Phosphorylation enhances recombinant HSP27 neuroprotection against focal cerebral ischemia in mice.

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Abbreviations: HSP27, heat shock protein 27; hHSP27, HSP27 purified from human lymphocytes; rHSP27, recombinant HSP27; prHSP27, phosphorylated rHSP27; BSA, bovine serum albumin; MCAO, middle cerebral artery occlusion; rCBF, regional cerebral blood flow; FITC, fluorescein isothiocyanate; PBS; phosphate-buffered saline; NeuN, neuron-specific nuclear protein; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling; 8-OHdG, 8-hydroxydeoxyguanosine; Iba-1, ionized calcium binding adapter molecule-1.

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Abstract

Heat shock protein 27 (HSP27) exerts cytoprotection against many cellular insults including cerebral ischemia. We previously indicated that intravenous injection of HSP27 purified from human lymphocytes (hHSP27) significantly reduced infarct volume following cerebral ischemia-reperfusion injury, while recombinant HSP27 (rHSP27) was less effective. Phosphorylation is important for HSP27 function, and hHSP27 was more highly phosphorylated than rHSP27. We hypothesized that MAPKAP kinase 2 *in vitro*-phosphorylated rHSP27 (prHSP27) might increase its brain protection. Mice underwent transient 1-h MCAO, and then received tail-vein injections of one of the following 1 h after reperfusion: hHSP27 as positive control, rHSP27, prHSP27, or bovine serum albumin (BSA) as control. We measured infarct volume, neurological deficits, neurological severity, physiological parameters, cell-death, oxidative stress, and inflammatory response. ~~Compared with BSA controls (34.4±2.8mm^3, n=5), infarct volume was reduced by 69% in the hHSP27 positive-control group (10.5±4.6mm^3, P<0.001, n=5), 19% following rHSP27 (27.8±4.2mm^3, P<0.05, n=5), and 49% following prHSP27 (17.5±4.5mm^3, P<0.001, n=9).~~ Compared with BSA controls (30.7±3.1mm^3, n=5), infarct volume was reduced by 67% in the hHSP27 positive-control group (10.1±4.6mm^3, P<0.001, n=5), 17% following rHSP27 (25.4±3.6mm^3, P<0.05, n=5), and 46% following prHSP27 (16.5±4.0mm^3, P<0.001, n=9). Compared to the rHSP27 and BSA-treated groups, prHSP27 also reduced functional deficits, and significantly suppressed apoptosis, oxidative stress, and inflammatory responses. Here, we showed the superior neuroprotective effects of phosphorylated HSP27 by administering prHSP27. prHSP27 may be a useful therapeutic agent to protect against acute cerebral ischemic stroke.

Key words: HSP27, MAPKAP kinase 2, phosphorylation, neuroprotection, focal ischemia.

INTRODUCTION

~~Cerebral ischemia is a medical emergency that can cause permanent neurological damage, complications, and disability. Neurological deficits could be associated with the site and size of infarctions or both. Cerebral ischemia is characterized by a reduced blood supply to brain tissue, production of free radicals, and activation of inflammatory cytokines and nitric oxide, leading to neuronal cell death (Warner et al., 2004). Cerebral infarct is the irreversible damage of neuronal cells. Thus, it is very important to begin the treatment as soon as possible during the acute phase of cerebral ischemia, before an infarct lesion, known as a penumbra, develops (Phan et al., 2002). In addition, it could be necessary to reduce the size of infarctions. Neuroprotection agents are designed to suppress free radicals, apoptosis, inflammation, and cytokines to salvage the penumbra (Ovbiagele et al., 2003). Clinical tests of cerebral infarction medical therapies with substances based on these premises are ongoing (Grotta et al., 2013). However, there are few compounds for which positive results were obtained (Sutherland et al., 2012).~~ Cerebral infarct is characterized by pan-necrosis, involving not only neurons but also glial cell and vascular elements, stemming from abrupt disruption of the cerebral blood supply, which may cause permanent neurological damage, complications, and disability. Immediate effective treatment during the acute phase of cerebral ischemia is critical, before an infarct lesion, known as a penumbra, develops (Phan et al., 2002). After focal ischemia, there are two different pathophysiologies of injury related to primary anoxic ischemic cell death and delayed secondary neuronal injury by reperfusion or reoxygenation (Hossmann, 2009). Secondary brain injury is characterized by production of free radicals, and activation of inflammatory cytokines and nitric

oxide, leading to neuronal cell death (Warner et al., 2004). Thus, neuroprotective agents that attenuate the cellular, and biochemical toxic responses after ischemia, have potential roles in ameliorating brain injury (Ovbiagele et al., 2003). Although clinical tests of neuroprotective compounds for cerebral infarction with substances based on these premises are ongoing (Grotta et al., 2013), there are few compounds for which positive results have been obtained (Sutherland et al., 2012). Thus, thrombolytic treatment is still the only beneficial treatment for acute primary ischemic injury (NINDS rt-PA stroke study group., 1995; Bluhmki et al., 2009).

There is recent growing evidence that heat shock protein 27 (HSP27) is a useful therapeutic molecule against various diseases (Vidyasagar et al., 2012). HSP27 belongs to a subfamily of small HSPs (:HSPB1), while HSP20 and αB-crystallin comprise another subfamily of small HSPs (:HSPB6, HSPB5) (Mymrikov et al., 2011). Small HSPs are responsible for binding improperly folded protein substrates and transferring them to ATP-dependent chaperones or to protein degradation machines, such as proteasomes or autophagosomes (Haslbeck et al., 2005; Vos et al., 2008). HSP27 suppresses caspase-3 or caspase-9 by inhibiting the release of cytochrome c from mitochondria (Stetler et al., 2008), resulting in cell protection by action of a radical scavenger and molecular chaperone (Concannon et al., 2003). Overexpression of HSP27 provides robust cellular protection against a variety of neurological insults and diseases including cerebral ischemia (An et al., 2008; Stetler et al., 2008; Badin et al., 2009; Van et al., 2010). Human HSP27 contains three major phosphorylation sites: Ser15, Ser78, and Ser82 (Stetler et al., 2012). There is also growing evidence for the cytoprotective effects of phosphorylated HSP27 against several pathological conditions (Benn et al., 2002; Kostenko et al., 2009; Stetler et al., 2012). We demonstrated that intravenously injected, human-derived HSP27 (hHSP27) reduced infarct volume in middle cerebral artery occlusion (MCAO) mice, and showed that the hHSP27 is more phosphorylated than recombinant HSP27 (rHSP27) and

forms more complexes with HSP20 and αB-crystallin. hHSP27 is a strong neuroprotective agent, however there are limited amounts of it, the costs for preparation are high, and the stability is low, creating problems in the use of hHSP27 in clinical trials for patients with stroke. Although, rHSP27 is much more convenient than hHSP27 for clinical trials, we showed that the dephosphorylated hHSP27 was less protective against ischemic brain injury. In the present study, we examined whether rHSP27 phosphorylated by MAPKAP kinase 2 protected ischemic brains in transient MCAO-treated mice.

