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Decreasing or increasing heat shock protein 72 exacerbates or attenuates heat-induced cell death, respectively, in rat hypothalamic cells

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ABSTRACT

Heat shock protein (HSP) 72 in serum was decreased to a greater degree in patients with serious heat stroke than in those with mild heat stroke. Thus, increased levels of HSP72 appeared to correlate with a better outcome for the patient. Nevertheless, the function of HSP72 in the heat-induced hypothalamic cell death has not been assessed. In this study, we found that increasing HSP72 levels with mild heat preconditioning or decreasing HSP72 levels with pSUPER plasmid expressing HSP72 small interfering RNA significantly attenuated or exacerbated heat-induced cell death in cultured primary hypothalamic cells, respectively. Our findings suggest that HSP72 plays a pivotal role in heat-induced cell death and may be associated with heat tolerance.

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1. Introduction

Evidence has accumulated to indicate that heat shock protein 72 (HSP72) promotes cell survival under various pathologic conditions by interacting with multiple components of caspase-dependent and -independent apoptotic pathways [1]. Exogenous HSP72 overexpression significantly reduced cerebral ischemia-induced neuronal loss in rats [2]. In contrast, knockout of HSP72 exacerbated infact volume in mice after focal cerebral ischemia [3]. These findings suggest that HSP72 has significant neuroprotective effects across multiple models of brain ischemia.

According to the description by Wang et al. [4], HSP27 may play a role in the response of patients to heat stroke. Many of the heat-stroke patients, displayed decreased serum levels of HSP72. The decreased response was more pronounced in those with serious heatstroke than in those with mild heatstroke. Evidence has accumulated to indicate that decreased heat tolerance is associated with hypothalamic impairment [5]. Thermal injury to the hypothalamus has been hypothesized to be the primary mechanism of multiple organ failure and mortality [6,7]. To our knowledge, evidence is not available whether cellular levels of HSP in the hypothalamus play a role in heat-induced cell death.

The present study was performed to investigate whether heat-induced cell death in rat primary cultured hypothalamic cells can be attenuated or exacerbated by increasing or decreasing HSP72, respectively. The cellular levels of HSP72 were elevated by mild heat preconditioning (MHP) [8–10] or lowered by gene silencing [11,12].

2. Materials and methods

2.1. Ethics statement

All animal procedures were performed in accordance to the guideline of the Ministry of Science and Technology of the Republic of China (Taipei, Taiwan) for Animal Care and approved by the Chi Mei Medical Center Animal Care Committee (protocol no: 100/20751). Animals were housed in specific pathogen-free facilities at the Chi Mei Medical Center (Tainan, Taiwan). All surgery was performed under anesthesia using isoflurane, and all efforts were made to minimize suffering. Human euthanasia of rats was

Abbreviations: HSP, heat shock protein; MHP, mild heat preconditioning; HI, heat injury; siRNA-HSP72, pSUPER plasmid expressing HSP72 small interfering RNA

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performed by carbon dioxide with a flow rate of 20% of chamber volume/min following the guidelines of the Ministry of Science and Technology of the Republic of China.

2.2. Construction of recombinant pSUPER plasmid expressing HSP72 siRNA

pSUPER vector (vector) (Oligo Engine, Seattle, WA, USA), which contains polymerase-III HI-RNA gene promoter, can direct the synthesis of siRNA-like transcripts. The target sequence for HSP72 (Gen Bank Accession No. NM_031971) was chemically synthesised (Tri-1 Biotech; Taipei, Taiwan) as complementary oligonucleotides [11]. ABLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) search of the human genome database was done to ensure that the sequence did not target other gene transcripts. The synthetic oligonucleotide siRNA-HSP72: 5′-GATCCCC**GGAGATCATCGCCAACGAC**TTCAAGAG A**GTCGTTGGCGATGATCTCC**TTTTTA-3′ and 3′-GGG**CCTCTAGTAGC GGTTGCTG** AAGTTCTCT**CAGCAACCGCTACTAGAGG**AAAAATTCGA-5′ was annealed and cloned downstream of HI promoter to construct recombinant pSUPER/siRNA-HSP72 plasmid (siRNA-HSP72). The cloned HSP72 target sequence was sequence-confirmed using a DNA sequencer (ABI Prism 377; Applied Biosystems, Foster City, CA, USA).

2.3. Cell culture and stable transfection

Primary cultured hypothalamic cells were obtained from embryonic (E18d) Sprague–Dawley rat fetuses. Briefly, the brains were removed and the hypothalamus were dissected out. The tissues were cut into fragments, and incubated in 0.2% trypsin for 20 min in 37 °C. Then, DMEM medium (Hyclone, Waltham, MA, USA) containing 10% fetal bovine serum (Hyclone) was added for trypsin activation, and the tissues were dissociated by mild mechanical trituration. About 1×10^5 cells in 1 mL were plated onto poly-D-lysine-coated 6-well plates for further culture. After 4 h, the DMEM medium was changed into Neurobasal™ medium (Gibco, Carlsbad, CA, USA) supplemented with 2% B-27 serum-free supplement (Gibco) and 0.5 mM L-glutamine in a humidified incubator containing 95% air and 5% CO_2 at 37 °C. Primary cell cultures were maintained for 8 days before experiments. Eight hours before hyperthermic injury, cells were transfected with pSUPER/siHSP72 plasmid and with pSUPER vector as control by using oligofectamine transfection according to the manufacturer's instruction. The positive clones were picked and expanded to establish cell lines, and stable transfection cell lines expressing HSP72 siRNA were determined by Western blot analysis.

