, with focusing complete after 96 kVh. Prior to 2-D, strips were incubated for 10 minutes in equilibration buffer (50 mM Tris-Cl, pH 6.8 containing 6 M urea, 2% SDS and 30% glycerol), first with 1% DTT and second with 2.5% iodoacetamide. Equilibrated strips were inserted onto SDS-PAGE gels (20-24 cm, 10-16%). SDS-PAGE was performed using the Hoefer DALT 2-D system (Amersham) following manufacturer’s instructions. 2-D gels were run at 20°C for 1.7 kVh, and then were silver stained as described by Oakley et al 1, but fixing and sensitization step with glutaraldehyde was omitted. B. Image analysis: Quantitative analysis of digitized images was carried out using the PDQuest software (BioRad) according to the protocols provided by the manufacturer. The quantity of each spot was normalized by total valid spot intensity. Protein spots were selected for significant expression variation deviated over two fold in expression level compared with control or normal sample.

MALDI-TOF analysis: Enzymatic digestion of protein in-gel spot was enzymatically digested in-gel in a manner similar to that previously described by Shevchenko et al 2 and using modified porcine trypsin. Gel pieces were washed with 50% acetonitrile to remove SDS, salt and staining, then dried to remove solvent, and finally rehydrated with trypsin (8-10 μg/μl) and...
incubated 8-10 h at 37°C. The proteolytic reaction was terminated by addition of 5 μl 0.5% trifluoroacetic acid. Tryptic peptides were recovered by combining the aqueous phase from several extractions of gel pieces with 50% aqueous acetonitrile. After concentration the peptide mixture was desalted using C18ZipTips (Millipore), and peptides eluted in 1-5 μl of acetonitrile. An aliquot of this solution was mixed with an equal volume of a saturated solution of cyano-4-hydroxycinnamic acid in 50% aqueous acetonitrile, and 1 μl of mixture spotted onto a target plate, and then protein analysis were performed using the MALDI-TOF (Amersham).
Table. Maldi-Tof isolated Hsp70 Tg mouse brain proteins with significantly altered levels after MCAO, identified by mass spectrometry.

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**Figure SI.** 2-D gel and MALDI-TOF screening of ischemic brain samples. 2-D gel of ischemic brain samples from sham (Sham, no injury), Wt and Hsp70 Tg mice. 2-D gels were generated, stained, and analyzed as described in text. Differentially expressed proteins are depicted by numbers. 6 proteins were found to be significantly different in Hsp70 Tg mice: 2 proteins showed upregulation and 4 proteins showed downregulation in 2-D analysis. The 7810 spot (corresponding to dynamin) showed significantly reduced expression in Hsp70 Tg mice compared to Wt mice after experimental stroke.
Figure SII. Dynamin-1 expression is increased following experimental stroke, and interacts with Hsp70 and Fas. Neuro2a (N2a) cells were treated with 17-AAG to induce Hsp70. Following OGD, 17-AAG led to neuroprotection in these cells. Cell viability (A) and cell death (B) assays using MTT and trypan blue, respectively, showed that 17-AAG reduced cell death (n=3/group; *P < 0.01). Immunoblots of Hsp70 and dynamin-1 in N2a cells subjected to 2 hours OGD show that treatment with 17-AAG induced Hsp70, and decreased expression of dynamin-1. C, Relative intensities of protein were quantified by NIH Image J software, and normalized to the intensity of β-actin. Expression of Hsp70 was increased, whereas dynamin-1 was decreased in 17-AAG compared vehicle (Veh) treatment (n=3/group; *P < 0.01).
**Figure SIII.** A, In brains of non-injured mice, MAP-2 and dynamin-1 stains show that dynamin colocalized to the cytosol in neurons, but not to astrocytes (GFAP) or microglia (CD11b) (scale bar=20 µm). B, Co-labeling shows that Dynamin-1, Fas and Hsp70 were all expressed in neurons (MAP-2) after ischemic stroke (scale bar=20 µm).
Figure SIV. Intracerebroventricular (ICV) injection of Texas Red conjugated Dyna. Representative brain sections of ICV injection of conjugated Dyna showed positive red fluorescent signals after dMCAO, indicating uptake by cells. This is compared to Veh treated brain, which showed no red signals ($n = 3$/group).
Figure SV. Lack of synergy between Hsp70 overexpression and dynamin inhibition. Dynasore (Dyna) failed to show protection against ischemic brain damage out to 14 days post dMCAO in Hsp70 Tg mice. A, Hsp70 Tg mice with Dyna had no significant effect in reducing infarct volume compared to Hsp70 Tg mice treated with vehicle (Veh; DMF). B, Behavioral tests showed no significant difference between Hsp70 Tg mice treated with Dyna and those treated with DMF ($n = 5-6$/group).
References
