Neuroscience 278 (2014) 113–121

PHOSPHORYLATION ENHANCES RECOMBINANT HSP27 NEUROPROTECTION AGAINST FOCAL CEREBRAL ISCHEMIA IN MICE

Y. SHIMADA,[a] R. TANAKA,[a]* H. SHIMURA,[b,c] K. YAMASHIRO,[a] T. URABE[b] AND N. HATTORI[a]

[a] *Department of Neurology, Juntendo University School of Medicine, Tokyo, Japan*

[b] *Department of Neurology, Juntendo University Urayasu Hospital, Chiba, Japan*

[c] *Institute for Environment and Gender Specific Medicine, Juntendo University School of Medicine, Chiba, Japan*

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 middle cerebral artery occlusion (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 (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 infarct is characterized by pan-necrosis, involving not only neurons but also glial cells 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, 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 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 the 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 der Weerd et al., 2010).

*Corresponding author. Address: Department of Neurology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo, Tokyo 113-8421, Japan. Tel: +81-3-3813-3111; fax: +81-3-58000547.
E-mail address: r_tanaka@juntendo.ac.jp (R. Tanaka).

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

http://dx.doi.org/10.1016/j.neuroscience.2014.07.073
0306-4522/

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 and Moens, 2009; Stetler et al., 2012). We demonstrated that intravenously injected, HSP27 purified from human lymphocytes (hHSP27) reduced infarct volume in transient 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.

EXPERIMENTAL 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

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 phosphate-buffered saline (PBS) and concentrated. The purities of the HSP20 and αB-crystallin proteins were both over 95% (Fig. 1D).

Recombinant HSP27 and phosphorylated recombinant HSP27

Phosphorylated rHSP27 (prHSP27) was generated *in vitro* from rHSP27(Acris Antibodies GmbH, Herford, Germany) using Recombinant Human active MAPKAP kinase 2 R&D Systems Inc, Minneapolis, USA). One milligram of rHSP27) was incubated with 20 μg of 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-glycerophosphate, 25 mM $MgCl_2$, 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 the Juntendo University. A total of 54 adults, 8-week-old, male C57BL/6 mice weighing 20–23 g were used in this study. All mice were housed under controlled lighting and provided with food and water *ad libitum*. Mice were anesthetized 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 ($n = 4$ per 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). One mouse was intravenously administered 50 μg of FITC-prHSP27 1 h after reperfusion ($n = 1$) 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 ischemic boundary zone (IBZ) adjacent to the ischemic core was examined with an LSM 510 confocal laser scanning microscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany).

Experimental protocol

Mice were subjected to transient 1-h MCAO and then one hour after reperfusion, the mice were randomly divided into seven groups, each of which received tail-vein injections of one of the following: bovine serum albumin (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/