EXPEIMENTAL PROCEDURES

HSP27 Antibodies

We previously generated two anti-HSP27 rabbit polyclonal antibodies: anti-HSP27-N1, against the 15-mer sequence MTERRVPFSLLRGPC at the N-terminal domain of human HSP27, and anti-HSP27-C1, against the 15-mer sequence CGGPEAAKSDETAAK at the C-terminal domain of human HSP27 (Teramoto et al., 2013).

Human Physiological HSP27 Preparation

~~Heparinized human peripheral blood (40 mL) was separated by density gradient centrifugation in Lympholyte-H (Cedarlane Laboratories Ltd., Hornby, Ontario, Canada) according to the manufacturer's instructions. Cells were lysed in lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 5 mmol/L EDTA, 0.5% sodium deoxycholate, and 0.1 mmol/L phenylmethylsulfonyl fluoride) with a Dounce homogenizer, and the lysate was centrifuged at 10,000×*g* for 1 h. The supernatant was applied to an HSP27-N1 antibody affinity column, which was then washed with lysis buffer. HSP27 was eluted by peptide antigen (10 mg/mL) for the HSP27-N1 antibody. The eluate was further applied to an HSP27-C1 antibody affinity column~~

~~and washed with lysis buffer. HSP27 was eluted by a 10 mg/mL excess amount of HSP27-C1 antibody peptide antigen. HSP27 was separated from the peptide with Amicon Ultra-10 centrifugal filter units (Millipore, Billerica, MA, USA). The purity of the hHSP27 protein was over 95%.~~ Lymphocytes were purified from heparinized human peripheral blood (40 mL) by density gradient centrifugation in Lympholyte-H (Cedarlane Laboratories Ltd., Hornby, Ontario, Canada) according to the manufacturer's instructions. hHSP27 was purified from lymphocytes by two HSP27 antibody affinity columns as previously described methods (Teramoto et al., 2013).

Human HSP20 and αB-Crystallin Preparation

We purified αB-crystallin (GI:227018373) and HSP20 (GI:21389433) from the flow-through fractions of the HSP27-N1 and -C1 antibody affinity columns using either an anti-HSP20 or αB-crystallin antibody affinity column, respectively. HSP20 and αB-crystallin were eluted with 1 M glycine buffer. The buffer was then exchanged to PBS and concentrated. The purities of the HSP20 and αB-crystallin proteins were both over 95% (Figure. 1D).

Recombinant HSP27 and Phosphorylated Recombinant HSP27

Phosphorylated recombinant HSP27 (prHSP27) was generated in vitro from recombinant HSP27 (rHSP27; Acris Antibodies GmbH, Herford, Germany) using Recombinant Human active MAPKAP kinase 2 R&D Systems Inc, Minneapolis.). One milligram of rHSP27) was incubated with 20 μg recombinant active GST-MAPKAP kinase 2 (46-end) and 30 nmol of ATP in a reaction volume of 1 ml containing 25 mM MOPS, 12.5 mM β-glycerolphosphate, 25 mM MgCl2, 5 mM EGTA, 2 mM EDTA, and 0.25 mM DTT at 30 °C for 3 h. GST-MAPKAP kinase 2 was eliminated using GSH-agarose beads.

Mice

All mouse procedures were approved by the Animal Care Committee of Juntendo University. A total of 54 adult, 8-week-old, male C57BL/6 mice weighing 20 to 23 g were used in this study. All mice were housed under controlled lighting and provided with food and water *ad libitum*. ~~Mice were subjected to transient 1-h MCAO followed by reperfusion as described previously (Hara et al., 1996).~~ Mice were anesthesized with 4.0% isoflurane (Abbott Japan Co.Ltd.,Tokyo, Japan) and maintained on 1.0-1.5% isoflurane in 70% N_2O and 30% O_2 using a small-animal anesthesia system. Mice were subjected to transient 1-h MCAO followed by reperfusion as described previously (Hara et al., 1996).

Measurement of serum prHSP27 levels

Blood (200 µl) was collected from the ophthalmic venous plexus before MCAO surgery and 15 min, or 1, 6, 12, or 24 h after reperfusion (n=~~2~~ 4per time point). Mouse serum prHSP27 levels were determined using a prHSP27 enzyme-linked immunosorbent assay (ELISA) kit (Abnova, Taipei, Taiwan).

Identification of Transition in Brain Parenchyma by Intravenous prHSP27 Injection.

prHSP27 was conjugated with fluorescein isothiocyanate (FITC) according to the manufacturer's protocol (KPL, Inc., MD, USA). Mice were intravenously administered 50 µg of FITC-prHSP27 1 h after reperfusion and then anesthetized with pentobarbital (50 mg/kg, i.p.) 30 min after the injection. The mice were sacrificed, and the brains were immediately removed, soaked in Tissue-TekH OCTTM Compound (SAKURA, Alphen aan den Rijn, The Netherlands), and frozen on liquid nitrogen. Frozen coronal sections (20 µm) were immediately, or after

incubation with Alexa Fluor® 555-conjugated anti-neuron-specific nuclear protein (NeuN; 1:100 Millipore), mounted with Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA). The sections were examined with an LSM 510 confocal laser scanning microscope. (Carl Zeiss MicroImaging GmbH, Göttingen, German.).

Experimental Protocol

Mice were subjected to transient 1-h MCAO and then one hour after reperfusion randomly divided into 7 groups, each of which received tail-vein injections of one of the following: BSA as control, hHSP27 as positive control, rHSP27 purified from *E. coli*, prHSP27, HSP20, αB-crystallin, or prHSP27 with HSP20 and αB-crystallin mixed in a ratio of 8 : 1 : 1. The HSPs were injected in doses of 50 μg/ mouse[18]. During this procedure, body temperature was maintained at 37.0±0.5°C with a heating pad. ~~Regional cerebral blood flow (rCBF) was measured by laser Doppler flowmetry before, during, and after MCAO, and before the mice were sacrificed. Twenty-four hours after reperfusion, the mice were anesthetized by intraperitoneal injections of 50 mg/kg pentobarbital and decapitated.~~ Regional cerebral blood flow (rCBF) was measured in the left temporal window by laser Doppler flowmetry (FLOW-C1; Omegawava Inc., Tokyo.Japan) before, during, and after MCAO, and before the mice were sacrificed. The tip of the laser Doppler probe was attached to the surface of the skull at a point 5mm to the left of and 3mm behind bregma (Tanaka et al., 2007). Twenty-four hours after reperfusion, the mice were anesthetized by intraperitoneal injections of 50 mg/kg pentobarbital and decapitated.