2.4. Western blot analysis

The cells were harvested from flasks, and lysed in a lysis buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 1 mM $MgCl_2$, 2.5 mM VO_4, 1 mM PMSF, 2.5 mM EDTA, 0.5% Triton X-100, 0.5% NP-40, 5 μg/mL of aprotonin, peptatin A, and leupeptin) for 60 min. Protein samples (500 μg) were separated on 10% or 12% SDS–PAGE gels, and transferred to Hybond-P PVDF membranes (Amersham Biosciences, Stockholm, Sweden). After blocking with 5% non-fat dry mild in TBS-T buffer (20 mM Tris, pH 7.6, 100 mM NaCl, 0.1% Twn-20) for 2 h at room temperature, the membranes were probed with a 1:1000 dilution of anti-HSP72 (DO-1, Santa Cruz Biotechnology, CA, USA) overnight at 4 °C, and a 1:5000 dilution of β-actin (AC-15, Sigma–Aldrich) to correct for differences in protein loading, followed by incubation in a 1:2000 dilution of secondary antibodies conjugated to horseradish peroxidase (Amersham Biosciences) for 1 h at room temperature. Protein bands were detected using ECL detection (Amersham Biosciences). All of the Western immunoblots were performed at least three times.

2.5. Simulated hyperthermic injury (HI) model

The primary hypothalamic cultured cells were put in a circulating 43 °C water bath for 120 min to induce HI, while the normothermic controls were kept in a 37 °C incubator for the equivalent time.

2.6. Mild heat preconditioning (MHP)

Eight hours before the start of HI, the cells were kept in a circulating 42 °C water bath for 30 min to induce HSP72 expression [8].

2.7. Cell viability

Cell viability was determined using an MTT (3-C-4.5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (Invitrogen, Carlsbad, CA, USA) in which the yellow tetrazolium salt catalyzed by nicotinamide adenine dinucleotide (NAD)-dependent dehydrogenase (in active mitochondria) to form purple formalin salt crystals. Cells were seeded into a 96-well plate. After HI, the MTT reagent (5 mg/mL in PBS) was added to each well and then incubated for 4 h. After the assay reagent had been removed, dimethyl sulfoxide (DMSO) was added to each well to solubilize the purple crystals. Plates were then immediately analyzed using an ELISA plate reader (Biotals Instruments, Winooski, VVT, USA) at 580 nm. Cell viability was defined relative to the ratio of normothermic control cells as follow: cell viability (%) = (value of optical density of treated sample)/(value of optical density of control × 100).

2.8. Flow cytometry

Apoptosis was analyzed using a kit (Annexin V-FITC kit, Beckman Coulter, Fullerton, CA) that measures the binding of a fluorescein-labelled annexin V to phosphatidylserine residues translocated on the cell surface soon after the induction of apoptosis and before nuclear breakdown occurs. After HI, cells were collected by trypsinisation and were suspended at a concentration of 1×10^6/mL in phosphate buffered saline. All subsequent steps were done on ice. The cells were resuspended in binding buffer and incubated with annexin V-FITC for 15 min in the dark. The cells were then re-pelleted and re-suspended in binding buffer, propidium iodide was added, and a flow cytometer (Beckman Coulter, Miami, FL, USA) was used for analysis: (A) viable cells do not stain with either reagent, (B) necrotic cells stain with both reagents, and (C) apoptotic cells stain with annexin V-FITC only.

2.9. Experimental groups

Six groups of hypothalamic cells were used in the present study (Fig. 1): (I) no mild heat preconditioning (MHP^-) and no hyperthermic injury (HI) ($MHP^- + HI^-$); (II) no MHP and HI ($MHP^- + HI^+$): the cells were kept in a circulating water bath of 43 °C for 120 min; (III) $MHP^+ + HI^+$: before the start of HI, the cells were kept in a circulating 42 °C water bath for 30 min and then allowed to recover at 37 °C for 8 h; (IV) $MHP^- + HI^+ +$ siRNA-$HSP72^+$ group: the MHP^- cells were transfected with pSUPER/siRNA-HSP72 8 h before the start of HI; (V) $MHP^+ + HI^+ +$ siRNA-$vector^+$ group: the MHP^+ cells were transfected with siRNA-vector 8 h before the start of HI; and (VI) $MHP^+ + HI^+ +$ siRNA-$HSP72^+$ group: the MHP^+ cells were transfected with siRNA-HSP72 8 h before the start of HI.

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