Measurement of Infarct Area and Volume

At various times following reperfusion, mice were anesthetized with pentobarbital (50 mg/kg,

i.p.) and decapitated. The brains were carefully removed and fixed in 4% parafomaldehyde for at least 2 days at 4°C and then overnight in 30% sucrose. To evaluate infarct area and volume, 9 consecutive coronal cryostat brain slices (20 µm) from forebrains were stained with cresyl violet, scanned with AxioVision software (Carl Zeiss MicroImaging GmbH, Göttingen, German), and measured using the ImageJ program (NIH, http://rsb.info.nih.gov/nih-image/). Measurements were made from 9 slices from each mouse and were conducted by researchers unaware of the treatment group. Brain swelling was calculated as follows: (ipsilateral hemispheric volume - contralateral hemispheric volume) / contralateral hemispheric volume x 100). In addition, infarct volume corrected for edema was calculated as follows: (1/1 + brain swelling) x infarct volume.

Neurological Evaluation

Neurological deficit score

Neurological functions were evaluated by the following modified scoring system: 0, no observable neurological deficits (normal); 1, failure to extend forepaw when entire body was lifted by the tail (mild); 2, circling to contralateral side (moderate); and 3, loss of walking or righting reflex (severe) (Hara et al., 1996). Four mice were tested in each group, and each mouse was subjected to 3 rounds of each test. Observers of the behavioral test were blinded to the groups to which the mice belonged.

Modified neurologic severity score

The neurological severity score was a composite of the motor (muscle status, abnormal movement), sensory (visual, tactile and proprioceptive), and reflex tests, similar to the contralateral neglect testing described in humans (Li et al., 2000). The neurological severity was

graded on a scale of 0 to 14 (normal score 0, maximal deficit score 14). One point was awarded for the inability to perform, or for abnormal task performance, or for the lack of a tested reflex.

TUNEL assay

To examine cell death, we detected *in situ* DNA fragmentation via terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL), carried out with an *in situ* cell death detection kit (TMR fluorescein, Roche Diagnostics GmbH) (Miyamoto et al., 2008).

Immunohistochemistry

Immunohistochemistry was performed on 20-µm, free-floating sections. ~~Sections were stained overnight using rabbit anti-cleaved caspase-3~~ Sections of forebrain and basal ganglia were stained overnight using rabbit anti-cleaved caspase-3 (1:200; Cell Signaling Technology, Inc.), mouse anti-8-hydroxydeoxyguanosine (8-OHdG; 1:100; Japan Institute for the Control of Aging, Shizuoka, Japan), and rabbit anti-ionized calcium binding adapter molecule-1 (Iba-1; 1:500; Wako Pure Chemical industries, Ltd., Osaka, Japan) antibodies. Sections were then incubated with biotinylated secondary antibodies (1:300; Vector Laboratories, Inc.) and subsequently processed with avidin-biotinylated peroxidase complex (Vectastain ABC kit; 1:400; Vector Laboratories, Inc.).

Cell counts and statistical analysis

~~In the immunohistochemical analyses, positively stained cells in the ischemic boundary zone (IBZ) adjacent to the ischemic core (0.25 mm^2) in 5 sections from each mouse were counted using AxioVision.~~ In the immunohistochemical analyses, positively stained cells in the IBZ adjacent to the ischemic core (0.25 mm^2) and basal ganglia in 5 sections from each mouse were

counted using AxioVision. All values are expressed as mean±SEM. One-way analysis of variance and *post hoc* Fisher's protected least significant difference tests were used to determine the significance of differences between the groups. *P* values less than 0.05 indicated statistical significance.

PAGE and Immunoblotting

SDS-PAGE experiments were performed with the NuPAGE Novex Bis-Tris Gel system according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The most frequently used SDS gel was a 4–12% gradient gel. Native–PAGE experiments were performed with the NativePAGE Novex Bis-Tris Gel System according to the manufacturer's instructions. The most frequently used native gel was a 4–16% gradient gel. To this solution, an additional detergent to be tested was added at a final concentration of 0.4% [1.0% in the case of *n*-octyl-β-d-glucoside (β-OG)] and incubated for 10 min prior to blue native–PAGE. To each lane of a native gel, 3–5 μg of protein were loaded. Anode buffer was made by diluting the 20×NativePAGE running buffer (Invitrogen, Carlsbad, CA), and the cathode buffer by mixing the NativePAGE running buffer with Cathode Buffer additive (Coomassie Blue G-250 dye, Invitrogen) according to the manufacturer's instructions. The gel was stained using the Colloidal Blue Staining Kit (Invitrogen). Block Ace (Daiichi Kogyo Seiyaku, Co., Ltd., Gifu, Japan) or PBS containing 0.05% Tween-20 (Sigma-Aldrich Co.) was used for blocking. Membranes with transferred proteins were incubated overnight with antibodies against HSP27-C1, p-HSP27 (Ser 15), p-HSP27 (Ser-78) or p-HSP27 (Ser-82) (Santa Cruz Biotechnology, Inc.). Immunoreactive bands were visualized with a Chromogenic Kit (Invitrogen).

RESULTS

Phosphorylation of rHSP27 by MAPKAP kinase 2

Phosphorylation of rHSP27 (GI:11036357) by MAPKAP kinase 2 at sites S15, S78, and S82 was confirmed by western blotting; the levels of phosphorylation were comparable to native, human-derived HSP27 (Figure. 1A). Native-PAGE and SDS-PAGE revealed that there was a larger amount of low molecular weight of HSP27 in the prHSP27 and hHSP27 preparations than in the rHSP27 and a smaller amount of high molecular weight HSP27 in the prHSP27 and hHSP27 preparations than in the rHSP27 (Figure. 1B, C). SDS-PAGE showed that the molecular weights of HSP27 in rHSP27, prHSP27, and hHSP27 were the same (Figure. 1C). Phosphorylation of HSP27 by MAPKAP kinase 2 decreased the amounts of large oligomeric HSP27 and increased the small oligomeric HSP27 (Figure. 1B).

Serum concentration of phosphorylated recombinant HSP27

We injected prHSP27 into the tail veins of mice 1 h following reperfusion from ischemia, and assessed the time course of serum prHSP27 levels at various times after the injection (Figure. 1E). Circulating levels of HSP27 in mice treated with BSA were not increased at any time point; the concentration of serum prHSP27 was 22.94±2.39 μg/ml after 15 min and 2.56±1.27 μg/ml after 60 min. prHSP27 was not detected at 6 h, 12 h, or 24 h after the injection.

Localization of (FITC)-hHSP27 in Brain Parenchyma after Injection.

To obtain direct evidence of prHSP27 localization in brain, we injected fluorescein isothiocyanate (FITC)-prHSP27 and made fresh frozen sections to preserve the FITC-prHSP27 signal. FITC-prHSP27 was diffusely localized in brain, including in neuronal marker protein NeuN-positive cells, indicating that FITC-prHSP27 was localized in neurons (Figure. 1F).

Physiological Parameters.

~~There were no significant differences in body weight or rCBF among the variously injected groups (Figure. 2A). As measured by laser Doppler, MCAO decreased the ipsilateral baseline rCBF by 47%–52%, but 24 h after reperfusion rCBF had recovered to 58%–63% of baseline under anesthesia.~~ There were no significant differences in body weight or rCBF among the variously injected groups (Figure. 2A).

Phosphorylated Recombinant HSP27 Reduces Infarct Volume and Decreases Neurological Deficits.

Ischemic mice were intravenously injected with BSA, hHSP27, rHSP27, prHSP27, HSP20, αB-crystallin, or prHSP27 with HSP20 and αB-crystallin (50 μg) 1 h after reperfusion. Infarct volumes were measured in cresyl violet-stained sections made 24 h after reperfusion (Figure 2B). ~~Infarct volume was reduced by 69% in mice treated with hHSP27 (10.5±4.6 mm^3, $P<0.001$, n=5), by 19% when treated with rHSP27 (27.8±4.2 mm^3, $P<0.05$, n=5), by 49% when treated with prHSP27 (17.5±4.5 mm^3, $P<0.001$, n=9), by 25% when treated with HSP20 (25.9±8.8 mm^3, NS, n=8), by 24% when treated with αB-crystallin (26.0±9.5 mm^3, NS, n=9), and by 44% when treated with prHSP27 with HSP20 and αB-crystallin (19.1±5.3 mm^3, $P<0.001$, n=8) vs. BSA-treated controls (34.4±2.8 mm^3, n=5) (Figure. 2C). prHSP27 had greater neuroprotective effects than rHSP27 ($P<0.01$), αB-crystallin ($P<0.05$) and HSP20 ($P<0.05$). These results demonstrate that phosphorylation of rHSP27 enhanced its neuroprotection against ischemic brain injury. We did not observe a synergy effect of combined administration of prHSP27 with HSP20 and αB-crystallin, which are also present in the hHSP27 preparation.~~ We calculated brain swelling and infarct volume corrected for brain swelling. Brain swelling

was reduced by 66% in mice treated with hHSP27 (4.2±5.1%, NS, n=5), by 21% when treated with rHSP27 (9.7±5.1%, NS, n=5), by 50% when treated with prHSP27 (6.2±3.6%, NS, n=9), by 29% when treated with HSP20 (8.7±4.8%, NS, n=8), by 27% when treated with αB-crystallin (9.0±5.8%, NS, n=9), and by 48% when treated with prHSP27 with HSP20 and αB-crystallin (6.4±4.2%, NS, n=8) vs. BSA-treated controls (12.3±6.9%, n=5) (Figure. 2D). Infarct volume corrected for edema was reduced by 67% in mice treated with hHSP27 (10.1±4.6 mm^3, P<0.001, n=5), by 17% when treated with rHSP27 (25.4±3.6 mm^3, P<0.05, n=5), by 46% when treated with prHSP27 (16.5±4.0 mm^3, P<0.001, n=9), by 23% when treated with HSP20 (23.7±7.8 mm^3, NS, n=8), by 22% when treated with αB-crystallin (23.8±8.7 mm^3, NS, n=9), and by 42% when treated with prHSP27 with HSP20 and αB-crystallin (17.9±4.9 mm^3, P<0.001, n=8) vs. BSA-treated controls (30.7±3.1 mm^3, n=5) (Figure. 2C). prHSP27 had greater neuroprotective effects than rHSP27 (P<0.01), αB-crystallin (P<0.05) and HSP20 (P<0.05). These results demonstrate that phosphorylation of rHSP27 enhanced its neuroprotection against ischemic brain injury. We did not observe a synergy effect of combined administration of prHSP27 with HSP20 and αB-crystallin, which are also present in the hHSP27 preparation.

The neurological deficit and severity scores of the hHSP27, prHSP27, and prHSP27 with HSP20 and αB-crystallin groups showed significantly better functional recoveries compared to the controls (neurological deficit score: hHSP27, prHSP27: P<0.01, prHSP27 with HSP20 and αB-crystallin: P<0.05; neurological severity score: hHSP27, prHSP27, prHSP27 with HSP20 and αB-crystallin: P<0.01); however, there were no significant differences among the hHSP27, prHSP27, and prHSP27 with HSP20 and αB-crystallin treatments (Figure. 3A, B).

Phosphorylated Recombinant HSP27 Suppressed Apoptotic Cell Death, Oxidative DNA Damage and Inflammatory Responses

~~The number of cells immunopositive for cleaved caspase-3 and the number of TUNEL-positive cells 24 h after reperfusion were significantly lower in the hHSP27 (P<0.001) and prHSP27 (P<0.01) groups than in the rHSP27 group. The number of cells immunopositive for 8-OhdG and Iba-1, markers of oxidative stress and inflammation, were significantly lower in the hHSP27 and prHSP27 (P<0.01) groups than in the rHSP27 group (Figure. 4A, B).~~ In the IBZ, the number of cells immunopositive for cleaved caspase-3 and the number of TUNEL-positive cells 24 h after reperfusion were significantly lower in the hHSP27 (P<0.001) and prHSP27 (P<0.01) groups than in the rHSP27 group. The number of cells immunopositive for 8-OHdG and Iba-1, markers of oxidative stress and inflammation, were significantly lower in the hHSP27 and prHSP27 (P<0.01) groups than in the rHSP27 group. On the other hand, in the basal ganglia, the number of cells immunopositive for cleaved caspase-3, 8-OHdG, Iba-1, and the number of TUNEL-positive cells 24h after reperfusion were not statistically different among these groups (Figure. 4A, B).

DISCUSSION

The Ser15, Ser78, and Ser82 positions of rHSP27 were phosphorylated *in vitro* by MAPKAP kinase 2, thereby enabling the prHSP27 to tend to form low molecular weight dimers or tetramers. The serum concentration of prHSP27 followed a very sharp time course, peaking at just 15 min post-injection and already very low after 1 h, suggesting that prHSP27 was rapidly degraded, excreted, or removed from intravascular serum. The blood brain barrier controls the passage of substances from the blood into the CNS. Usually, injected proteins are hampered from reaching brain neurons by the tight regulation of this barrier; however, we observed

peripherally injected FITC-prHSP27 in neurons, indicating that FITC-prHSP27 crossed the barrier and then entered neurons. Intravenous injection of prHSP27 after MCAO significantly reduced infarct volume and neurologic deficit and severity scores, in addition to suppressing apoptotic cell death, oxidative DNA damage, and inflammatory responses. Thus, prHSP27 demonstrated neuroprotective effects against ischemic reperfusion injury, as did the hHSP27. On the other hand, rCBV, measured by laser Doppler flowmetry, did not differ among the variously treated groups.

Phosphorylation of HSP27 affects its functions. Ser15, Ser78, and Ser82 of HSP27 are phosphorylated by MAPKAP kinase 2/3 and dephosphorylated by protein phosphatase 2A (Cairns et al., 1994). HSP27 tends to form variable size oligomers with apparent molecular masses of up to 800 kDa (Kostenko et al., 2009). Phosphorylated HSP27 has a tendency to form dimers or tetramers (Kato et al., 1994; Rogalla et al., 1999; Hayes et al., 2009). The WDPF domain of HSP27 is involved in small aggregate formation, the αB-crystallin domain is involved in large polymer formation, and the flexible domain helps to maintain the molecule's solubility (Gusev et al., 2002; Lelj-Garolla et al., 2005). HSP27 function depends in large part upon its phosphorylation (Stetler et al., 2009; 2010). In the high molecular weight oligomeric, non-phosphorylated form, HSP27 functions primarily as a chaperone (Knauf et al., 1994), and has been best characterized in the targeting of aberrantly folded proteins to inhibit aggregate formation (Jakob et al., 1993). In the lower molecular weight oligomeric, phosphorylated form, HSP27 can function as an inhibitor of cell death, targeting both upstream and downstream of mitochondrial signaling (Hollander et al., 2004). Thus, in our study the cytoprotective activity of rHSP27 was increased by phosphorylation. Thus, we demonstrated the superior neuroprotective effects of phosphoryled HSP27 by administering prHSP27 itself.

The BBB controls the passage of substances from the blood into the CNS. The BBB is composed of a microvascular endothelium, basal lamina, pericytes, astrocytes, and neurons, and regulates receptor mediated transcytosis, passive diffusion, and transportation carriers, via tight junctions (Abbot et al., 2010). In our previous study, we showed that intravenously administered human HSP27 crossed the BBB of the ischemic side, and rapidly entered neurons on the ipsilateral side of ischemia (Teramoto et al., 2013). In this study, we found that prHSP27 also rapidly entered neurons on the ischemic side within 30min in the same way as hHSP27. A possible mechanism for this occurrence may be barrier dysfunction that might occur via transient tight junction opening or via changes in transport systems. However, further studies are needed to elucidate the mechanism by which phosphorylated HSP27 enters neurons.

In our previous study, mass spectrometric analysis revealed that a high molecular weight hHSP27 multimer contained HSP27 and small amounts of αB-crystallin and HSP20, while the hHSP27 dimers and tetramers contained only HSP27 without αB-crystallin and HSP20. The amount of HSP27 contained in the hHSP27 was more than 20 times that of αB-crystallin and HSP20 (Teramoto et al., 2013). Previous studies showed that because of similarities in structure and other properties, HSP27, αB-crystallin, and HSP20 are co-purified (Kato et al., 1994); moreover, HSP20 and αB-crystallin are also neuroprotective (Zeng et al., 2010; Li et al., 2012). Thus, there exists the possibility that the αB-crystallin and HSP20-parts of the hHSP27 complex influence its effects on brain protection. To test this, we examined whether intravenous injection of αB-crystallin, HSP20, or prHSP27 with αB-crystallin and HSP20 affected brain protection. HSP20 or αB-crystallin alone slightly reduced infarct volume, but did not affect recovery of neurologic deficits or reduce the neurologic severity score compared to control. Moreover, we did not observe additional neuroprotective effects of prHSP27 when combined with HSP20 and αB-crystallin compared to prHSP27 alone.

A significant reduction in cerebral infarct volume, improved neurological deficits, and suppressed cell death by the inhibition of caspase activation were observed following the treatment of ischemic mice with prHSP27. prHSP27 is far superior to hHSP27 in terms of productivity and cost effectiveness. There are, however, several questions still to be resolved. First, we applied a single shot intravenous injection of prHSP27, but we should examine whether a frequent administration protocol will produce superior effects and extend the therapeutic time window. Second, long term tissue side effects, such as tumorigenesis, should be analyzed. Third, we should also examine the effects of co-administration of tissue-plasmionogen activator and HSP27. Although these questions need to be considered in the near future, the current study suggests that intravenous injection prHSP27 may be a useful therapeutic agent to protect against acute cerebral ischemic stroke.

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Figure Legends

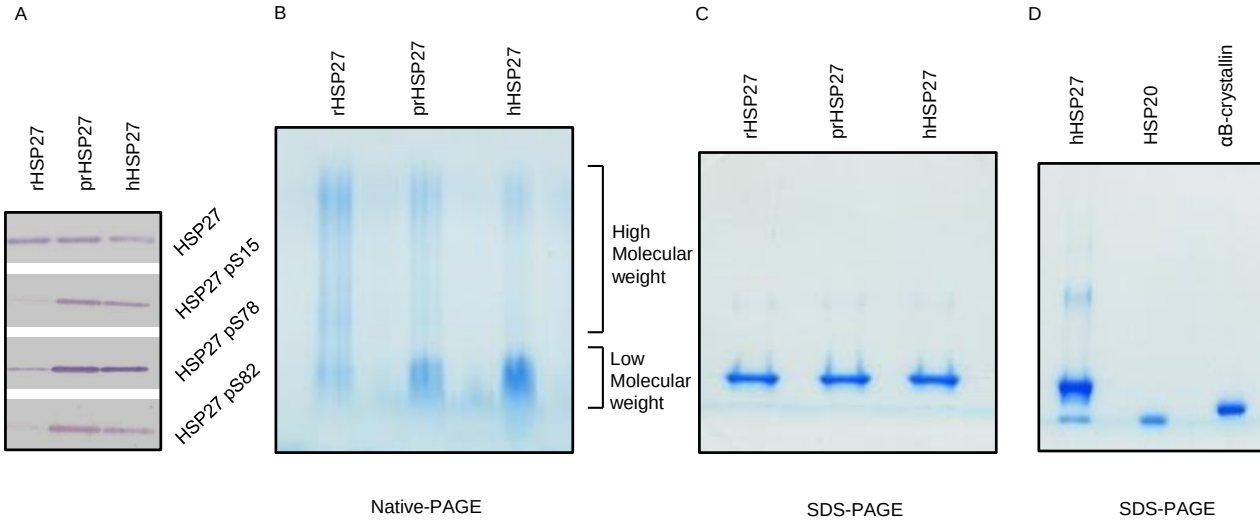
Figure. 1. Phosphorylation of rHSP27 by MAPKAP kinase 2 and purification of HSP20 and αB-crystallin. **(A)** Western blots of rHSP27, prHSP27 and hHSP27 proteins immu**nostained** with anti-phosphorylated S15 HSP27, S78 HSP27, and S82 HSP27 antibodies. **(B-D)** rHSP27, prHSP27 and hHSP27 proteins were separated by native-PAGE (**B**), and SDS-PAGE (**C**), hHSP27, HSP20, αB-crystallin proteins were separated by SDS-PAGE (**D**), and stained with Coomassie brilliant blue. **(E)** The time course of serum phosphorylated recombinant HSP27 level**s**. Mouse serum prHSP27 level measured in blood from ophthalmic venous plexus. Data are mean±SEM of two mice in each group. Pre, before operation; Reperfusion, reperfusion after 1 h of middle cerebral artery occlusion and immediate intravenous injection of prHSP27 or BSA; 15 min, 1, 6, 12, 24 h after reperfusion and injection. **(F)** Localization of injected FITC-prHSP27 in the penumbra of ischemic mouse brains. FITC-prHSP27 (green); NeuN, a neuronal marker protein (re); and merge. FITC, fluorescein isothiocyanate. Scal**e** bar = 20 μm.

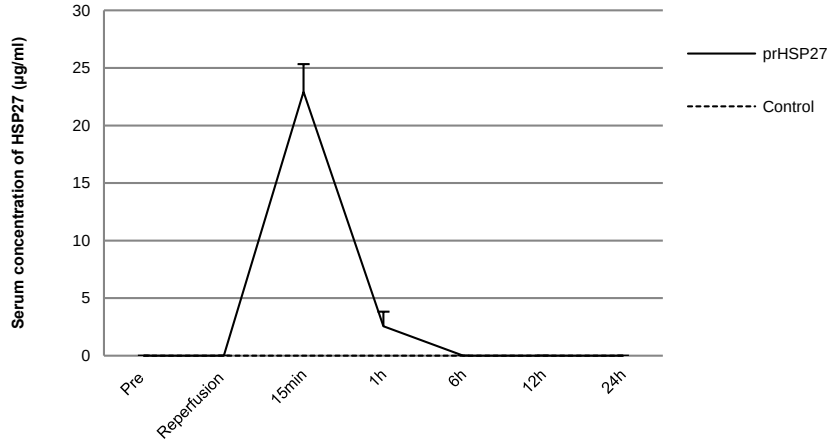
Figure. 2. Phosphorylated recombinant HSP27 (prHSP27) decreased brain infarct size. **(A)** Body weight and regional cerebral blood flow (rCBF) before the operation (Pre); during middle cerebral artery occlusion (During); and 24 h after injection (24 h) of BSA (Control, n=5), human HSP27 (hHSP27, n=5), recombinant HSP27 (rHSP27, n=5), phosphorylated rHSP27 (prHSP27, n=9), HSP20 (n=9), αB-crystallin (n=8), or prHSP27 with HSP20 and αB-crystallin (n=8). Data

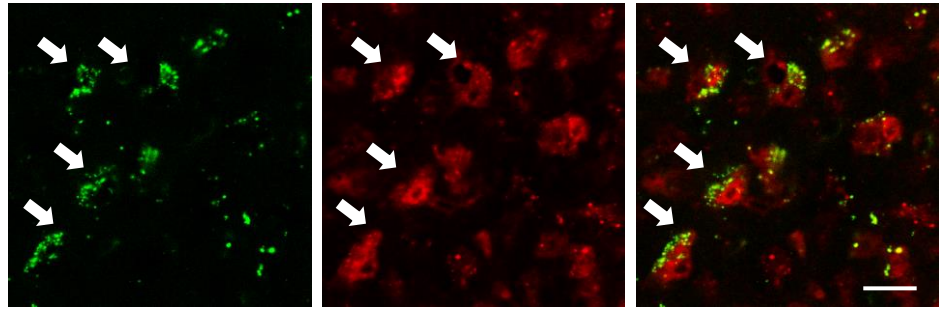
are mean±SEM. **(B)** Representative photomicrographs of infarct areas stained with cresyl violet in each group 24 h after injection of the above substances. Infarct areas are circumscribed with dotted lines. Scale bar = 2 mm. **(C)** Infarct volumes in each group. Data are mean±SEM (n=same as above) $^{*}P<0.05$, $^{***}P<0.001$ vs. controls.

Figure. 3. Phosphorylated recombinant HSP27 improved neurological scores. **(A-B)** Neurological deficit **(A)** and severity **(B)** scores 1 h following middle cerebral artery occlusion (post MCAO, beige) and 24 h following reperfusion (24 h, black) in mice treated with BSA (control, n=5), hHSP27 (n=5), rHSP27 (n=5), prHSP27 (n=9), HSP20 (n=9), αB-crystallin (n=8), and prHSP27 with HSP20 and αB-crystallin (n=8). Data are means±SEM.

Figure. 4. prHSP27 suppressed apoptotic cell death, oxidative DNA damage, and the inflammatory response. **(A)** Photomicrographs of cleaved caspase-3 immunostaining, TUNEL staining, and 8-OHdG- and Iba-1- immunostaining in the infarct boundary zones in mice treated with hHSP27, rHSP27, and prHSP27 24 h after reperfusion. Scale bars = 100 μm. **(B)** Number of cleaved caspase-3-, TUNEL-, 8-OHdG-, and Iba-1-positve cells in the hHSP27, rHSP27, and prHSP27 groups. Data are mean±SEM of five mice in each group. $^{*}P<0.05$, $^{**}P<0.001$ vs. rHSP27.


A
rHSP27
prHSP27
hHSP27
HSP27
HSP27 pS15
HSP27 pS78
HSP27 pS82
B
rHSP27
prHSP27
hHSP27
High Molecular weight
Low Molecular weight
Native-PAGE
C
rHSP27
prHSP27
hHSP27
SDS-PAGE
D
hHSP27
HSP20
αB-crystallin
SDS-PAGE

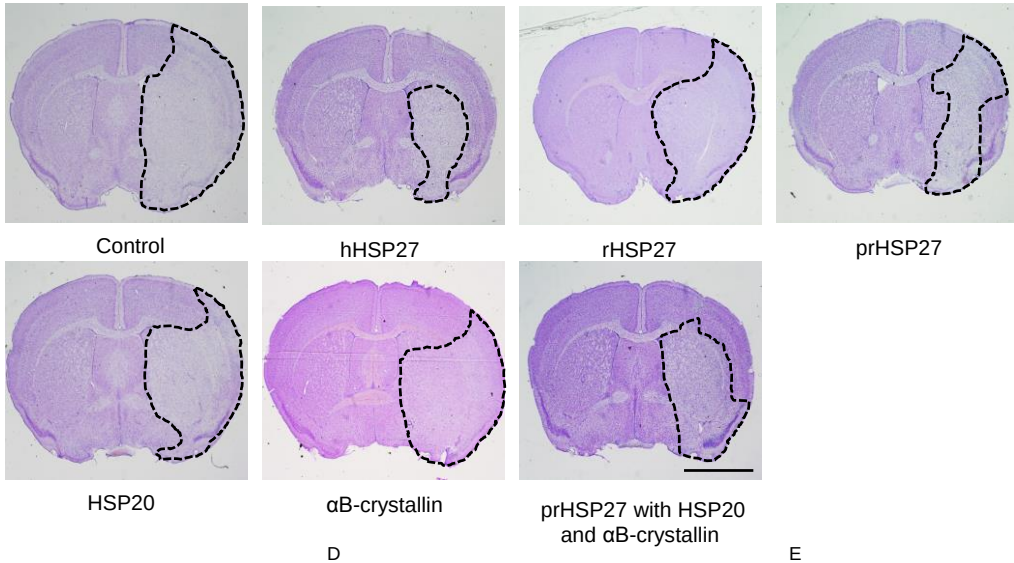

E
Serum concentration of HSP27 (μg/ml)
30
25
20
15
10
5
0
Pre
Reperfusion
15min
1h
6h
12h
24h
prHSP27
Control


F
prHSP27
NeuN
Merge

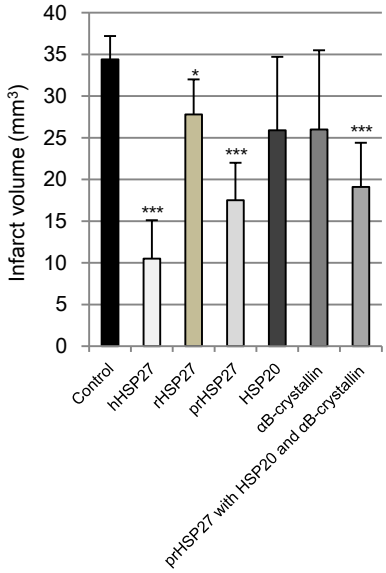
A

Parameter		Substance injected						
BW (g)								prHSP27 with
		Control	hHSP27	rHSP27	prHSP27	HSP20	αB-crystallin	HSP20 and αB-crystallin
	Pre	20.4±0.4	20.8±0.7	20.9±0.7	20.5±0.8	20.2±0.8	19.9±0.7	20.5±1.5
	24h	18.3±0.4	18.7±0.4	18.2±0.8	18.6±1.1	17.7±0.6	17.9±1.3	18.3±1.6
rCBF(ml/min/100g)								
	Pre	66.7±4.7	67.2±4.6	67.4±3.0	69.4±1.5	68.6±1.1	70.2±1.5	69.2±1.3
	During	35.5±2.6	32.5±2.6	34.8±2.2	34.7±1.4	35.9±1.2	35.7±1.3	35.1±1.5
	24h	40.4±3.1	42.3±3.0	42.6±2.6	39.5±2.1	41.6±1.2	40.4±2.1	41.6±3.7

B



C



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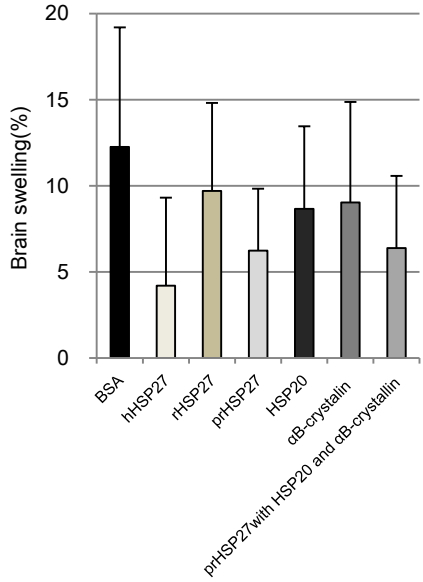

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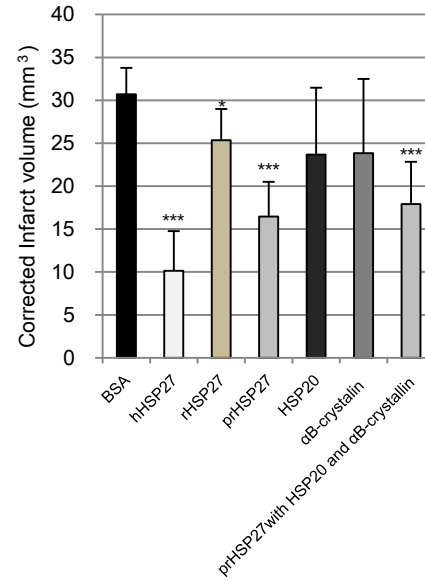
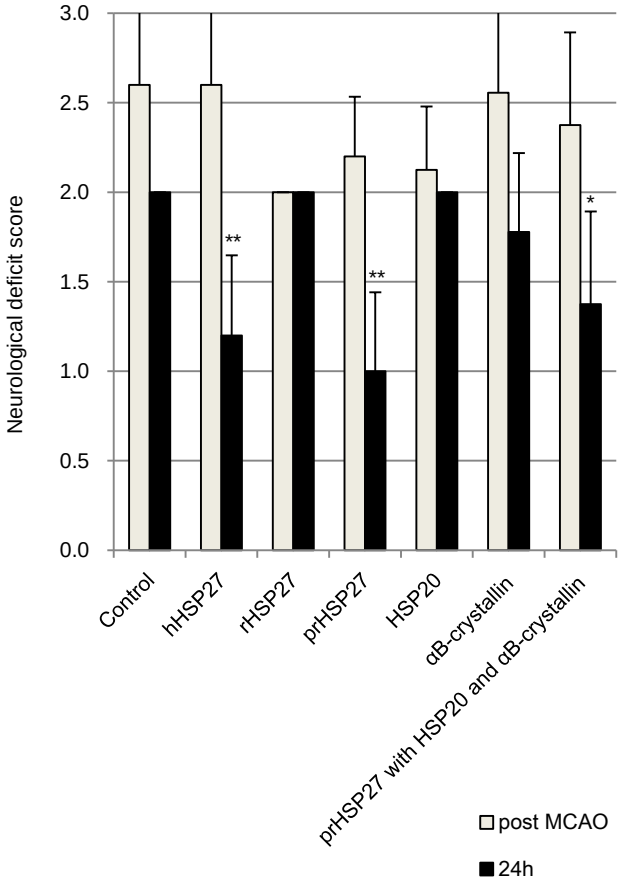
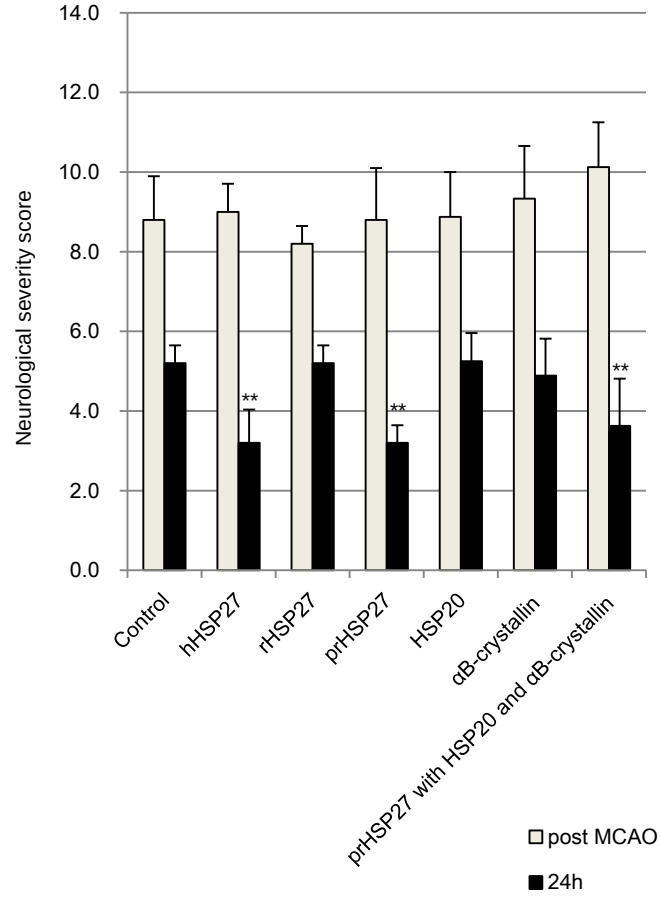


A


Neurological deficit score
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
**
*
Control
hHSP27
rHSP27
prHSP27
HSP20
αB-crystallin
prHSP27 with HSP20 and αB-crystallin
post MCAO
24h

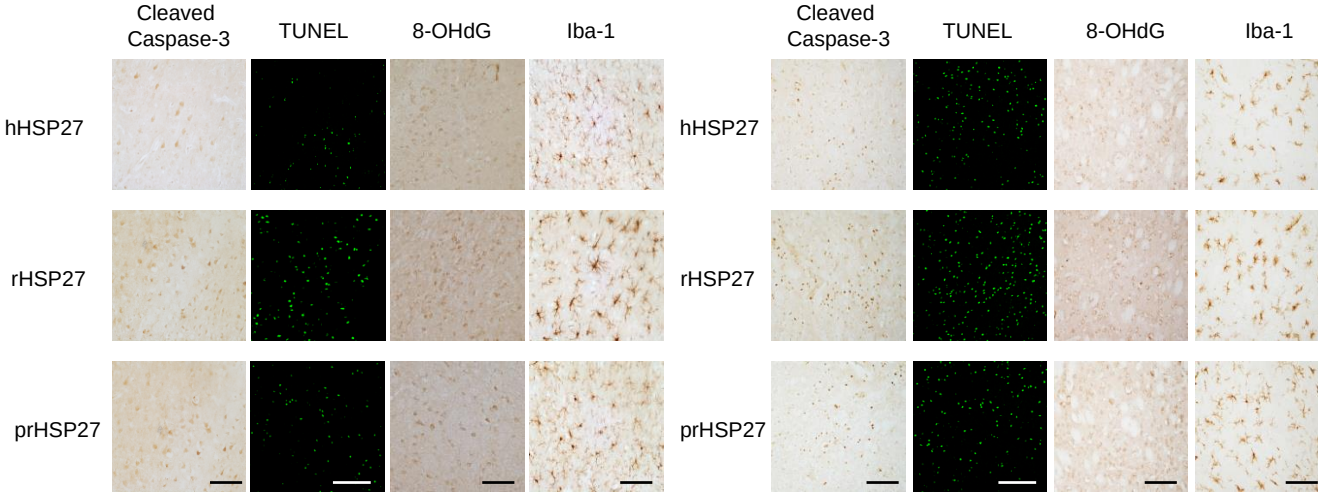
B


Neurological severity score
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
**
**
**
Control
hHSP27
rHSP27
prHSP27
HSP20
αB-crystallin
prHSP27 with HSP20 and αB-crystallin
post MCAO
24h

A

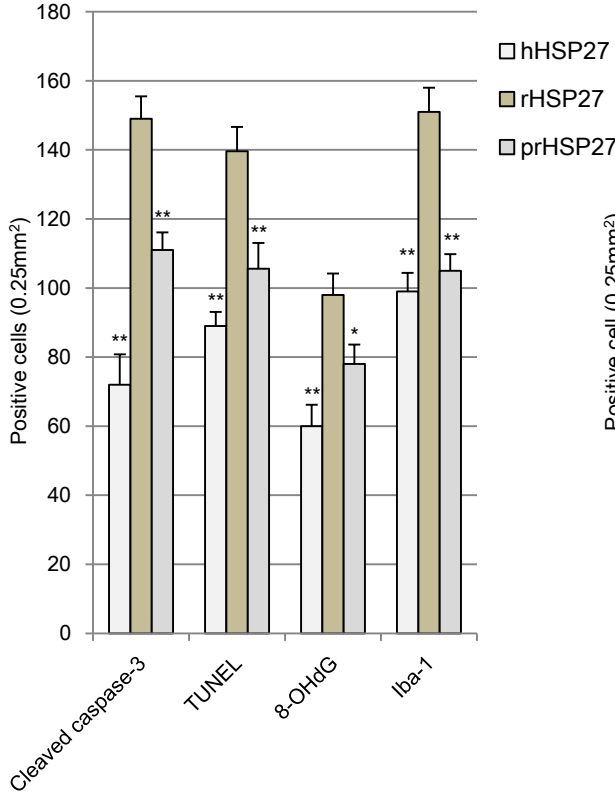
IBZ

Basal ganglia



B

IBZ



Basal ganglia